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Production, optimization, and physicochemical characterization of biodiesel from seed oil of indigenously grown *Jatropha curcas*

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With the growing demand for vegetable oils, alternative non-edible feedstocks like *Jatropha curcas* seed oil have gained interest for biodiesel production. The study aimed to comprehensively evaluate the physicochemical properties and biodiesel production potential of locally produced *J. curcas* seeds in Pakistan. Two different approaches were applied: a chemical synthesis approach involving acidic pretreatment and alkaline transesterification, and a biosynthetic approach using a lipase-producing strain of the *Bacillus subtilis* Q5 strain. The microbial biosynthesized biodiesel was further optimized using the Plackett–Burman design. The physicochemical properties of the *J. curcas* methyl esters were analyzed to assess their suitability as biodiesel fuel. Initially, the raw oil had a high free fatty acid content of 13.11%, which was significantly reduced to 1.2% using sulfuric acid pretreatment, keeping the oil to methanol molar ratio to be 1:12. Afterward, alkaline transesterification of purified acid-pretreated seed oil resulted in 96% biodiesel yield at an oil to methanol molar ratio of 1:6, agitation of 600 revolutions per minute (RPM), temperature 60°C, and time 2 h. Moreover, alkaline transesterification yielded ~98% biodiesel at the following optimized conditions: oil to methanol molar ratio 1:6, KOH 1%, time 90 min, and temperature 60°C. Similarly, the *Bacillus subtilis* Q5 strain yielded ~98% biodiesel at the following optimized conditions: oil: methanol ratio of 1:9, agitation 150 RPM, inoculum size 10%, temperature 37°C, and n-hexane 10%. The fuel properties of *J. curcas* seed biodiesel are closely related to standard values specified by the American Society for Testing and Materials (ASTM D6751–20a), indicating its potential as a viable biodiesel fuel source.

KEYWORDS

acid pretreatment, alkaline transesterification, biodiesel, *Jatropha curcas*, two-step biodiesel production

1 Introduction

The consecutive decline in fossil fuel reserves and increase in global warming caused by the extensive combustion of petro-diesel have turned the focus of related industries and the scientific community to the utilization of renewable bioenergy resources such as biodiesel. Biodiesels are mono-alkyl esters that are biodegradable, non-toxic, and environmentally friendly green energy resources (Soudagar et al., 2018). It is acquired from vegetable or microalgae oils and animal fats. It can be effectively used as a substitute to petro-diesel alone and in the blended form with petro-diesel. The biodiesel blend possesses characteristics similar to those of petro-diesel but with comparatively less hazardous emissions of particulate matter such as hydrocarbons and oxides of nitrogen, sulfur, and carbon, like nitric oxide (NO), nitrogen dioxide (NO₂), nitrous oxide (N₂O), sulfur dioxide (SO₂), and carbon monoxide (CO), respectively. The blend is evidenced to have lower toxicity, higher safety, and reduced CO₂ emissions (Koh and Ghazi, 2011; Mofijur et al., 2019; Khan et al., 2020; Soudagar et al., 2020; Soudagar et al., 2020).

Both edible and non-edible oils are used for biodiesel. The use of edibles for biodiesel production is controversial due to the risk of diverting farmland for biofuel production and the detriment of food supply, while non-edible oils have no food versus fuel competition, and therefore, their use for biodiesel production is increasing worldwide (Liu et al., 2019). To date, several non-edible oil feedstocks have been used for biodiesel production, but *Jatropha curcas* is a non-edible feedstock and is considered an ideal option for biodiesel production due to its ability to thrive both in tropical and subtropical climates in marginal and non-agricultural wastelands. It originated from North America and Central America, and now also inhabits South Africa, Southeast Asia, India, and China. *J. curcas* belongs to the Euphorbiaceae family, has a life span of 50 years, and contains 27%–40% oil in its seeds (Nahar and Ozores-Hampton, 2011; Reddy et al., 2015). However, *J. curcas* seeds are also toxic to humans and animals due to toxic components such as phorbol esters and curcumin (Leung et al., 2010; Mofijur et al., 2012).

Biodiesel can be produced using different methods, but transesterification using methanol has remained the most widely used method compared to the direct use of oil or biodiesel blending, pyrolysis, and micro-emulsion (Meher et al., 2013). In transesterification, the triglycerides react with an alcohol to form fatty acid alkyl esters. However, it has been found that alkaline transesterification efficiency relies on the free fatty acid (FFA) composition of feedstocks. It has been reported that alkaline transesterification is not recommended for feedstocks having 3%–40% FFAs. So the alternate option is to use both acidic and alkaline catalysts for higher FFAs. Acid pretreatment can esterify FFAs, and after decreasing FFAs to an acceptable range, i.e., 1%–2%, its product can further be used for alkaline transesterification to obtain a higher biodiesel yield (Chung et al., 2008). The homogenous alkaline catalyst produces soap on reacting with FFAs, and hence, the quantity of catalyst required for transesterification is reduced, resulting in an incomplete reaction and complicated downstream processing (Marchetti et al., 2007; Bohlouli and Mahdavian, 2019).

Several studies have been conducted to increase the yield of biodiesel and improve its fuel properties. Various factors have been considered during biodiesel optimization, such as the oil-to-methanol molar ratio, agitation, temperature, and time. Optimization of these

factors is necessary to decrease the process cost and increase the biodiesel yield (Micic et al., 2015). To the best of our knowledge, it is the first comprehensive assessment of locally produced *J. curcas* seed oil in Pakistan for its physicochemical properties, and biodiesel production via chemical and lipase-mediated transesterification. This study gives a detailed assessment of locally produced *J. curcas* seed oil for biodiesel production and its optimization. In the present study, *Jatropha* oil (JO) was characterized and used for biodiesel production. The optimization of various factors, including the oil-to-methanol molar ratio, time, amount of the catalyst, and temperature, was investigated for the chemical synthesis of biodiesel.

2 Materials and methods

In the current study, various physicochemical properties of raw oils and their methyl esters have been analyzed to investigate their suitability as biodiesel fuel. The initial free fatty acid (FFA) contents of raw oil were 13.11%, which were reduced by acid pretreatment. Afterward, alkaline transesterification of purified acid-pretreated seed oil was carried out. Moreover, the oil-to-methanol molar ratio, amount of KOH, temperature, and time were optimized for alkaline transesterification to achieve a high biodiesel yield.

2.1 Sample collection

J. curcas seeds, which were primarily produced in District Swabi, were supplied by a local supplier from Lahore, Pakistan. An amount of 10 kg of seeds were supplied for oil extraction, and 3.2 L of oil was obtained using a mechanical oil expeller unit in Rawalpindi, Pakistan. Seeds were sun-dried and subjected to mechanical oil extraction using an oil expeller. Oil was filtered to remove solid impurities. The crude oil was then heated at 105°C for 2–3 h to remove moisture and stored in the dark for further use. The oil yield was calculated using the following formula. Figure 1 shows the expeller unit used for the mechanical extraction of *Jatropha* oil.

$$\text{Oil yield (\%)} = \left(\frac{\text{Oil extracted in litres}}{\text{Total weight of seeds in kg}} \right) \times 100.$$

2.2 Microbial biosynthesis of biodiesel

As the lipase enzyme, in the presence of alcohol as a reaction medium, carries out transesterification of fatty oils, the previously isolated lipase-producing *Bacillus subtilis* Q5 strain, which was shown to be tolerant to a high methanol concentration and toxic *J. curcas* oil (Rana et al., 2019), was used for the production of biodiesel from seed oil using the whole-cell method described by Rana et al. (2019). Microbial cell mass was generated by the growing strain Q5 in Luria broth (LB) for 24 h. Cells were then pelleted by centrifugation. Approximately 1 ml of pelleted cells were then added into the reactor containing *J. curcas* oil and mobilized within the oil layer to avoid direct methanol exposure. Methanol was then added dropwise along with 300 µl of n-hexane as an emulsifier. The mouths of the flasks were closed tightly with rubber corks in order to restrict any evaporation of methanol from the reaction mixture. After 48 h,



TABLE 1 Percentage volumetric yield of biodiesel in response to conditions specified by the Plackett–Burman design for each run.

