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Concomitant strain of *Bacillus vallismortis* BR2 and *Escherichia coli* Khodavandi-Alizadeh-2 for Biocatalytic Synthesis of Fatty Acid Methyl Ester from Waste Oil Feedstock

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ABSTRACT

Bacillus vallismortis BR2 and Escherichia coli Khodavandi-Alizadeh-2 lipases (E.C.3.1.1.3) were used to produce fatty acid methyl ester (FAME), a sustainable source of fuel. The lipase activity was measured using the titrimetric method after it was extracted from a solid fermented substrate in phosphate buffer. The use of Central Composite Design to optimize condition parameters was examined, while qualitative and quantitative assessments of FAME samples were performed using GC-MS with MSD in scan mode and selective ion monitoring. Lipase activity peaked at 24 h and then declined as the incubation time went on. The independent variables, such as pH, temperature, agitation, incubation time and enzyme quantity, all had an effect on biodiesel yield since they were all significant in the rate of biodiesel yield. FAME yield increased significantly after adding 1 to 2 mL of enzyme and a pH range of 4.57143 to 7.42857, but thereafter declined. The chromatograms indicated a peak of cis-10-Heptadecanoic acid methyl ester with concentrations of 39.95 mg/L and 58.95 mg/L in the FAME molecules. The viscosity (3.67 m3/s), specific gravity (0.813 g/cm3), flash point (102.70 °C), cetane number (55.52), and pour point (-24 °C) of the fuel were also measured. The synthesized biodiesel from the spent oil through the synergic enzymes were found to be a simple, effective, and sustainable fuel production process, as well as a potential means of eliminating pollution caused by haphazard waste cooking oil disposal.

Keywords: Bacillus vallismortis BR2, Concomitant Lipase, Escherichia coli Khodavandi-Alizadeh-2, Fatty Acid Methyl Ester, Waste cooking Oil.

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Introduction

The sources of non-renewable energy are natural gas and petroleum (about 90% of the global energy production that powers the economy). Due to the unavailability and exhaustibility of this fossil fuel as a result of increasing population and industrialization, another substitute like waste cooking oil (WCO) is hereby sourced [1,2]. Biodiesel is a fuel replacement because it is environmentally safe and benign, and it can be blended or used directly with conventional gasoline in uncontaminated diesel engines. It contains no sulfur, no aromatics and possesses a higher cetane number (> 47), with 10%–11% oxygen by weight, as in contrast with petrol [3], thereby minimizing the emission of hydrocarbons, carbon monoxide and particle pollution in the exhaust gas. Biodiesel is usually synthesized by transesterification of pure vegetable oils with short-chain alcohols using alkaline catalysts. A high-quality food-grade vegetable oil is needed with a low level of free fatty acids (FFA), leading to low biodiesel conversion and difficulties in glycerol separation, so saponification is avoided.

Several reports have been made in regard to the utilization of lipase from single strains of organisms such as Alternaria sp. MGGP 06, Aspergillus niger ATCC 1015; Candida rugosa PV 0514; Aspergillus niger F7-02; Aspergillus terreus [4-8] but a coexisting mixture of such enzymes is being reported to enhance the specificity of the enzyme.

Response surface methodology (RSM) has been widely applied in biodiesel production and as an assemblage of statistical and mathematical techniques useful for patterning and evaluating problems in which a desired response is controlled by some variables [8]. Variable factors include substrate molar ratio, pH, temperature, agitations optimized for biodiesel synthesis using immobilized lipase in biomimetic silica, or flow rate, substrate molar ratio, and temperature using a lipase-catalyzed packed-bed reactor for steady biodiesel production [2]. Several variables influence the transesterification reaction, including catalyst concentration, presence of water, substrate molar ratio of alcohol and free fatty acid content in oil feedstock, time, agitation speed, and temperature. In order to make environmentally friendly and economically viable biodiesel, an understanding of the factors is very important [9-10].

This present study is aimed at optimizing the synthesis of biodiesel from a cheaper waste oil using the

concomitant lipase of *Bacillus vallismortis* BR2 and *Escherichia coli* Khodavandi-Alizadeh-2 strains. The relative effects of some condition parameters and their concentrations were determined. GC-MS was used to analyze the chemical constituents of biodiesel, and the fuel characteristics were also checked for conformity to American Society for Testing Materials (ASTM) standards.

Materials and Methods

Materials

Potential microorganisms were isolated from soil samples collected from a mechanic workshop in Mowe, Ogun State (Longitude 6.811°N and Latitude 3.437°E) and McPherson University, Seriki Sotayo, Obafemi Owode Local Government Area, Ogun State, Nigeria.

Methods

Sample Collection

The soil sample was obtained from an oil-polluted site at Mowe, Obafemi Owode, and McPherson University, Seriki Sotayo, Ogun State. Soil samples and waste cooking oil were packed in sterile bottles and taken to the laboratory.

Isolation of lipase producing bacteria

Isolation of potential bacterial isolates that can degrade petroleum was carried out using the serial dilution and the pour plate methods on Nutrient Agar and was incubated at 37 °C for 24 h. Pure cultures of the isolates were maintained on slants at 27 °C and sub-cultured for fourteen consecutive days to conserve their efficaciousness.

Lipase Production on Solid-State Fermentation (SSF)

Solid-state fermentation was used to produce the lipase. The medium used to be made up of a specific wheat offal solid substrate (10 g). The petri dish media was wet with 15 mL distilled water, autoclaved for 15 min at 121 °C, and allowed to cool. It was inoculated with 0.5 mL of three-day-old broth cultures of *E. coli* Khodavandi-Alizadeh-2 and *B. vallismortis* BR2 and incubated for 24-96 h at 37 °C. One (1) gram of culture was placed in a test tube with 10 mL of phosphate buffer (mixed) and centrifuged for 10 minutes at 4000 rpm. The extracellular enzyme (supernatant) was collected in sterile test tubes and utilized to determine lipase activity. The enzyme activity was determined using the titrimetric method with olive oil as a substrate according to Pualsa *et al.,* [11]. The enzyme (0.1 mL) was added to an emulsified olive oil substrate (buffered with 0.1 M potassium phosphate pH 7) and incubated for 15 minutes at 37 °C. Fatty acid was extracted using a 1.0 mL acetone-ethanol (1:1) solution, and the amount liberated was determined using a titration against 0.1 N NaOH with phenolphthalein indicator until light pink coloring appeared. A blank reading (no enzyme) was taken.

