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Biodiesel production from the microalgae *Nannochloropsis gaditana*: Optimization of the transesterification reaction and physicochemical characterization

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ABSTRACT

The current environmental scenario has encouraged the study of renewable and competitive alternative feedstocks for biodiesel production. Microalgae present a significant opportunity as a feedstock that does not require arable land and can be cultivated using wastewater. The paper investigates the optimization of biodiesel production from microalgae *Nannochloropsis gaditana* bio-oil using response surface methodology and also analyzes the main physicochemical properties of the bio-oil. It was confirmed that this methodology is suitable for materials with low homogeneity. The model is also adequate for determining optimal experimental conditions, resulting in a FAME content of 87.25 %. The biodiesel's physicochemical properties were analyzed. It was discovered that the high degree of unsaturation in the microalgae bio-oil's chemical structure resulted in a narrower temperature range for its application compared to other vegetable sources.

1. Introduction

The energy industry has shown clear evidence of the urgent need for change over the past decade due to fuel price inflation and the environmental impact of global warming. As fossil resources become scarcer, attention has turned to more environmentally friendly alternatives, such as natural-based biodiesel. Biodiesel, defined by ASTM D6751 [1], refers to mono-alkyl esters of fatty acids derived from vegetable oils and animal fats. Biofuels offer several advantages over petroleum-based fuels. They are more efficient, have lower sulfur and aromatic content, better lubricity, higher cetane number, higher flash point, and a positive energy balance [2,3]. Furthermore, biofuels are renewable, portable, non-toxic, non-flammable, biodegradable, and reduce most regulated exhaust emissions. Additionally, using vegetable-based sources can help grow rural economies and reduce reliance on petroleum-based fuels, ultimately lowering the cost of the final product [4]. However, biodiesel has limitations due to poor flow properties at low temperatures, inadequate storage performance caused by low oxidative stability, and potential NOx emissions, especially when used in older engines without new emission reduction technologies [2].

The four main categories of biodiesel are based on the feedstock used: 1st generation, which uses edible crops; 2nd generation, which uses non-edible crops that require arable land; 3rd generation, which is derived from microalgae, bacteria, fungi, etc.; and 4th generation, which uses genetically modified microalgae [5]. The 1st generation satisfies over two-thirds of the world's bioenergy needs and comprises all arable crops [4,6]. The use of plant resources for energy production has raised concerns about competition with food sources and water supplies. This highlights the problem of water and food scarcity in today's world [7,8]. Increased production costs and higher food prices have resulted. The 2nd generation of plant-based energy sources was developed to address the limitations of the 1st generation. This group includes animal fats, agricultural waste, and oil waste [3,9]. These products are easy to access and do not require investment in cultivation or harvesting since they are waste primary products. This reduces the overall process cost. However, their availability is not fixed and may be insufficient. Additionally, they require pre-treatment to remove any impurities present in the waste [7]. Third-generation biodiesel uses microalgae-derived biomass, which has the advantage of not requiring arable land. Microorganisms can grow in wastewater and absorb nutrients and impurities, making them useful for purifying polluted water [5,10]. The third generation of microorganisms is becoming more popular due to its advantages over the first and second

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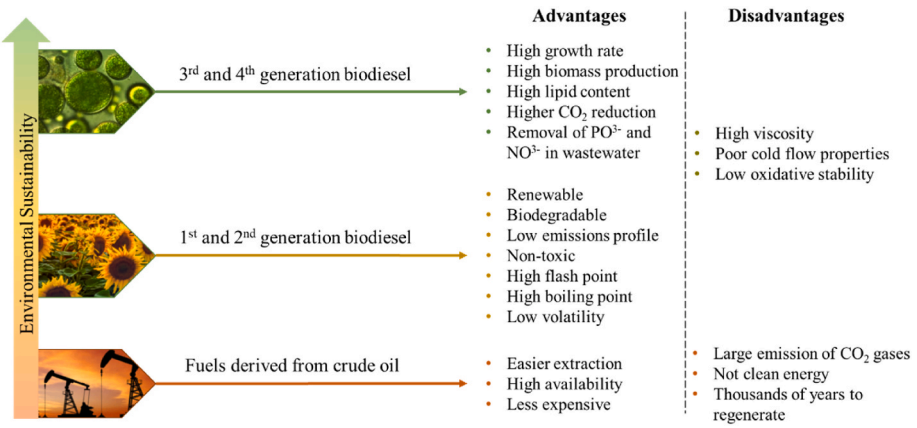

Fig. 1. Advantages and disadvantages of the different feedstocks used in the production of diesel and biodiesel.