Run	Factor 1 A: molar ratio	Factor 2 B: agitation RPM	Factor 3 C: inoculum size ml (%)	Factor 4 D: temperature (celsius)	Factor 5 E: n-hexane %	Percentage volumetric yield of biodiesel (%)
1	3.00	300.00	10	37.00	6.00	0
2	3.00	150.00	10	37.00	6.00	0
3	9.00	150.00	30	37.00	6.00	80
4	3.00	150.00	30	55.00	10.00	0
5	9.00	300.00	30	37.00	10.00	80
6	9.00	150.00	30	55.00	6.00	80
7	6.00	225.00	20	46.00	8.00	70
8	6.00	225.00	20	46.00	8.00	70
9	9.00	300.00	10	55.00	6.00	80
10	9.00	300.00	10	55.00	10.00	84
11	9.00	150.00	10	37.00	10.00	98
12	6.00	225.00	20	46.00	8.00	70
13	3.00	300.00	30	37.00	10.00	75
14	3.00	150.00	10	55.00	10.00	0
15	3.00	300.00	30	55.00	6.00	0

the flask’s mixture was poured into a separating funnel and kept overnight to separate different layers. The upper yellowish methyl ester layer (biodiesel layers) was collected in screw cap glass vials and

stored. The produced biodiesel was then confirmed by both Fourier transform infrared spectroscopy (FTIR) and gas chromatography coupled with mass spectrometry (GCMS) analysis.

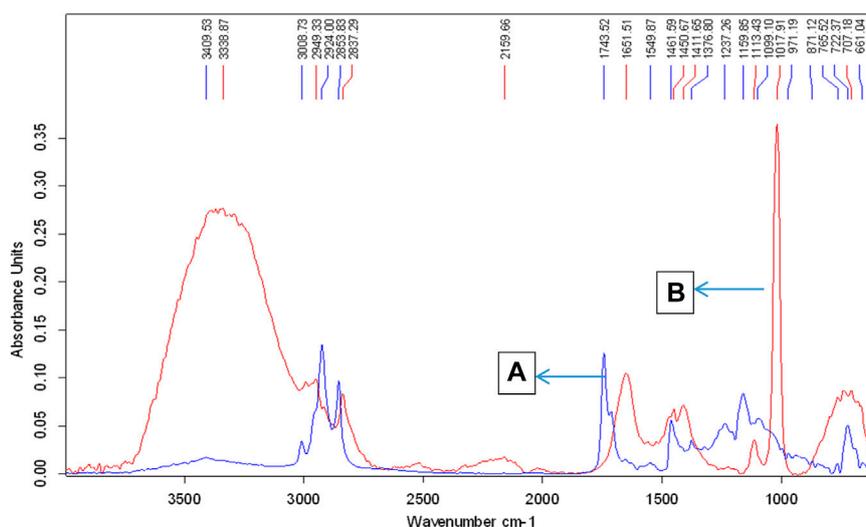


FIGURE 2

ATR-FTIR spectra of crude jatropha oil and FAMES of jatropha oil produced by microbial biological synthesis. Peaks denoted by letters (A,B) represent jatropha oil and biodiesel, respectively.

TABLE 2 FAMES composition of biodiesel biosynthesized by *Bacillus subtilis* Q5 from *J. curcas* seed oil identified by GC-MS.

Systemic name	Common name	Molecular formula
Acetic acid methyl ester	Methyl ethanoate	C ₃ H ₆ O ₂
Butyric acid methyl ester	Methyl butanoate	C ₅ H ₁₀ O ₂
Capric acid methyl ester	Methyl decanoate	C ₁₁ H ₂₂ O ₂
Butanoic acid methyl ester	Methyl butyrate	C ₅ H ₁₀ O ₂
Decanoic acid methyl ester	Methyl decanoate	C ₁₁ H ₂₂ O ₂
Dodecanoic acid methyl ester	Methyl dodecanoate	C ₁₃ H ₂₆ O ₂

2.3 Optimization of parameters for microbial biosynthesis of biodiesel

Biodiesel production via the Q5 strain was statistically optimized for *Jatropha* oil using five factors, namely, temperature, agitation (rpm), inoculum size, oil: methanol ratio, and n-hexane percentage with respect to oil, which were optimized using the Plackett–Burman design in Stat-Ease Design-Expert software version 7.0. Experiments were performed according to conditions given in Table 1, and a total of 15 runs by the Plackett–Burman design and response (percentage volumetric yield of biodiesel) were recorded.

2.4 Two-step transesterification of *J. curcas* seed oil

The crude jatropha oil was brown. The amount of FFA in JO (Table 3) was determined, as previously described (Ghadge and Rahman, 2006). *J. curcas* seed oil had an initial acid number of 26 ± 0.02 mg KOH/gram seed oil corresponding to the 13.11%

FFA level, which is exceedingly above the acceptable limit (1%–2%) for the alkaline transesterification reaction. The main goal was to complete the conversion of FFA and triacylglycerides (TAG) into biodiesel using a two-step process. Therefore, FFA was converted to esters by esterification, as discussed in the following paragraphs.

2.5 Acid-catalyzed esterification of *J. curcas* seed oil

Esterification was used to convert seed oil FFA to esters using sulfuric acid with methanol, according to Veljković et al. (2006), with little modifications. In the esterification step, JO and methanol were used in a 1:12 molar ratio. Initially, 100 mL JO was added to three-necked round bottom flasks (250 mL) equipped with a reflux condenser, thermometer, and rubber corks, and heated up to 60°C. Absolute methanol and sulfuric acid (2% in accordance with the final volume of seed oil) were premixed together, heated, and then added to the seed oil in the flasks and continuously mixed at 600 revolutions per minute (rpm) to homogenize the mixture. The acid-catalyzed esterification (ACE) batch reaction was maintained at 60°C for 2 h on a heating mantle. After the completion of the ACE process, the mixture was completely separated into different layers using a separating funnel. After the separation of the oil layer, the sulfuric acid catalyst was washed out and removed by neutralization with calcium oxide (CaO). Approximately 20 mL of the hot 1% CaO solution was added to the pretreated oil mixture. The mixture was centrifuged at 1,744 g for 10 min, resulting in CaSO₄ formation that was precipitated out and settled down. This washing was repeated twice to properly remove the acid catalyst. The supernatant containing catalyst-free pretreated oil was decanted and centrifuged again at the aforementioned conditions to separate water. The product obtained after ACE was again dried in an oven for 2–3 h and

TABLE 3 Fuel properties of *J. curcas* seed oil and biodiesel (ASTM D6751–20a 2020; ASTM D975–20c 2020; Ong et al., 2011; Patil and Deng, 2009).