The activity of lipase was measured using:

$$\mathrm{U/mL} = \frac{[(\text{mL NaOH for sample} - \text{mL NaOH for blank}] \text{ x N}}{\text{Amount of Lipase (mL) x Reaction time}} \quad \text{Eq. 1}$$

Where N= Normality of NaOH

One unit of lipase is defined as the amount of lipase needed to hydrolyze 1 mol of fatty acids from triglycerides equivalent per minute under the assay conditions.

Fatty Acid Methyl Ester (Biodiesel) from WCO

Mixed lipases from *Escherichia coli* Khodavandi-Alizadeh-2 and *Bacillus vallismortis* BR2 were used to produce biodiesel. Muslin cloth was used to treat waste cooking oil (WCO) to remove dirt and other undesired particles that could interfere with the biodiesel synthesis process. Thus, the WCO is being refined for improved performance. In three sequential methanol additions for biodiesel manufacture using waste cooking oil, the substrate molar ratio of oil to methanol (3:1) was employed. The reaction was co-catalyzed by free lipase of the two distinct microorganisms (1 mL each) at 45 °C for 200 rpm in a shaking incubator during varied reaction time intervals of 24 h, 48 h, and 72 h, respectively. After 72 h of reaction time, the mixture was put into a 100 ml separating funnel to separate the fatty acid methyl ester and placed in a sterile vial for analysis.

Concurrent lipase from E. coli Khodavandi-Alizadeh 2 and B. vallismortis BR2 used to Optimize Biodiesel Production

Design of experiments

Response surface methodology (RSM) provided by Design-Expert software 13.0 was used to create and optimize the biodiesel synthesis (Stat-Ease Inc., Minneapolis, USA). The catalyzed transesterification reaction variable was investigated using the central composite design (CCD), a common RSM design technique. The six identified independent factors are pH (4–8), agitation (100–300 rpm), incubation period (24–96 h), substrate molar ratio (1:1–1:4 methanol: oil), enzyme amount (1–3 ml), and temperature (35–55 °C). A five-level central composite matrix was used, and 13 runs were performed in random order. To circumvent lipase inhibition, methanol was added in three steps for biodiesel production utilizing modifier's approach of Fan *et al*. [12]. The first batch was made at the start of the reaction, and the others were made at 16 h, 32 h, and 48 h into the reaction, which took 96 h in a 100 mL conical flask and was incubated in a shaker incubator for 96 h. The sample was poured into a 100 mL screwedcapped se parating funnel at the end of the reaction. The upper layer was used to calculate the % yield using Nazir *et al*. [13]. Following that, gas chromatograph-mass spectroscopy (GC-MS) analysis was performed as previously reported.

Analysis of Biodiesel

Yield of biodiesel estimation

The FAME yield (percent) was a dependent variable that was defined as the weight of methyl ester divided by the expressible weight of oil. Equation 2 below shows how the yield of FAME in the samples was estimated.

$$\text{Yield (Y)} = \frac{\text{methyl ester weight x 100\%}}{\text{Weight of oil}} \quad \text{Eq. 2}$$

Fatty Methyl Ester (Biodiesel) Gas Chromatography (GC) Analysis

Sigma Aldrich provided a SUPELCO 37-Component FAME MIX (Catalog Number: LRAC1814), which had 37 FAMES components. Before calibration, the MS was auto-tuned to perfluorotributylamine (PFTBA) by checking the abundance of m/z 69, 219, 502 and other instrument optimum and sensitivity settings. To ensure low-level detection of the target constituents, the levels of FAMES in the sample were determined using GC-MS in selective ion monitoring (SIM) and Scan mode.

Preparation of Samples

The samples were diluted by a factor of 5 (100 uL sample + 400 uL n-Hexane) in vials before GC analysis. An Agilent 6890N gas chromatograph coupled to a 5973C inert mass spectrometer (with triple-axis detector) with an electron-impact source (Agilent Technologies) was used. The stationary phase of separation of the compounds was the HP-5 capillary column coated with 5% Phenyl Methyl Siloxane (30m length x 0.32mm diameter x 0.25m film thickness) (Agilent Technologies). The carrier gas was hydrogen, used at a constant flow of 1.48 mL/min at an initial nominal pressure of 1.49 psi and an average velocity of 44.22 cm/sec. Each sample (1µL) was injected in splitless mode at 300 °C injection temperature. The purge flow rate to the spilled vent was 15.0 mL/min for 0.75 min, for a total flow of 16.67 mL/min, with gas saver mode turned off. The oven was set at 100 °C for 5 mins before ramping up to 250 °C at a rate of 7 °C per minute (2 min). With a three-minute solvent delay, the overall run time was 28 mins. The mass spectrometer was run in electron-impact ionization mode at 70eV with a 230 °C ion source, 150 °C quadrupole, and 300 °C transfer line. Following calibration, the samples were examined, and corresponding FAME concentrations were calculated using the calibration curve equation, if detected based on a retention time match of the FAME standard. The quantity (g/mL). The following formula is used to determine the amount of FAME in the sample (g/mL):

$$\text{g/mL of FAME} = \frac{\text{(Total Amount (Ug/mL) x DF x 1g)}}{1000000\text{ug}} \quad \text{Eq. 3}$$

where DF = 5

g/mL was multiplied by 100 to get g/100mL = % (w/v) FAME

Characterizations of Biodiesel

The fatty acid methyl ester samples from the optimal experimental point were characterized for cloud point, pour point, flash point, viscosity, cetane number, and specific gravity to see if the standards set by the American Society for Testing Materials (ASTM) and the European Union (DIN EN-14214) were met.