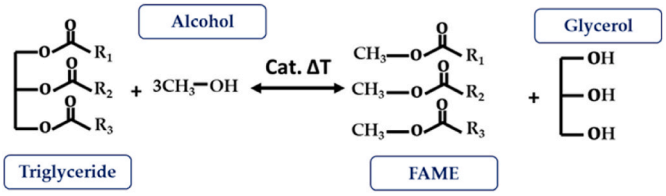

Fig. 2. Biodiesel production through transesterification reaction [5].

generations. For example, it does not require arable land and can treat contaminated water. Additionally, it has a high growth rate and high lipid content [11]. The 4th generation is expected to include biomass derived from genetically modified microalgae. This offers the possibility of modifying the molecular structure of the microorganism [12–14]. Fig. 1 summarizes the benefits of vegetable-based fuels and the differences between generations of biodiesel. Microalgae have advantages over vegetable sources, including a high growth rate, high biomass production per culture, higher lipid content, and greater reduction of greenhouse gases (GHG), phosphate, and nitrate compounds [15,16].

In 2016, the European Federation of Transport and Environment evaluated the CO2 emissions of biofuels compared to fossil fuels [17]. The data shows that fossil fuels emit 94.1 g CO2/MJ, while 1st generation biofuels emit over 100 g CO2 eq./MJ due to land-use change emissions. According to 2022 world energy supply ranking, biofuels from edible crops accounted for 9.03 % of the total [18]. Using microalgae could have helped free up land, reduce food costs, and prevent the emission of nearly 9 % of CO_2 eq./MJ caused by land use change.

In the early 1990s, researchers including P. G. Roessler et al. [19] investigated the potential of microalgae for biodiesel production. They found that these microorganisms were capable of accumulating lipids, with a lipid content of up to 60 % relative to the total cell mass. Microalgae can modify their compositionby changing cultivation parameters such as salinity, temperature, light intensity, O2 concentration, agitation, light/dark cycle, or nutrients (carbon, nitrogen, phosphorus …) [20–22]. After selecting the microalgae strain, it must be cultured for optimal growth, harvested and dried to obtain the corresponding biomass. The bio-oil, which is primarily composed of triglycerides, is extracted through solvent-based processes [23–26]. The transesterification reaction is the technique used to convert bio-oil into biodiesel. This reaction involves the reaction of a triglyceride with an alcohol under heat conditions in the presence of a catalyst. The main product of this reaction is modified fatty acids, with glycerol as a secondary product (Fig. 2).

The structure of triglyceride consists of three fatty acids (FA) linked by a glycerol molecule. The transesterification reaction occurs independently for each FA, resulting in a stepwise reaction. Fatty acids with one or more double bonds are less stable against oxidation. Unsaturation promotes hydrolytic degradation and oxidative damage to the carbon atoms adjacent to the bond [27,28]. Vegetable oils have carbon chains ranging from 8 to 20 carbons, while microalgae predominantly have chains of 16–24 carbon atoms. Microalgal FAs usually contain more double bonds than crop oils, with chains having 5 or 6 double bonds [29,30].

For basic transesterifications, simple alcohols like methanol (MeOH) or ethanol (EtOH) are commonly used. This results in fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE), respectively [31–33]. The catalyst type can also differ between heterogeneous, homogeneous, or enzymatic, in addition to the acidity (acidic or basic) [34–36]. Industrially, biodiesel (FAME) is obtained by using MeOH in the presence of a basic catalyst such as KOH or CH3ONa [10,31]. If the bio-oil feed has a high free fatty acid (FFA) content, it is necessary to perform esterification before use. This process involves reducing the FFA content through transesterification reaction with an acid catalyst. It is recommended to perform the esterification reaction when the FFA concentration is greater than 2–4 mg KOH/g [37]. Moisture is also a crucial factor to consider during the conversion process. This helps to avoid a secondary saponification reaction that can negatively impact the final product's quality and reduce the conversion yield [2].

To produce high-quality biodiesel from vegetable sources, it is important to optimize the transesterification reaction. Table 1 provides a list of research conducted on optimizing this reaction for biodiesel production.

To optimize the process, it is necessary to select independent input variables such as the bio-oil to alcohol ratio, reaction temperature, reaction time, and catalyst amount. Finally, one or more output variables are selected to analyse the impact of the variation in input parameters. Previous studies on microalgae biodiesel production have used a simple experimental design (TR) [38,39,42,43,45]. This methodology involves changing one variable while keeping the other parameters constant, which requires a large number of experiments. Several microalgae strains, including *C. protothecoides* [38,45], *N. gaditana* [39], and *C. vulgaris* [42,43], have been optimized for biodiesel production using this approach. However, this process is time-consuming and resource-intensive due to the significant number of experiments required.