Property	Unit	<i>J. curcas</i> oil	Biodiesel ^c	Biodiesel ^b	Biodiesel standard ASTM D6751–20a
Acid number ^a	mg KOH/g	26 ± 1.7	0.45 ± 0.1	0.24 ± 0.1	0.5 max
FFA ^a	%	13.11 ± 0.9	N.D	N.D	—
Saponification number ^a	mg/g	213 ± 5.1	N.D	N.D	—
Ester value ^a	mg/g	186.6 ± 3.7	N.D	N.D	—
Glycerin ^a	%	10.1	N.D	N.D	0.240 max
Cloud point ^a	°C	5 ± 1.0	3.8 ± 0.4	3 ± 0.1	–3 to 12
Pour point ^a	°C	4 ± 0.2	2 ± 0.1	2.3 ± 0.1	–15 to 10
Peroxide value ^a	Meq O ₂ /kg	1.91 ± 0.01	1.43 ± 0.1	1.41 ± 0.1	—
Boiling point ^a	°C	390 ± 1.5	261 ± 1.0	—	>201.85
Specific gravity ^a	kg/m ³	0.916 ± 0.1	0.88 ± 0.02	0.87 ± 0.1	0.87–0.90
Density	g/cm ³	0.917 ± 1.5	0.866 ± 0.1	0.834 ± 0.02	0.860–0.900
Odor	—	Agreeable	Agreeable	Agreeable	—
Color	—	Brown	Light brown	Light brown	—
Percentage yield	%	32.5	98	98	—

^aValues presented as mean ± SD.

^bBiodiesel produced by a biosynthetic approach; N.D, not determined.

^cBiodiesel produced by a chemical approach.

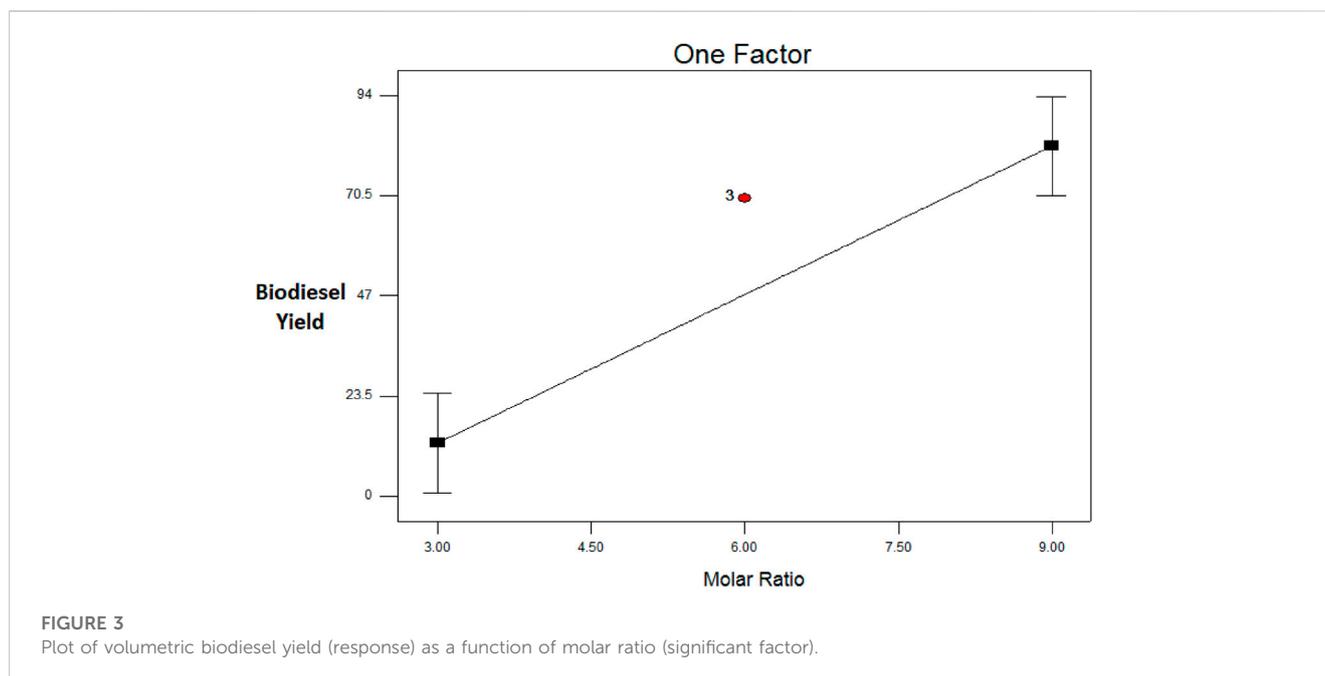


FIGURE 3

Plot of volumetric biodiesel yield (response) as a function of molar ratio (significant factor).

cooled down at 25°C. Finally, the pretreated JO was treated with anhydrous sodium sulfate to absorb any remaining moisture completely. The acid number of acid pretreated oil was determined and found to be 2.4 ± 0.01 mg KOH per gram of pretreated oil that was corresponding to an FFA level of 1.2%, which is within the acceptable limits for alkaline transesterification. This product was further used in an alkaline transesterification reaction.

2.6 Alkaline transesterification of *J. curcas* seed oil

Alkaline transesterification of acid-pretreated JO was carried out, as described by Berchmans and Hirata (2008) and Veljković et al. (2006), with slight modifications. KOH, 2% w/w (2% in accordance with a final volume of seed oil), was dissolved in methanol (1:6 oil-to-methanol molar ratio) and added to the

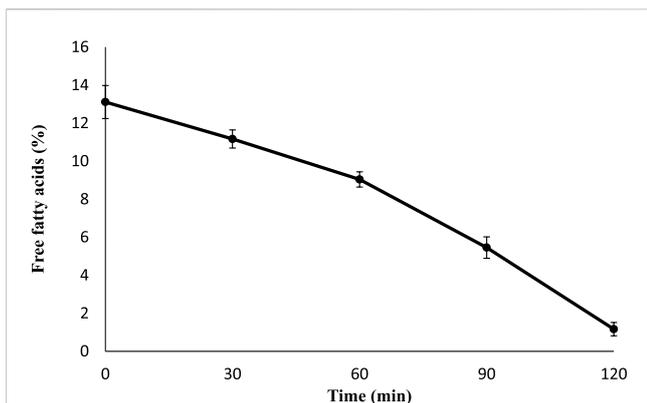


FIGURE 4
 Acid-catalyzed pretreatment of *J. curcas* seed oil FFA with concentrated sulfuric acid. The data are presented as mean ± SD. The acid pretreatment was carried out in triplicate independently, and the conditions were 2% sulfuric acid, 1:12 oil-to-methanol molar ratio, 60°C of temperature, time of 120 min, and agitation at 600 rpm. The FFA percentage was investigated after each 30 mins. The free fatty acid reduction was statistically analyzed using one-way ANOVA, and multiple comparisons were carried out by Tukey's post-test (significance: $p < 0.05$).

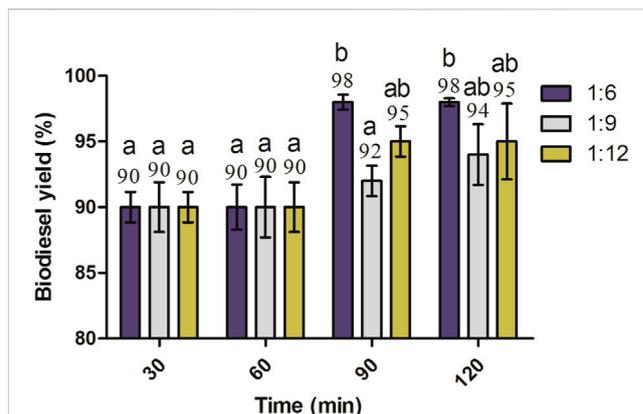


FIGURE 6
 Effect of molar ratios and reaction time on the biodiesel yield of *J. curcas* seed oil maintained at 1% KOH, 600 rpm, and 60°C. All the setups were carried out as independent experiments in triplicate. The data are presented as mean ± SD. One-way ANOVA and multiple comparisons were carried out by Tukey's post-test. The alphabets on the bars show significant and non-significant differences. Similar alphabets on different bars show non-significant differences, whereas different alphabets show a significant difference. The alphabets "ab" on bars represent a non-significant difference between bars annotated by "a" and "b" (significance: $p < 0.05$).