Measurement of Viscosity

The viscosity of the samples was determined using a viscometer according to the ASTMD 445 protocol. A screw on the vertical pole was used to raise the viscometer to its greatest level. The viscometer was lowered until the spindle was submerged to the spindle's mark and reading taken within 60 s.

Pour-Point Determination

The level mark was filled with two grams of the sample. The test jar was immersed in a bath of crushed ice and the cork carrying the test thermometer was carefully closed. The test jar was inspected every three (3) min by placing it in a horizontal position for a few seconds before returning it to cool. When the oil surface remained upright for 5 s without sagging, it was considered to be at the pour point. The thermometer was inserted at this point and allowed to cool for 10 s before taking the oil's temperature.

Flash Point Determination

Using an ASTM D 93 flash tester, the sample's flashpoint was determined to be 120 °C as outlined in ASTM D 93. A closed-cup test that retains the fumes created and effectively simulates the situation where an ignite source is mistakenly introduced into a container. The oil was placed in a cup, which had a tight-fitting lid. Heat was applied to the cup and the oil. Apertures in the lid were then opened to allow air into the cup, and the ignition source was dipped into the vapors to see if a flash occurred. The flashpoint was determined by heating the oil in a cup and then inserting a tiny flame slightly above the liquid surface. The flash/ignition temperature is recorded as the flash point.

Determination of Specific Gravity

A 25 ml crystal clear specific gravity bottle was weighed, filled, and reweighed with the sample (W1). After washing and drying the density bottle, the sample was replaced with water, and the weight was recorded as W2. The specific gravity (S.G.) was calculated using the following formula:

$$\text{S.G.} = \frac{W1 - W0}{W2 - W0}$$

W0 = Initial weight of density bottle

Analytical Statistics

The response surface methodology of Design-Expert Software 13 was used to analyze the experimental data collected from the CCD. The ideal point between the

response variable (FAME yield) and the independent variables of transesterification is predicted using a second-order polynomial equation model. The coefficients of determination (R2) and the regression coefficient significant were used to assess the fit model's quality (analysis of variance [ANOVA]). By keeping two of the independent variables fixed while changing the other three, response surfaces and contour plots were created using the quadratic polynomial equation obtained through regression analysis of experimental data.

Results and Discussion

Lipase-producing Bacterial Isolation and Identification

From the soil samples, four microorganisms were recovered. The most promising microbe was chosen and identified by morphological, biochemical, and molecular characterization utilizing 16S r sequencing-based. Because of the abundance of lipids, oil-contaminated soil is thought to be a perfect habitat for lipase-producing bacteria. These lipids are used as a source of nutrition by the bacteria that live there [14]. Jenisha & Renuga [15] described the occurrence of lipase positive strains from comparable sources in isolate samples taken from various habitats. The lipase activity of the *Escherichia coli* strain detected in soil samples from the mechanic's workshop was greater. This adds to the evidence that the biological system's state and behavior are influenced by the environment [16]. The identification of *Escherichia coli* and *Bacillus vallismortis* in petroleum-contaminated locations is consistent with prior research by Giwa and Ibitoye [17]; Rong et al. [18], which discovered *E. coli* and *Bacillus* sp. in similar conditions among other microbes. The microbes were cultured on wheat offal and lipase was produced by solid state fermentation. The organisms' development on a wheat offal substrate matches previous study by D'hoe et al., [19]; Ji et al., [20], in which wheat offal was used to synthesis enzymes including amylase and lipase.

Polymerase Chain Reaction (PCR) and PCR-amplified DNA Identification

The DNA was amplified using standard PCR and illuminations (Not shown), and then seen on an agarose gel electrophoresis under UV light. The molecular weight was determined using a 1 kb DNA ladder, and the amplicon size was around 1500 bp. Table 1 shows the distribution of species detected in the DNA bacterium isolate library based on an investigation of a PCR product sequence in the non-redundant nucleotide database of the National Center for Biotechnology Information (NCBI). *Escherichia coli* Khodavandi-Alizadeh-2 and *Bacillus vallismortis* BR2 were identified as the isolates with accession numbers MN186856 and MK880631, respectively. The highest percentages of identity were 84 percent and 74%, respectively. On agarose gel electrophoresis of the template DNA amplified by PCR, the base pair of the isolated microorganisms was indicated in comparison to the PCR marker (Not shown). The gene template had the same molecular weight as the gene itself, at 1500 bp. Figures 1 and 2 show the phylogenic trees for the query organisms *Escherichia coli* Khodavandi-Alizadeh-2 and *Bacillus vallismortis* BR2 as well as nearby species. The organisms' nucleotide sequences matched those of *Escherichia coli* and *Bacillus vallismortis* in the NCBI database. Using the nucleotide sequence as a query, a gene bank and phylogenetic trees indicating comparable organisms were generated.