Response surface methodology (RSM) is mathematical technique that evaluates the effect of each parameter (or input variable) and their interactions with the response (or output parameter). It provides an alternative to reduce the number of runs. RSM has been applied in biodiesel production, mainly focusing on feedstocks with simple and highly homogeneous materials such as 1st and 2nd generation vegetable oils like palm, sunflower, soybean, neem, and waste cooking oils. Recent studies have used this methodology to analyse complex and less

Table 1
Design of experiments for the optimization of biodiesel production from vegetable oils.

Feedstock	Experimental Design	Independent factors	Outputs	Ref.
Chlorella prototheocoides	TR	Alcohol ratio (molar), temperature (°C), time (h)	Conversion rate (%)	[38]
Nannochloropsis gaditana	TR	Alcohol ratio (mL/g), temperature (°C), time (h)	Conversion rate (%)	[39]
Palm oil	TR	Catalyst calcination temperature (°C), catalyst (%), alcohol ratio (molar), temperature (°C), stirring speed (rpm)	FAME yield (%)	[40]
Rice bran	TR	Catalyst (g), alcohol ratio (ml)	Conversion rate (%)	[41]
Chlorella vulgaris	TR	Alcohol ratio (mL), temperature (°C), catalyst (%), time (min)	FAME yield (%)	[42]
Chlorella vulgaris	TR	Alcohol ratio (molar); temperature (°C), time (min)	FAME yield (%)	[43]
Waste cooking oil	TR	Alcohol ratio (molar), temperature (°C), time (min)	FAME content (%)	[44]
Chlorella prototheocoides	TR	Alcohol ratio (molar), temperature (°C), catalyst (%)	Conversion rate (%)	[45]
Jatropha curcas *Elaeis guineensis* (palm)	RSM	Alcohol ratio (molar), catalyst (%)	FAME/FAEE content (%)	[46]
Sunflower oil	RSM	Alcohol ratio (molar), temperature (°C), catalyst (g)	Viscosity (cSt)	[47]
Refined soybean oil (RSO)	RSM	Alcohol ratio (molar), temperature (°C), catalyst (%), ultrasound power (W)	Conversion rate (%)	[48]
Pistacia lentiscus	RSM	Alcohol ratio (molar), temperature (°C), catalyst (%)	FAME yield (%)	[49]
Food grade refined palm oil (RPO)	RSM-CCD	Alcohol ratio (molar), temperature (°C) time (h), catalyst (%)	Conversion rate (%)	[36]
Neem oil	RSM-CCD	Alcohol ratio (molar), temperature (°C), time (h), catalyst (%), stirring speed (rpm)	Conversion rate (%)	[9]
Palm oil	RSM-CCD	Alcohol ratio (ml), time (min), catalyst (%)	FAME yield (%)	[50]
Rubber seed oil	RSM-CCD	Alcohol ratio (molar), temperature (°C), time (min), catalyst (%)	Conversion rate (%)	[51]
Chlorella vulgaris + castor oil	RSM-CCD	Alcohol ratio (mL), temperature (°C), catalyst (%)	Conversion rate (%)	[52]
Chlorella prototheocoides	RSM-CCD	Alcohol ratio (mol/mol), temperature (°C), residence time (min), pressure (bar), water content (wt%)	FAME yield (%)	[53]
Chlorella variabilis	RSM-CCD	Alcohol ratio (molar), temperature (°C), time (min), catalyst (wt%)	FAME yield (%)	[54]
Spirulina platensis	RSM-CCD	Alcohol ratio (mL/g), temperature (°C), co-solvent ratio (mL/g), time (min), water content (%)	FAME yield (%)	[55]
Brassica campestris	RSM-CCRD	Alcohol ratio (molar), temperature (°C), time (min), catalyst (%)	Conversion rate (%) Viscosity (mm^2/s) Cetane number	[56]
Spirogyra crassa	RSM-CCRD	Alcohol ratio (molar), temperature (°C), time (min), catalyst (%)	FAME yield (%)	[57]
Palm oil	RSM-BBD	EFI (V/cm), time (min)	Conversion rate (%)	[58]
Palm oil	RSM-BBD	Alcohol ratio (molar), temperature (°C), time (min)	FAME yield (%)	[34]
Nannochloropsis gaditana	RSM-BBD	Alcohol ratio (mL/g), catalyst (%), hexane/SL ratio (mL/g)	FAME yield (%)	[59]

homogeneous materials, such as microalgae oils. In most cases, researchers preferred the central composite design (CCD) as the experimental design when dealing with fewer than four independent variables. CCD was used by Nan et al. [53], Nirmala et al. [54], and Mohamadzadeh et al. [55] to optimize the transesterification reaction and achieve the highest FAME yield in microalgae, such as *C. prototheocoides*, *C. variabilis*, and *S. platensis*. Beyene et al. [52] used RSM via CCD to optimize the production of biodiesel from a mixture of castor oil and the microalgae *C. vulgaris*.