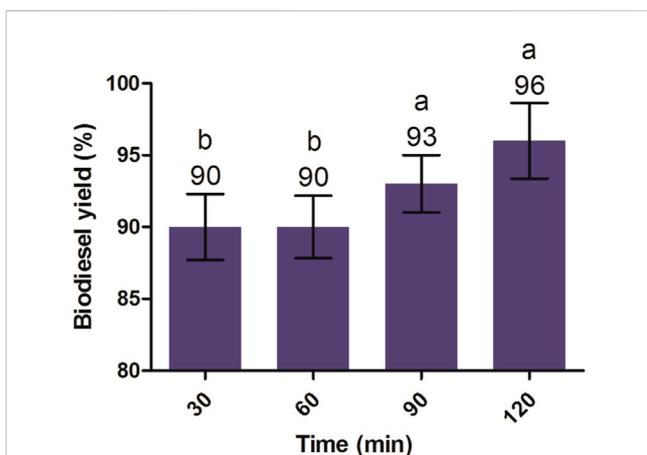


FIGURE 5
 Alkaline transesterification of *J. curcas* seed oil was carried out at a 1:6 oil-to-methanol molar ratio, 2% KOH (alkaline catalyst), 600 rpm, and 60°C temperature. All of the setups were carried out as independent experiments in triplicates. The data are presented as mean ± SD. One-way ANOVA and multiple comparisons were carried out by Tukey's post-test. The lowercase alphabets on adjacent bars show significant and non-significant differences. Similar alphabets on adjacent bars show non-significant differences, whereas different alphabets show a significant difference (significance: $p < 0.05$).

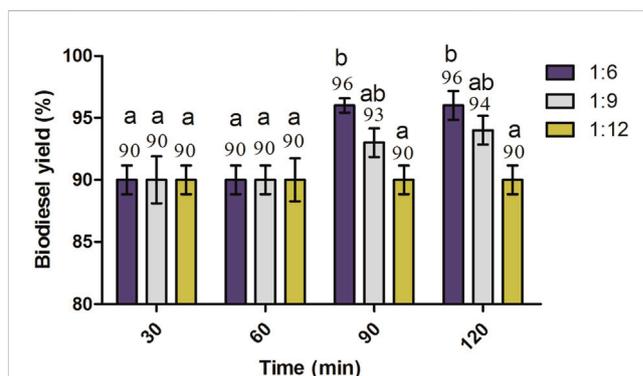


FIGURE 7
 Effect of molar ratios and reaction time on the biodiesel yield of *J. curcas* seed oil maintained at 1.5% KOH, 600 rpm, and 60°C. All of the setups were carried out as independent experiments in triplicate. The data are presented as mean ± SD. One-way ANOVA and multiple comparisons were carried out by Tukey's post-test. The alphabets on the bars show significant and non-significant differences. Similar lowercase alphabets on different bars show non-significant differences, while different alphabets show a significant difference between them. The alphabets "ab" on bars represent a non-significant difference between bars annotated by "a" and "b" (significance: $p < 0.05$).

same reactor, as described previously. The mixture was heated to 60°C and stirred at 600 rpm for 2 h; afterward, it was completely separated into different layers using a separating funnel.

After the completion of the reaction, the product (biodiesel) was poured into the round bottom flask of the distillation apparatus, and its temperature was adjusted to 65°C. The distillation apparatus was stopped when methanol collection in

the flask on the other side of the distillation tube was stopped. KOH was removed through neutralization and washing with acidified distilled water. For this purpose, 100 mL of the 1% dilute sulfuric acid hot solution was then added to the transesterified mixture and centrifuged at the aforementioned conditions; the resultant product (potassium sulfate) was precipitated out, settled down, and removed from the mixture.

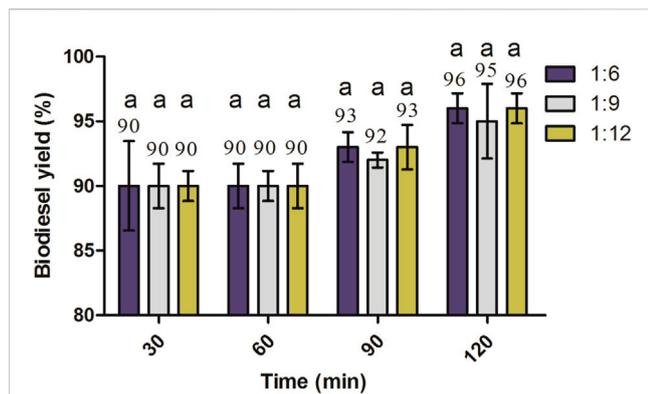


FIGURE 8

Effects of oil-to-methanol molar ratios and reaction time on the biodiesel yield of *J. curcas* seed oil maintained at 2% KOH, 600 rpm, and 60°C. All of the setups were carried out as independent experiments in triplicate. The data are presented as mean \pm SD. One-way ANOVA and multiple comparisons were carried out by Tukey's post-test. The alphabets on bars show significant and non-significant differences. Similar alphabets on different bars show non-significant differences, whereas different alphabets show a significant difference (significance: $p < 0.05$).

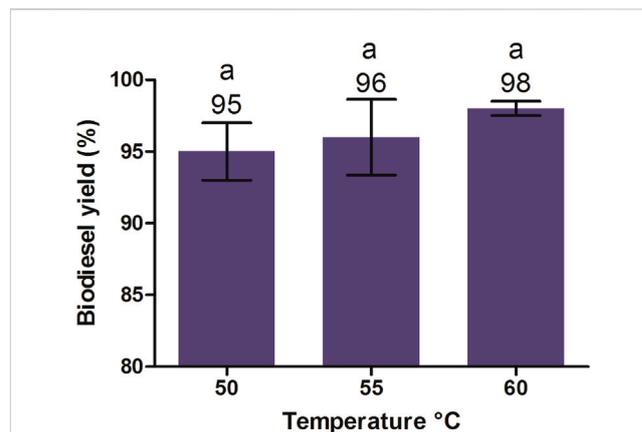


FIGURE 10

Effect of different temperatures on the biodiesel yield of *J. curcas* seed oil maintained at the 1:6 oil-to-methanol ratio, 1% KOH, 600 rpm, and a reaction time of 90 min. All of the setups were carried out as independent experiments in triplicate. The data are presented as mean \pm SD. One-way ANOVA and multiple comparisons were carried out by Tukey's post-test. The alphabets on the bars show significant and non-significant differences. Similar alphabets on different bars show non-significant differences, whereas different alphabets show a significant difference (significance: $p < 0.05$).