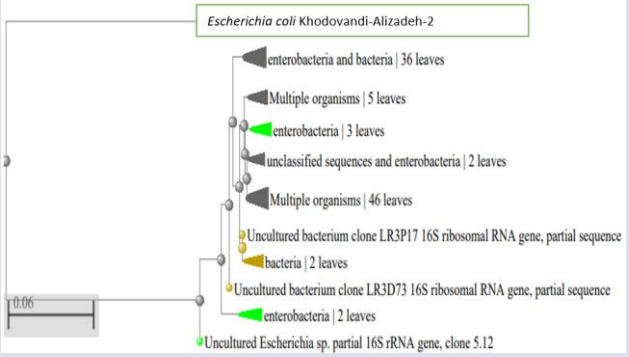

Figure 1: Phylogenic tree showing the query organism *Escherichia coli* Khodavandi-Alizadeh-2 and close species

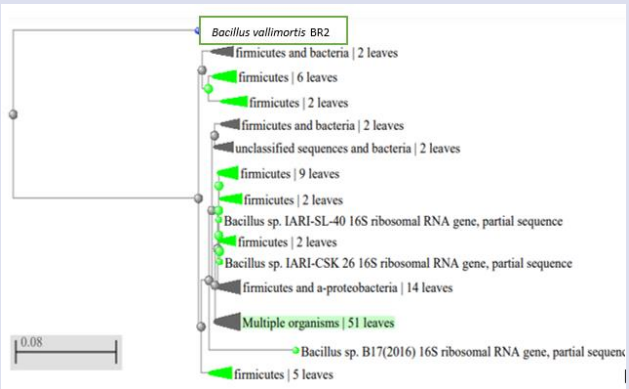

Figure 2: Phylogenic tree showing the query organism *Bacillus vallismortis* BR2 and close species

Lipase Activity Determination

Lipase activity was determined using the titrimetric method according to Pualsa et al. [11] approach, which was described in section 3.3.2, and the lipase activity was estimated using Equation 1. The lipase activity increases after 24 h and diminishes as the incubation duration increases (Fig 3). The lipase activity was measured using the titrimetric method with olive oil (10% v/v) as a substrate, emulsified with gum Arabic (5% w/v) in 0.1M potassium phosphate pH 7.0 and 0.1ml of enzyme, then incubated at 37 °C for 15 minutes. After 24 h, the lipase activity rose, which is consistent with Çağatay and Aksu [21] findings on lipase produced from waste cooking oil. The application of complicated composites design was offered by Stat-Ease Inc.'s Design Expert software 13.0 (Minnepolis, USA). Independent variables such as pH, temperature, agitatio

n, and others influenced the increase and reduction of bi odiesel output, as they all have a substantial impact on th e rate of biodiesel yield. Mohammed et al., [22] used a si milar methodology and came to the same conclusions as the study.

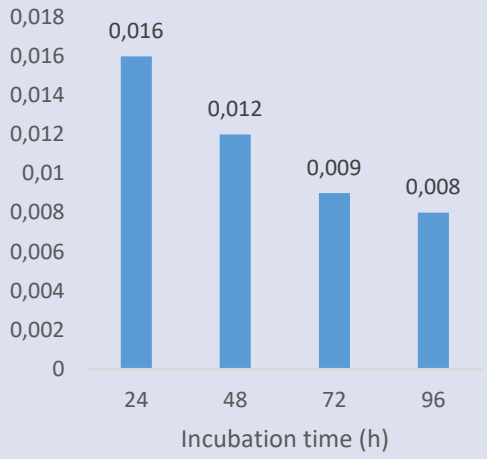

Figure 3: Activity of concomitant lipase of *Escherichia coli* Khodavandi-Alizadeh-2 and *Bacillus vallismortis* BR2 on Solid State Fermentation medium

Optimization of Biodiesel

Statistical model analysis

Figure 4 shows the results of the 13 experimental runs performed using the Design Expert 13.0 software. The outcome was studied and a second polynomial equation was fitted (Equation 1).

$$Y = \beta o + \Sigma 3i=1\ \beta iXi + \Sigma 3i=1\ \beta iiXi2 + \Sigma\Sigma 3i<i=1 \beta ijXiXj \quad \text{Eq. 1}$$

Where Y is response (Lipase production); β (0=intercept, i=linear, ii=quadratic and ij=interaction) and Xi, Xj (i=1, 4; j=1, 4; I ≠ j represent the coded independent variables) are the model coefficients.

Final Equation in Terms of Coded Factors

$$\begin{aligned}\text{FAME Yield} = {} & +23.00 + 0.0000x1 + 2.24x2 + 2.24x3 + \\ & 2.56x4 - 3.51x5 - 4.15x6 + 0.0000x1x2 + 0.0000x1x3 \\ & + 0.0000x1x4 + 0.0000x1x5 + 0.0000x1x6 + \\ & 0.0000x2x3 + 0.0000x2x4 + 0.0000x2x5 + \\ & 0.0000x2x6 + 0.0000x3x4 + 0.0000x3x5 + \\ & 0.0000x3x6 + 0.0000x4x5 + 0.0000x4x6 + \\ & 0.0000x5x6 - 4.90x12 + 3.47x22 + 1.84x32 + 3.27x42 \\ & + 0.2041x52 + 6.33x62\end{aligned} \quad \text{Eq. 2}$$

The best fit model, rather than the linear or cubic models, was suggested by the Statistical Model Fit Summary (which comprises of a sequential model sum of squares and a lack of fit tests). Although the cubic model has a smaller standard error than the quadratic model, the cubic model's presence of multiple aliased terms renders it unsuitable for representing the study. The data value in the quadratic model was aligned to the unit slope, indicating that it was the best model to capture the factor affecting biodiesel yield.

Table 1: Experimental design and response (biodiesel yield) of different experimental runs. Response of different experimental runs

		Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Response 1
Std	Run	A: pH	B: Temperature °C	C: Agitation rpm	D: Substrate Molar ratio mL	E: Enzyme Quantity mL	F: Incubation Time h	Biodiesel Yield %
3	1	6	35	200	2.5	2	60	28
11	2	6	45	200	2.5	2	24	45
10	3	6	45	200	2.5	3	60	18
12	4	6	45	200	2.5	2	96	32
13	5	6	45	200	2.5	2	60	23
4	6	6	55	200	2.5	2	60	35
7	7	6	45	200	1	2	60	27
5	8	6	45	100	2.5	2	60	24
2	9	8	45	200	2.5	2	60	11
6	10	6	45	300	2.5	2	60	31
1	11	4	45	200	2.5	2	60	11
8	12	6	45	200	4	2	60	35
9	13	6	45	200	2.5	1	60	29

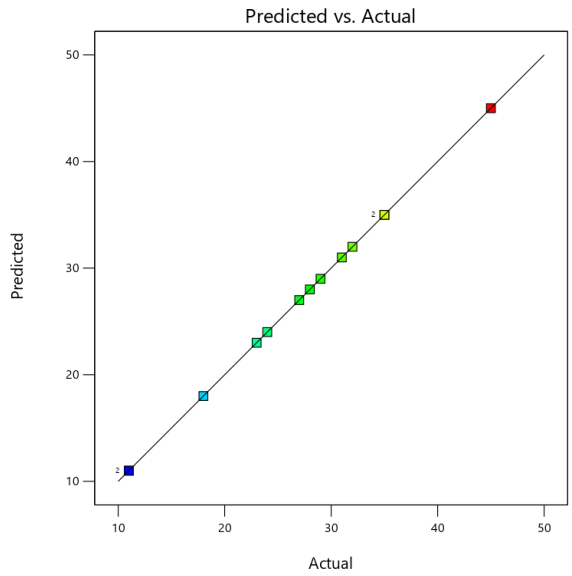

Figure 4: Model Fit Profile of Quadratic Model

FAME yield as a function of operational variables

FAME yield as a result of the main effect variables

The statistical analysis of the experiment range tested identified pH, temperature, agitation, and incubation time as significant determinants on the biodiesel yield response, as shown in Fig.5, although enzyme quantity and substrate molar ratio were the least significant factors. As the pH rises, so does the yield (fig.5a). Temperature had an effect on yield, with yield decreasing around 45 °C. Because the optimum yield was at 200 rpm and increasing agitation would have produced more yield, agitation has minimal effect on yield. Because the yield declined as the enzyme quantity was increased, the substrate molar ratio had no effect on yield.

The second-order models can be plotted as interactive contour and response surface graphs for each response, representing the biodiesel yield as a function of four of the six factors at the operating condition's center point value; thus, Fig 6 to 8 shows the interactive contour and response surface plot for the fitted model of the biodiesel molar yield.

The simultaneous dependency of FAME yield on enzyme quantity and reaction pH is shown in Figure 6. Temperature, agitation, substrate molar ratio, and incubation duration were all set to 45 °C, 200 rpm, 2.5 mL, and 48 h. FAME yield increased by 44 percent when the enzyme quantity was increased from 1 mL to 2 mL and the pH was elevated from 4.57143 to 7.42857, but subsequently declined as the enzyme quantity was increased from 2 mL to 3 mL. This means that the optimum yield can be obtained with 1 to 2 mL of enzyme and a pH range of 4.45143 to 7.42857.

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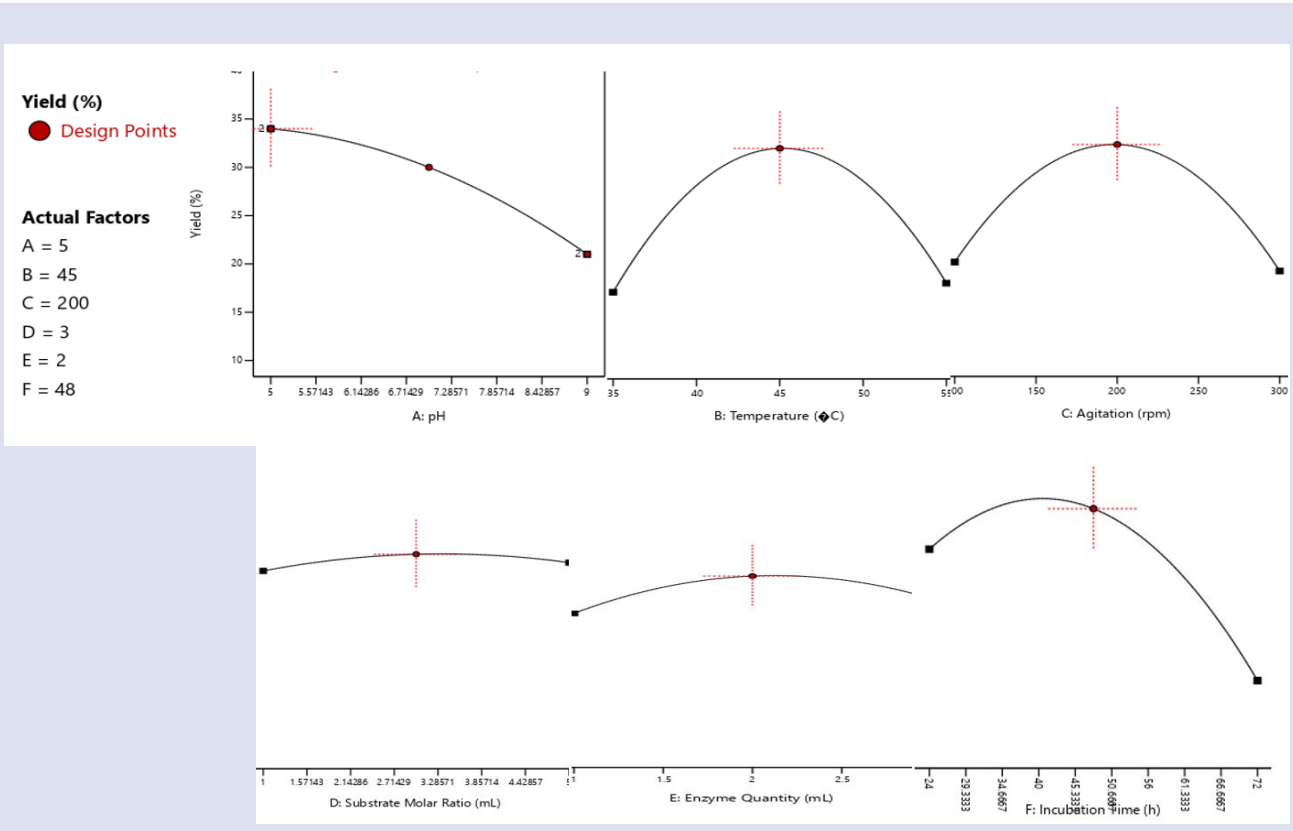