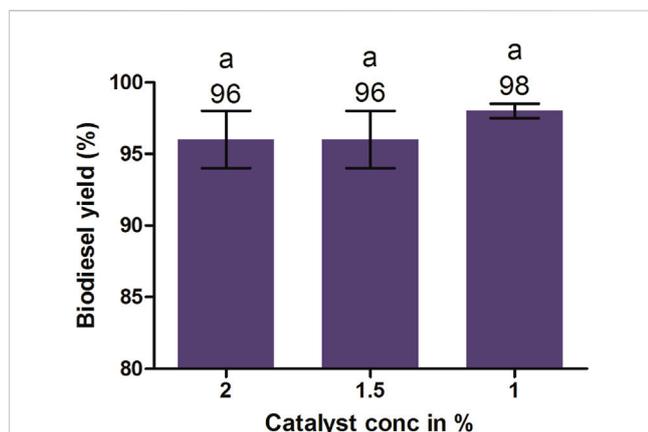


FIGURE 9

Effects of catalyst concentrations on biodiesel yield maintained at 1:6 molar ratios, 600 rpm, 60°C, and 90 min. All of the setups were carried out as independent experiments in triplicate. The data are presented as mean \pm SD. One-way ANOVA and multiple comparisons were carried out by Tukey's post-test. The alphabets on the bars show significant and non-significant differences. Similar alphabets on different bars show non-significant differences, whereas different alphabets show a significant difference (significance: $p < 0.05$).

This step was performed twice to remove any remaining catalyst. The catalyst-free product was again centrifuged at the aforementioned conditions to separate water from the final product. The water-free product was passed through anhydrous Na_2SO_4 on a Whatman filter paper to remove any remaining moisture. The volume of fatty acid methyl esters (FAMES) was noted. The FAME yield was calculated using the following equation:

$$\text{FAME yield (\%)} = \left(\frac{\text{Weight of FAME in grams}}{\text{Total weight of oil in grams}} \right) \times 100.$$

2.7 Optimization of alkaline transesterification

Parameters affecting the alkali-catalyzed transesterification reaction were optimized using the method of Patil and Deng (2009) with few modifications. An alkali-catalyzed transesterification reaction setup was used to determine the optimum oil-to-methanol molar ratio, amount of catalyst, and time at 600 rpm and 60°C. For batch setup, 25 mL of preheated JO was used in each batch reaction. The catalyst, along with methanol, was preheated at a desired temperature, added to the oil in a batch setup, and stirred at 600 rpm for 2 h. The reactions were conducted at different molar ratios of oil to methanol (1:6, 1:9, and 1:12), amount of the catalyst (1%, 1.5%, and 2%), and reaction times (30, 60, 90, and 120 min). After confirming the optimum parameters, the effect of different temperatures (50°C, 55°C, and 60°C) on biodiesel yield was evaluated at the optimized molar ratio, amount of KOH, and reaction time.

2.8 Physicochemical analyses of oil and biodiesel

Fuel properties of oil and biodiesel samples, obtained from chemical transesterification reactions, were carried out using standard procedures according to Mehta et al. (2015) and Onukwuli et al. (2017), with some modifications, as discussed in the following paragraphs.

2.8.1 Acid number and free fatty acid content

The acid number (AN) of JO, acid pretreated oil, and biodiesel was determined by adding 5 g of a sample in a flask and mixing it with 25 mL of absolute ethanol and 2–3 drops of phenolphthalein.

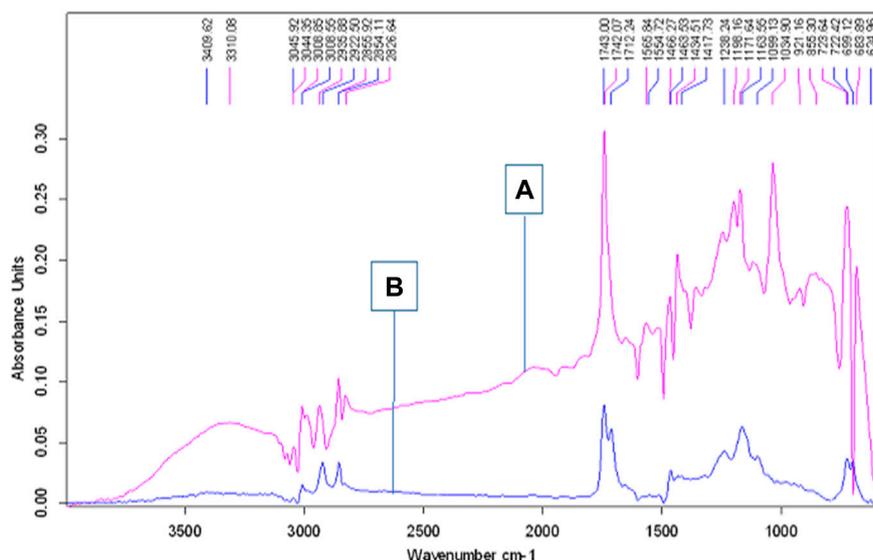


FIGURE 11

FTIR spectra of crude *J. curcas* oil and its alkaline catalyzed FAMES; the upper peaks denoted by the letter “(A)” show biodiesel, and the lower peaks labeled with the letter “(B)” represent JO spectra.

TABLE 4 Fatty acid composition of *J. curcas* seed oil identified by GC-MS.

Systemic name	Common name	Molecular formula	MW	RT	Area %	Height %
Octanoic acid	Caprylic acid	C ₈ H ₁₆ O ₂	144	8.90	0.24	0.77
Nonanoic acid	Pelargonic acid	C ₉ H ₁₈ O ₂	158	8.90	0.24	0.77
n-Decanoic acid	Capric acid	C ₁₀ H ₂₀ O ₂	172	11.95	0.25	1.23
Dodecanoic acid	Lauric acid	C ₁₂ H ₂₄ O ₂	200	14.63	1.59	5.23
Undecanoic acid	Undecylic acid	C ₁₁ H ₂₂ O ₂	186	14.63	1.59	5.23
Tridecanoic acid	Tridecylic acid	C ₁₃ H ₂₆ O ₂	214	14.63	1.59	5.23
Tetradecanoic acid	Myristic acid	C ₁₄ H ₂₈ O ₂	228	16.95	3.54	9.51
n-Hexadecanoic acid	Palmitic acid	C ₁₆ H ₃₂ O ₂	256	19.08	6.62	10.35
Pentadecanoic acid	Pentadecylic acid	C ₁₅ H ₃₀ O ₂	242	19.08	6.62	10.35
Eicosanoic acid	Arachidic acid	C ₂₀ H ₄₀ O ₂	312	19.08	6.62	10.35
Octadecanoic acid	Stearic acid	C ₁₈ H ₃₆ O ₂	284	21.15	51.60	24.39
11-Eicosenoic acid	Gondoic acid	C ₂₁ H ₄₀ O ₂	324	22.67	17.08	19.45
13-Docosenoic acid	Erucic acid	C ₂₂ H ₄₂ O ₂	338	24.33	12.58	19.46

MW, molecular weight; RT, retention time (min).

They were heated at 65°C in a water bath for 10 min under continuous shaking, allowed to cool, and titrated against 0.1 normal KOH solution until a permanent pink color appeared. The amount of KOH solution needed for the reaction was calculated. The acid number (AN) was determined using the following formula.

$$AN = \left(\frac{\text{mL of KOH} \times \text{Normality of KOH} \times 56.1}{\text{Weight of sample}} \right) = \text{mg of } \frac{\text{KOH}}{\text{g}}$$

where 56.1 represents the molecular weight of KOH (g/mol).

The FFA contents of JO and acid-pretreated oil were determined by the following formula. The percentage of FFA in a sample is always approximately half of the acid number.

$$\% \text{ FFA} = AN \times (0.503).$$

2.8.2 Saponification number

The saponification number was determined by adding 2 g of JO in a 100-mL Erlenmeyer flask, followed by the addition of 25 mL of

TABLE 5 FAME composition of biodiesel produced from *J. curcas* seed oil identified by GC-MS.