Figure 5(a- f): Plots of main variable effects of the FAME yield

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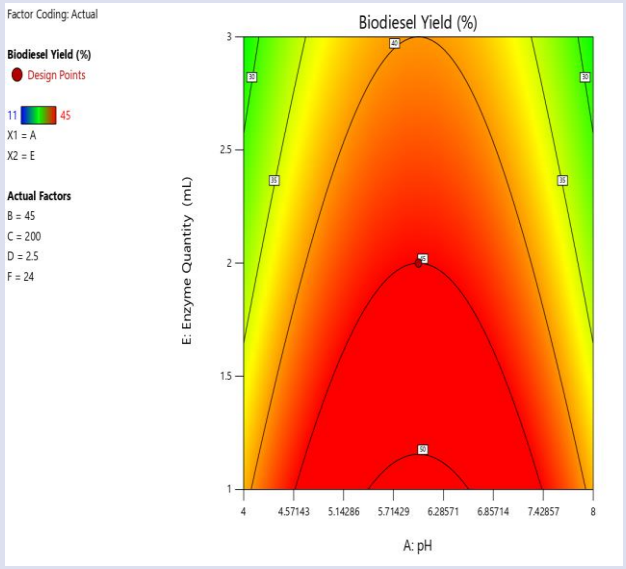

Figure 6: Interactive contour plot of biodiesel yield as function of Enzyme Quantity and pH: temperature (45° C); Agitation (200 rpm); Substrate molar ratio (2.5 ml); Incubation time (24 h)

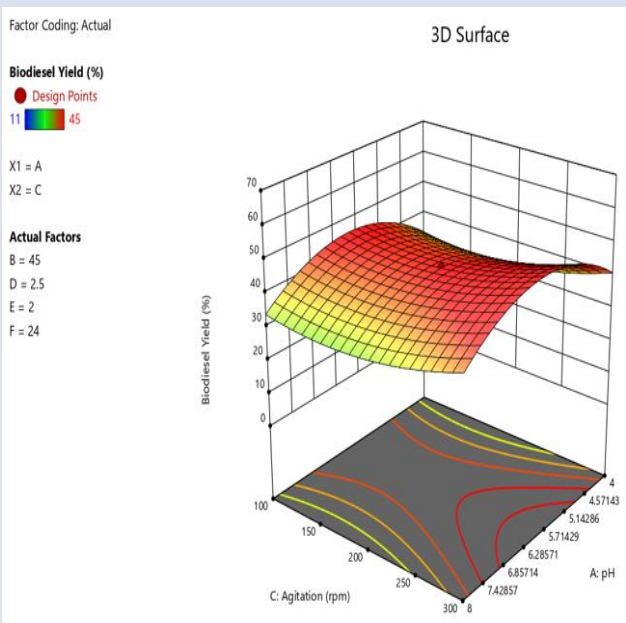

Figure 7: Response surface plot of biodiesel yield as function of Agitation and pH: temperature (45° C); Substrate molar ratio (2 ml); Enzyme Quantity (2 ml); Incubation time (24 h)

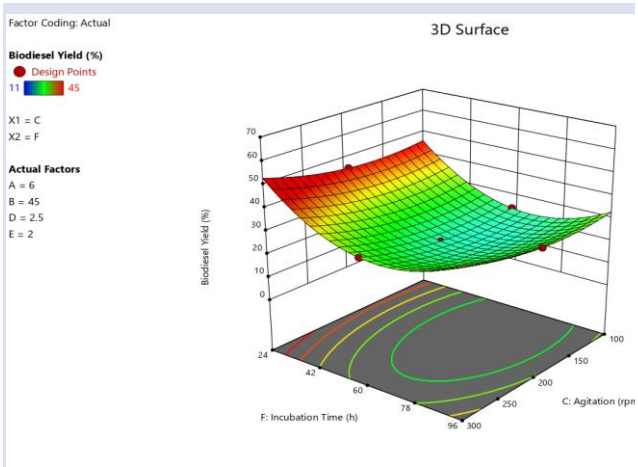

Figure 8: Response surface plot of biodiesel yield as function of Incubation time and Agitation: pH (6); temperature (45° C); substrate molar ratio (2.5 ml); Enzyme Quantity (2 ml)

Concurrent Lipases Catalyze the Transesterification of Waste Cooking Oil (WCO)

FAME produced by mixed lipase from *E. coli* Khodavandi-Alizadeh-2 and *B. vallismortis BR2* in the transesterification of WCO and methanol to a control experiment (without enzyme) (Not shown). Mixed lipase from *E. coli* Khodavandi-Alizadeh-2 and *B. vallismortis* BR2 were used in the flask's upper layer. The third layer of the flask contained fatty acid methyl ester, followed by glycerol, and the third layer was crude enzyme sediments. Two separate layers were visible in the control. The unreacted methanol is on top, and the oil is on the bottom.

Some FAME compounds, such as methyl stearate, cis-10-Heptadecanoic acid methyl ester, Oleoic acid methyl ester, Eladic acid methyl ester, Palmitic acid methyl ester, 9,12-Octadecanoic acid methyl ester, Arachidonic acid methyl ester, 13-Docosenoic acid methyl ester, and Lignoceric acid methyl ester, indicated that the free fatty acids were able to convert to methyl esters. This was observed in the study of Aboelazayem *et al*. [23], where several of these FAME compounds were discovered in the WCO after gas chromatography examination of free fatty acids. It also demonstrated that opportunistic bacteria with lipase-producing capacity, such as *E. coli* and *B. valllismortis*, can make biodiesel from waste cooking oil. The use of enzyme catalysts can eliminate the need for chemical catalysts, making the process more cost-effective and ecologically benign.

Fatty Acid Methyl Ester Analysis by Gas Chromatography (GC)

The fatty acid methyl ester profile yielded 64.3 mg/L and 74.85 mg/L concentration components of the FAMES from waste cooking oil, respectively. Tables 3 and 4 indicate the Fatty Acid Methyl Ester components of examined samples as determined by GCMS. As shown in Figures 9 and 10, the gas chromatograms of the two samples indicated 39.95 mg/L and 58.95 mg/L, respectively, when the peak area of the cis-heptadecanoic acid methyl ester was taken into account.

Table 2: Profile of WCO Fatty Acid Methyl Ester (FAME) of mixed lipase by *Eschericia coli* Khodavandi-Alizadeh-2 and *Bacillus vallismortis* BR2 from one of the Experimental Run 2

FAMES Compound	Retention Time	Response	Conc. (Unit)	Dev. (min)
Methyl myristoleate	12.441	1087	2.85 mg/L	48
Palmitic acid, methyl ester	16.030	22424	9.55 mg/L	91
Cis-10-Heptadecenoic acid Methyl ester	16.885	12899	39.95 mg/L	59
9,12-Octadecadienoic acid Methyl ester	18.307	1447	3.25 mg/L	72
Oleic acid Methyl ester	18.385	3925	2.70 mg/L	100
Elaidic acid, methyl ester	18.296	1860	0.65 mg/L	42
Methyl stearate	18.385	3925	3.95 mg/L	5
13-Docosenoic acid, methyl ester	23.051	535	0.75 mg/L	19
Lignoceric acid, methyl ester	25.106	201	0.65 mg/L	19
TOTAL			64.3 mg/L	

Table 3: Profile of WCO Fatty Acid Methyl Ester (FAME) of lipase by *Escherichia coli* Khodavandi-Alizadeh-2 and *Bacillus vallismortis* BR2 from one of the Experimental Run 11

FAMES Compound	Retention Time	Response	Conc. (Unit)	Dev. (min)
Methyl myristoleate	12.441	497	1.30 mg/L	24
Palmitic acid, Methyl ester	16.030	1357	4.85 mg/L	86
Cis-10-Heptadecenoic acid Methyl ester	16.941	9037	58.95 mg/L	58
9,12-Octadecadienoic acid Methyl ester	18.307	865	1.95 mg/L	40
Oleic acid Methyl ester	18.385	1835	1.25 mg/L	100
Elaidic acid, Methyl ester	18.085	209	0.05 mg/L	16
Methyl stearate	18.385	1835	1.85 mg/L	1
Arachidonic acid Methyl ester	19.663	211	0.85 mg/L	30
13-Docosenoic acid, Methyl ester	22.862	1293	1.85 mg/L	60
Lignoceric acid, methyl ester	25.128	611	1.95 mg/L	19
TOTAL			74.85mg/L	

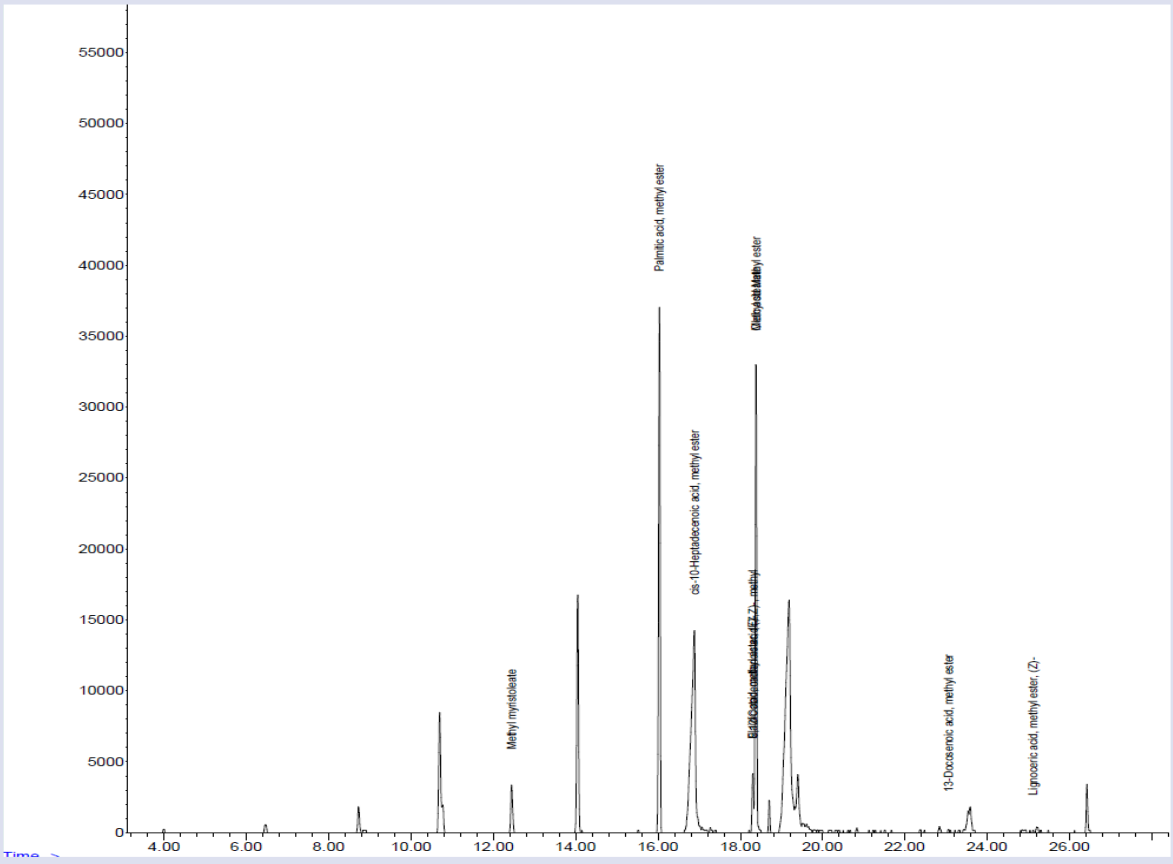

Figure 9: Gas Chromatography of WCO Fatty Acid Methyl Esters from mixed lipase of *Escherichia coli* Khodavandi-Alizadeh-2 and *Bacillus vallismortis* BR2 from Experimental (Run 2)