Systemic name	Common name	Molecular formula	MW	RT	Area %	Height %
Dodecanoic acid methyl ester	Methyl laurate	C ₁₃ H ₂₆ O ₂	214	11.87	0.56	1.02
Methyl tetradecanoate	Methyl myristate	C ₁₅ H ₃₀ O ₂	242	14.35	2.01	4.08
Palmitic acid methyl ester	Methyl palmitate	C ₁₇ H ₃₄ O ₂	270	16.15	4.48	4.25
Octadecenoic acid methyl ester	Methyl linoleate	C ₁₉ H ₃₄ O ₂	294	16.33	0.04	0.15
Palmitic acid methyl ester	Methyl palmitate	C ₁₇ H ₃₄ O ₂	270	16.55	1.40	1.39
Methyl octadecanoate	Methyl stearate	C ₁₉ H ₃₈ O ₂	298	18.37	4.71	15.04
11-Eicosenoic acid methyl ester	Stearyl acrylate	C ₂₁ H ₄₀ O ₂	324	20.31	16.90	10.43
Arachidic acid methyl ester	Methyl icosanoate	C ₂₁ H ₄₂ O ₂	326	20.54	7.04	6.96

MW, molecular weight; RT, retention time (min).

0.5 normal alcoholic KOH solution. The same procedure was carried out for B (blank) without the addition of an oil sample. The flask was connected to the reflux condenser and placed in a water bath, and saponification was completed by gently and steadily boiling for an hour with periodic shaking. After 1 h, a few drops of phenolphthalein were added to it, and the solution was titrated against 0.5 normal hydrochloric acid until the disappearance of the pink color (endpoint). The saponification number of JO was determined using the following formula:

$$\text{SP Number} = \left(\frac{56.1 (H^{\circ} - S^{\circ}) \times \text{Normality of hydrochloric acids}}{\text{Weight of sample}} \right),$$

where “H” is the number of hydrochloric acid concentrations used for the blank in mL, “S” is the amount of hydrochloric acid used for the sample in mL, and N represents the normality.

2.8.3 Ester value and percentage of glycerin

Ester value is the number of milligrams of KOH needed to saponify the esters present in 1 g of substance and is determined by subtracting an acid number from the saponification number.

$$\text{Ester value (EV)} = (\text{Saponification number (SN)} - \text{Acid number (AN)}).$$

During the saponification reaction, the triacylglycerol is hydrolyzed in the presence of KOH to produce potassium salts of soap and glycerin. The percentage of glycerin can be obtained using the following formula.

$$\% \text{ Glycerine} = (\text{Ester value} \times 0.054664).$$

2.8.4 Peroxide value

It was determined as described by the Association of Official Analytical Chemists' (AOAC) method (Society, 1988). *J. curcas* seed oil/biodiesel (5 g) was added to the flask, and 30 mL of the acetic acid and chloroform mixture (at a molar ratio of 3:2) was added to it with swirling to dissolve oil in it. A measure of 0.5 mL of the potassium iodide solution (saturated) was added to the mixture and swirled for 1 min in the dark until its color turned into a light brown color. Afterward, 30 mL of distilled water was added to it and titrated against 0.1 normal Na₂S₂O₃ until the solution turned pale yellow. Then, 1 mL of the 1% starch indicator was added and further titrated against 0.1 N Na₂S₂O₃ until the disappearance of the blue color. For

the blank, the same procedure was used without adding a sample. The peroxide value is expressed as the milli equivalent of peroxide oxygen per kg of the sample, as given as follows:

$$\text{Peroxide value} = \left(\frac{(S^{\circ} - B^{\circ}) \times N \times 100}{\text{Weight of sample (g)}} \right),$$

where S° represents the test sample titrated, B° represents the blank sample titrated, and N represents the normality of Na₂S₂O₃.

2.8.5 Specific gravity and density

A 25-mL dried, clean bottle was weighed either empty or with water. The oil and biodiesel samples were added separately in it and weighed. All the steps were carried out at a similar temperature (30°C). Specific gravity (kg/m³) was calculated using the following equation:

$$\text{Specific gravity} = \left(\frac{A^{\circ} - B^{\circ}}{C^{\circ} - B^{\circ}} \right).$$

where A° represents the weight (in grams) of the bottle along with oil, B° represents the weight (in grams) of the empty bottle, and C° represents the weight (in grams) of the bottle with water. The density was measured using a pycnometer.

2.8.6 Boiling point determination

A measure of 10 mL of the biodiesel sample was taken in a test tube, and a thermometer was inserted into it. After that, the temperature of the magnetic plate gradually increased. The point at which biodiesel started to bubble was recorded as the boiling point.

2.8.7 Cloud point and pour point

Approximately 30 mL of oil and biodiesel samples were poured into separate jars and placed in the cooling jacket in a water bath. A thermometer was fixed in each jar, and the jars were observed at different intervals without disturbing the liquids. The temperature at which clouds of crystals appeared was recorded as the cloud point. For the pour point, approximately 20 mL of each sample was placed in a beaker, sealed with corks, and incubated in the refrigerator to solidify. After solidification, the samples were kept in the open, and the temperature at which each sample melted and started flowing was recorded as the pour point.

2.9 Chemical characterization of *J. curcas* seed oil and biodiesel

J. curcas seed oil and biodiesel produced by the chemical transesterification method were analyzed using both FTIR and GCMS.

2.10 Chemical analysis

FTIR spectroscopy (Bruker, model Tensor 27, with software version Opus 65, and equipped with Zn-Se ATR) was performed. A measure of 5 μL of seed oil or its FAMES was loaded at the sample injector, and FTIR spectra for all samples ranged between 4,000 and 400 cm^{-1} .

Gas chromatography and mass spectrophotometry (GC-MS; model GCMS-QP5050A; Shimadzu Europe) were performed with a thermal conductivity detector (TCD) for the analysis of both oil and biodiesel samples with the following conditions: a measure of 2 μL of the sample was injected into the GC column using the split mode. Separation was performed on a capillary column DB-5 (column length: 30 m, internal diameter 0.25 mm, and thickness 0.25 μm). The initial temperature was 50°C, ramped at 5°C/min to 80°C, 10°C–300°C, and kept at 300°C for 18 min. The injector was set at 250°C with a column flow rate of 1.8 mL/min for seed oil and 1 mL/min for biodiesel. Helium gas was used as a mobile phase with a flow rate of 1 mL/min, and a split ratio for oil was 1/25 and for biodiesel was 1/48, while TCD was set at 300°C. The *m/z* scanning of MS was in the range of 50–650, with electron impact (EI) at 70 eV.

2.11 Statistical analyses

Each batch was conducted in triplicate, and the FAMES obtained from each setup were analyzed individually in triplicates to calculate the average values of experimental data. One-way ANOVA and multiple comparisons were carried out by Tukey's post-test (significance: $p < 0.05$).

3 Results and discussions

The mechanical extraction resulted in 32.5% crude oil, which is near the previously reported extraction yields (de Oliveira et al., 2009).