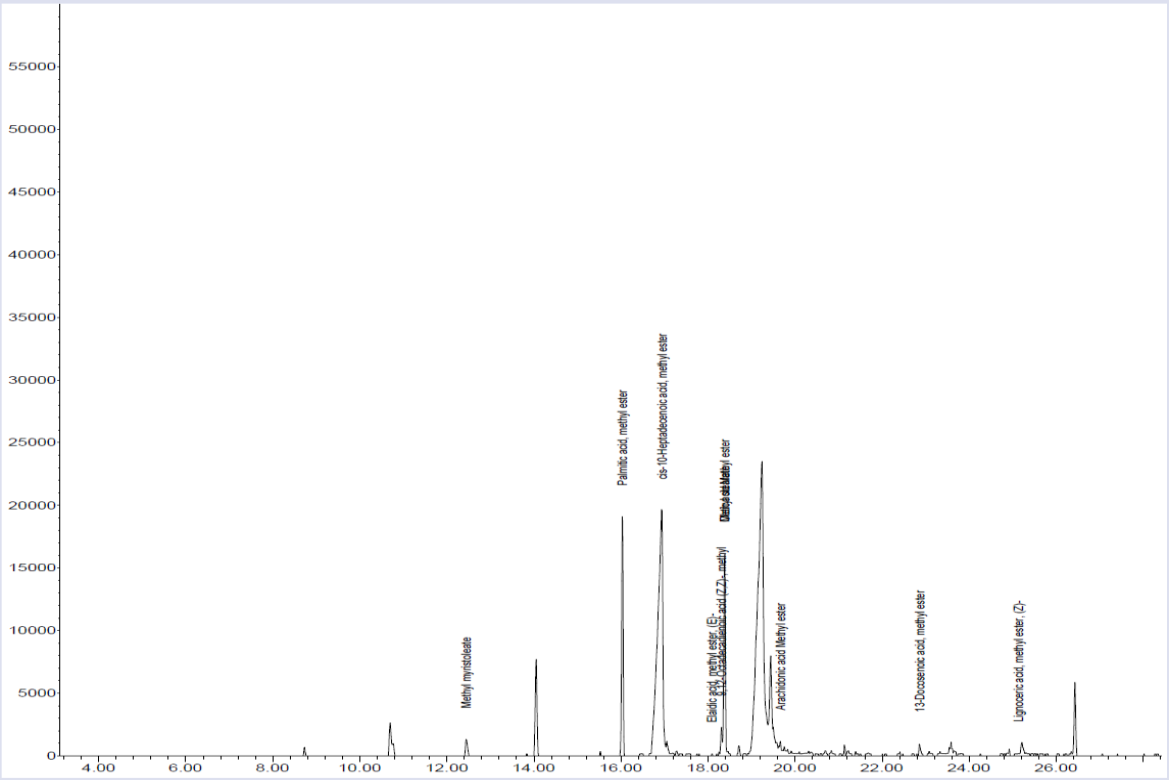

Fig. 10 Gas Chromatography of WCO Fatty Acid Methyl Esters from mixed lipase of *Escherichia coli* Khodavandi-Alizadeh-2 and *Bacillus vallismortis* BR2 from experimental (Run 11)

The Fuel Properties of Biodiesel (Fame)

The fuel parameters of fatty acid methyl ester (biodiesel) are compared to those of the biodiesel standard in Table 4. The current findings revealed that the transesterification method enhanced the viscosity, flash point, and pour point of the gasoline. When the flash point, pour point, cloud point, cetane number, and viscosity of the biodiesel were compared to those of the biodiesel standard, it was discovered that the transesterification procedure improved these attributes. The cetane number (CN) is one of the most important qualities of biodiesel fuels that indicates the ignition characteristics and quality of motor power, and it correlates to Huang *et al.* [24] study as well as the ASTM and EU regulations (DIN EN-14214). A property's flash point was substantially improved, as a higher flash point prevents any type of fire outbreak. The biodiesel yield can also be used in cold weather due to reduced pour point and cloud point features.

Table 4 Properties of Biodiesel (FAME) from Waste Cooking Oil

Property	Unit	Run A	Run B	ASTM standard D6751-02	EU Standard EN-14214
Specific gravity	g/cm3	0.946	0.818	0.880	0.886-0.90
Viscosity	m2/s	3.80	4.20	1.9 – 6.0	3.5 – 5.0
Flash point	°C	105.84	102.90	> 130	> 120
Cloud point	°C	0.00	0,.00	summer -4 winter -1	-
Pour point	°C	-25	-24	15-18	-
Cetane		51.40	52.65	>47	>

ss

Conclusion

The excessive use of fossil fuels depletes reserves, and continuing greenhouse gas emissions as a result of global climate change have an impact on the country's socioeconomic development. As a result, renewable energy sources are being used as a substitute. With the constant depletion of fossil fuels, the biotransformation of domestic spent cooking oil into a value-chain product such as biofuel is critical, particularly for Sub-Saharan African countries.

The goal of this study was to use a concurrent hydrolyzing enzyme to convert waste cooking oil into fatty acid methyl ester and to optimize the parameter circumstances to favor good output. As quantitative and qualitative assessments of the biofuel samples were subjected to instrumentation utilizing Gas Chromatography-Mass Spectrometry, a major statistical tool was used to collaborate on the efficient operations. The rate of biodiesel yield was affected by all variables, including pH, temperature, agitation, and enzyme concentration. The FAME properties found were within ASTM and EU guidelines.

Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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