3.1 Microbial biosynthesis of biodiesel

FAMES produced using the *Bacillus subtilis* strain Q5 were analyzed by FTIR and GCMS. Biodiesel is a mono-alkyl ester of long-chain fatty acids; thus, its IR spectrum should contain peaks of ester groups, which are observed at the frequency (cm^{-1}) range of 1,735–1,750. The infrared spectra of the biodiesel produced showed peaks at 1,742.07, 1,198.16, and 1,034.90 cm^{-1} that were only present in the biodiesel, while crude *Jatropha* oil has no such bands in this region, which showed the presence of esters in the

sample, thus confirming the production of biodiesel (Figure 2). GCMS chromatogram data (Table 2; Supplementary Figure S2) revealed that the total fatty acid methyl ester content of the produced biodiesel was >98%, which is in accordance with the biodiesel quality standards specified by ASTM D6751–20a (Table 3).

3.2 Optimization of parameters for microbial biosynthesis of biodiesel using the Plackett–Burman design

The Plackett–Burman design was used for statistical optimization of biodiesel production using the strain Q5 and selecting five factors, namely, temperature, agitation (rpm), inoculum size, oil: methanol ratio, and n-hexane percentage with respect to oil, which were optimized. The highest volumetric yield of biodiesel 98% was obtained with run 11 with an oil: methanol ratio of 1:9, agitation speed of 150 RPM, inoculum size 10%, temperature 37°C, and n-hexane 10%. ANOVA analysis of the design gave an F-value of 20.34, which depicted that the experimental model generated by the design was significant. The F-test on each factor was also applied by the software application to identify the significant factors. Values of Prob > F less than 0.0500 indicated that factors are significant. In this case, the molar ratio was a significant factor with a Prob > F value of 0.0001. The molar ratio affected the response, i.e., the percentage volumetric yield of biodiesel, positively, which means increasing the molar ratio increases the yield (Figure 3). The results are in alignment with those reported by Rana et al. (2019) and Rana et al. (2022).

3.3 Acid pretreatment and alkaline transesterification of *J. curcas* seed oil

The JO had a high FFA content of 13.11% (Table 3), which is higher than the acceptable value for alkaline transesterification. The alkaline transesterification of high FFA-containing JO is considered complicated due to higher soap formation, loss of catalyst, and loss in product yield. The higher FFA content causes saponification of oil and requires the extra addition of an alkali catalyst for transesterification (Canakci and Van Gerpen, 2001). So in order to decrease the FFA before alkaline transesterification, acid pretreatment was used to reduce the FFA acids present in JO. The FFA was significantly reduced ($p < 0.05$) from 13.11% to 1.2% after 120 min of pretreatment (Figure 4).

After acid pretreatment, the purified product was subjected to alkaline transesterification and processed at 2% KOH (w/v), an oil-to-methanol molar ratio of 1:6, time of 2 h, 60°C temperature, and agitation at 600 rpm. The resultant amount of methyl esters of JO was determined at different reaction times, but the highest biodiesel yield (96%) was obtained after 2 h of reaction with no significant increase compared to the methyl esters obtained at 30, 60, and 90 min (Figure 5). Meher et al. (2006) also reported similar findings, in which the karanja produced the highest biodiesel yield of 97% at an oil-to-methanol molar ratio of 1:6 using the KOH catalyst in alkaline transesterification.

3.4 Optimization of alkaline transesterification of *J. curcas* seed oil

The alkaline transesterification process depends upon certain factors such as the oil-to-methanol molar ratio, amount of the catalyst, temperature, and time. Therefore, in order to enhance the biodiesel yield, different oil-to-methanol molar ratios, amount of KOH, and duration of time were investigated at 60°C, as mentioned previously. Stoichiometrically, 1 mol of oil requires 3 mol of alcohol for transesterification reaction completion. However, the 1:3 ratio of oil to methanol results in the formation of a unique and opaque layer due to the presence of unreacted triglycerides. Second, as transesterification is a reversible reaction, an excess of alcohol is required to proceed with the reaction in a forward direction (Leung and Guo, 2006). In practice, a higher amount of methanol is needed for higher conversion efficiency of oil (to ensure that the reaction completes). The conversion efficiency is defined as the increased yield of biodiesel in terms of percentages. Therefore, the biodiesel optimization setup was started at a molar ratio of 1:6 (oil: methanol) (Freedman et al., 1984; Fukuda et al., 2001). The optimum catalyst concentration and oil-to-methanol molar ratio are highly necessary for a complete and efficient transesterification process, and a slight variation in these parameters can result either in an incomplete reaction or may produce unnecessary products, leading to a lower biodiesel yield. Moreover, increasing the oil-to-methanol molar ratio may further complicate the purification due to more dissolution of glycerol in biodiesel, which ultimately decreases its yield (Ali et al., 2015). The highest biodiesel yield was obtained at 1% KOH, a molar ratio of 1:6 of oil to methanol, at 90 as well as 120 min reaction time, and incubated at 60°C (Figure 4). The catalyst is the most important factor in biodiesel production. It provides the available active site for the biodiesel reaction to occur. No further increase in biodiesel yield occurred when the catalyst amount was increased beyond 1%. This may be due to the possibility of some of the products' solubility or absorption onto the excess amount of the catalyst surface (Kamel et al., 2018). Initially, there was no significant difference in biodiesel yields obtained at 30 and 60 min. However, at 90 and 120 min, the 1:6 molar (oil-to-methanol) ratio produced significantly higher ($p < 0.05$) yields than those obtained at 30 and 60 min. The biodiesel yield recorded for a 1:6 molar ratio was significantly higher ($p < 0.05$) than 1:9 at 90 min and 60°C (Figure 6).

A similar pattern was also observed for alkaline transesterification at 1.5% KOH, and the biodiesel yield of a ratio of 1:6 was higher than that of 1:9 and 1:12 at 90 min and 120 min reaction times incubated at 60°C (Figure 7). Here, at 1.5% KOH, the biodiesel yield of a 1:6 molar ratio was significantly higher ($p < 0.05$) than that of 1:12 at 90 min and 120 min reaction times.

Moreover, at 2% KOH, the same biodiesel yield was obtained at 1:6 and 1:12 molar ratios of oil to methanol (Figure 8), and the yield was found to be slightly higher compared to the 1:9 molar ratio, with no significant increase. The methyl ester yield for all molar ratios incubated at 60°C and 2% KOH was found higher at 120 min compared to other reaction times, with no significant increase ($p > 0.05$).

It is evident from the aforementioned results that the highest biodiesel yield was achieved at 1% KOH, an oil-to-methanol molar ratio of 1:6, and reaction time 90 min. Furthermore, it was also noted

that an increase or decrease in the amount of KOH from 1% resulted in a lower biodiesel yield (Figure 9). The lower concentration than the optimum limit causes an incomplete transesterification reaction, ultimately producing lower methyl esters. Therefore, the catalyst concentration must be enough to complete the reaction. The excess amount of catalyst beyond its optimum limit reacts with triglycerides and free fatty acid-producing emulsion, which leads to the formation of a viscous gel or emulsion. It also makes the glycerol layer separation difficult, ultimately resulting in the loss of ester yields (Dorado et al., 2004). Similar findings were reported by Berchmans et al. (2010) and Rashid et al. (2008), achieving a biodiesel yield of 97% and 96% from the transesterification of JO and rapeseed oil, respectively, with 1% KOH catalyst.

From the cost and processing time perspectives, the ideal and the highest biodiesel yield was obtained at a molar ratio of 1:6 at 1% KOH catalyst, temperature 60°C, and 90 min of reaction time, with an optimum biodiesel yield of 98%. For standard biodiesel, the permissible level of glycerol is 0.24% in the final product, and to achieve this standard, the biodiesel yield must be $\geq 97.7\%$ (Van Gerpen et al., 2004). The current study assumed the optimal molar ratio of oil to methanol as 1:6 in order to obtain a higher ester yield (Figure 7). No significant change in the biodiesel yield was observed when the oil-to-methanol molar ratio higher than 1:6 was used. The results showed similarities with the reports of Meher et al. (2013) and Usta (2005), where the highest yield of ester from methanolysis of *P. pinnata* and tobacco seed oil at the 6:1 alcohol-to-oil molar ratio, respectively, was reported. Similarly, 96% yield was obtained from the transesterification of rapeseed oil using a molar ratio of methanol to oil of 6:1 and 1% KOH as a catalyst (Rashid et al., 2008).

Similarly, the effect of different temperatures (50°C, 55°C, and 60°C) on biodiesel yield was evaluated at the aforementioned optimized conditions (1:6 molar ratio, reaction time 90 min, and 1% KOH). The reaction temperature above the boiling point of methanol was ignored because at higher temperatures, the saponification rate for glycerides of oil remains faster than that of transesterification. In addition, temperatures above the boiling point of methanol cause excessive evaporation and loss of methanol from the reaction mixture, which leads to an incomplete reaction and loss of ester formation. As shown in Figure 10, the highest yield for biodiesel was achieved at 60°C, followed by 55°C and 50°C, with no significant difference. The increase in temperature until the optimum limit (60°C) increased the conversion efficiency of oil into its methyl esters and any decrease from the optimum temperature caused a decrease in the methyl ester yield. A similar effect was observed by Chitra et al. (2005) and Rashid et al. (2008) during the transesterification of JO and rapeseed oil, respectively.

3.5 Physicochemical properties of oil- and chemical-mediated and biosynthesized biodiesel

The quality of seed oil and its methyl esters is expressed in terms of physicochemical properties such as the acid number, saponification number, peroxide value, specific gravity, pour point, and cloud point that were analyzed in the present study (Table 3). The least temperature at which biofuels can flow is called

the pour point whereas the temperature at which the wax first appears when fuels are cooled is called the cloud point (Coutinho et al., 2002). The determined values of the pour point and cloud point of *J. curcas* biodiesels are closely in line with ASTM D6757 (Table 3). The acid number shows the amount of FFAs present in fuel oil. If the acid number for biodiesel is higher than 0.5 mg KOH g⁻¹ FAME, it causes corrosion of the fuel engine. In the present study, the acid pretreatment of the oil reduced the acid number of oil from 26 ± 1.7 mg KOH per g of oil to 2.4 ± 0.01 mg KOH per gram of pretreated oil. The acid number of biodiesel was 0.45 mg KOH g⁻¹ FAME, which is below the critical value and is considered safe for fuel engine operation (Table 3). Similarly, the acid number of biosynthesized biodiesel was 0.24 ± 0.1 mg KOH/g. The saponification number is an index of the mean molecular mass of fatty acids present in seed oil. The FFA, saponification number, ester value, specific gravity, density, and acid number of *Jatropha* oil were 13.11%, 213 ± 5.1 mg/g, 186.6 mg/g, 0.916 ± 0.1 kg/m³, 0.917 ± 1.5 g/cm³, and 26 mg/KOH g, respectively. Similarly, the peroxide value, specific gravity, density, cloud point, and pour point of chemically transesterified FAMES were 1.43 Meq O₂/kg, 0.88 ± 0.02 kg/m³, 0.866 ± 0.1 g/cm³, 3.8°C, and 2°C, respectively. On the contrary, the peroxide value, specific gravity, density, cloud point, and pour point of lipase-mediated FAMES were 1.41 ± 0.1 Meq O₂/kg, 0.87 ± 0.01 kg/m³, 0.834 ± 0.02 g/cm³, 3°C ± 0.1°C, and 2.3°C ± 0.1°C, respectively. The present study showed that transesterification improved the fuel quality of oil with respect to the aforementioned properties. All of the physicochemical characteristics determined in the present study may slightly vary from other studies due to differences in habitats of feedstock, geography, and soil conditions but are in close coherence with the standards for biodiesel specified by ASTM D6751–20a and EN 14214 (ASTM D6751–20a 2020; ASTM D975–20c 2020; Demirbas, 2009).

3.6 Chemical composition of *J. curcas* seed oil and produced biodiesel

The FTIR spectra for JO and its FAMES obtained at conditions of 1% KOH, 1:6 molar ratio of methanol to oil, reaction time 90 min, and temperature 60°C are presented in Figure 8. The FAMES were confirmed, as described previously (Rana et al., 2019). One major and two minor stretches of ester bands observed at 1742.07, 1198.16, and 1034.90 were only present in the biodiesel, confirming FAMES (Figure 11). The peaks at 1163.55 and 1198.16 correspond to C–O stretches for esters and are only present in FAMES, confirming the biodiesel produced from JO.

The chemical composition of JO and its FAMES was further analyzed by GC-MS. The GC-MS analysis showed 13 different saturated and unsaturated fatty acids in JO (Table 4; Supplementary Figure S1). Additionally, approximately eight different kinds of saturated and unsaturated FAMES were observed in the total ion chromatogram (Table 5; Supplementary Figure S3). All of these fatty acids and FAMES were confirmed using library match software provided by the National Institute of Standards and Technology (NIST). The GC-MS analysis also confirmed the presence of five saturated, two monounsaturated, and four poly-

unsaturated FAMES in the alkali-catalyzed biodiesel of JO (Table 5; Supplementary Figure S3). Usually, saturated and mono-unsaturated fatty acids or their esters are considered ideal for increasing energy yield and oxidative stability. However, oil or biodiesel containing mono-unsaturated fatty acids or their esters are prone to solidification at low temperatures. The fuels or oils containing polyunsaturated fatty acids or their esters have very good cold flow properties but are vulnerable to oxidation. This tendency of fuel oil or biodiesel harms fuel storage and combustion (Imahara et al., 2006). The determined components were in accordance with the reported composition of JO (Akbar et al., 2009). Here, in this case, the composition of fatty acids and their FAMES may vary from other reports due to different geological variations, soil conditions, and seed varieties. De Oliveira et al. (2009) also support the same statement.

4 Conclusion

The study conducted concludes that the seeds of *J. curcas* contain a high content of oil that can be used for the production of high-quality biodiesel. However, *J. curcas* seed oil contains a high free fatty acid content of approximately 13.11%, which hinders the process of biodiesel production using alkali catalysts. This study depicts a successful implementation of a two-step transesterification process, in which, at the first step, the acid pretreatment with sulfuric acid reduced the FFA amount in oil to 1.2%. In the second step, the low FFA-containing pretreated oil was subjected to alkali-based transesterification to yield biodiesel. The study conducted was successful in optimizing the alkali transesterification process to achieve 98% biodiesel yield. Moreover, biosynthesis of biodiesel through a bacterial whole-cell approach is also a feasible option to supersede the chemical-mediated method and overcome the issue of free fatty acids in *Jatropha* oil. The maximum biodiesel yield obtained via lipase-mediated transesterification was 98%. The properties of the produced biodiesels satisfied the standards required for good-quality biodiesel. All the results obtained confirmed that *J. curcas* seed oil can be a viable option for both biosynthetic and two-step transesterification processes and can find successful applications in the field of biofuel production.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

Conceptualization, writing the original draft, formal analysis, methods: AH, ML, and QR. Investigation, resources, reviewing and editing, data validation, and data curation: AK, WS, HK, SK, AS, FH,

SA, AI, and MaB. Reviewing and editing, data validation, and data curation: TS, TD, and MoB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenrg.2023.1225988/full#supplementary-material>

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