Macías-Sánchez et al. [59,60] conducted two studies to optimize the FAME yield of direct transesterification of wet *N. gaditana* biomass. In the first study, they optimized temperature and reaction time using a simple experimental design without RSM [60]. Later, they applied RSM-CCD to optimize the catalyst concentration, alcohol, and hexane ratio on the same material, using the optimal conditions of temperature and reaction time obtained in the first study [59].

The aim of this work is to optimize the production of biodiesel from the bio-oil extracted from the microalgae *Nannochloropsis gaditana (N. gaditana)* through transesterification. The study focused solely on microalgae bio-oil, not wet biomass. All independent variables, including alcohol ratio, temperature, and reaction time, were optimized in a single study. A physicochemical characterization was performed to fully understand the resulting product.

Table 2
Input variables and levels for optimizing the MBO transesterification reaction.

Input variables	Factor	Variable levels				
		−2 (−α)	−1	0	+1	+2 (+α)
Bio-oil:methanol ratio	A	1:3	1:6	1:9	1:12	1:15
Reaction temperature (°C)	B	30	50	70	90	110
Reaction time (min)	C	30	67.5	105	142.5	180

Table 3
Design of experiments and responses.

Std. order	Run order	Factorial input variable			Responses, Y		
		A	B	C	Actual	Predicted	
					Y (%)	Y (%)	Error (%)
11	1	0	−2	0	81.81	84.18	2.90
18	2	0	0	0	78.52	78.90	0.49
2	3	1	−1	−1	86.99	88.87	2.16
6	4	1	−1	1	72.69	68.89	5.24
10	5	2	0	0	70.35	69.88	0.67
4	6	1	1	−1	70.37	67.05	4.72
9	7	−2	0	0	33.44	37.44	11.95
13	8	0	0	−2	50.90	52.80	3.74
3	9	−1	1	−1	35.21	35.45	0.66
8	10	1	1	1	62.08	64.63	4.11
1	11	−1	−1	−1	67.09	60.95	9.15
7	12	−1	1	1	65.57	60.11	8.33
14	13	0	0	2	55.80	57.48	3.02
12	14	0	2	0	53.22	54.42	2.25
5	15	−1	−1	1	68.31	68.05	0.37
15	16	0	0	0	77.44	78.90	1.88
17	17	0	0	0	77.72	78.90	1.52
16	18	0	0	0	78.35	78.90	0.70

2. Materials and method

2.1. Bio-oil characterization

N. gaditana was chosen because of its high lipid content, which makes it a promising source for bio-oil production. This strain of microalgae has been reported to accumulate up to 68 % (%wt.) lipid content in the best cases [61]. The bio-oil used in this study (MBO) was provided by Neoalgae (Gijon, Spain).

The MBO's TAN was measured with a Metrohm 848 Tritino PLUS (accuracy ±0.01 mg KOH/g) following ASTM D664 to determine if esterification was necessary. The water content was also measured with a Metrohm 899 Karl Fischer coulometer (accuracy ±0.1 ppm).

The SL was determined using the method outlined by Callejón et al. [39]. The analysis provides the SL content per unit of lyophilized MBO and the lipid profile. This is achieved through a transesterification reaction with a solution of MeOH and acetyl chloride.

The lipid profile was analyzed using gas chromatography with flame ionization detector (GC-FID) following the UNE-EN 14103 standard after the transesterification reaction with acetyl chloride. The equipment used was a Clarus 690 (PerkinElmer) with an Elite-WAX column (30 m × 0.25 mm x 0.25 μm). The oven temperature was programmed in the following sequence: (1) hold at 60 °C for 2 min, (2) heat from 60 °C to 200 °C at 10 °C/min, (3) heat from 200 °C to 240 °C at 5 °C/min, (4) hold at 240 °C for 7 min. Hydrogen was used as the carrier gas, and the detector temperature was set at 250 °C with a flow rate of 2 mL/min. The injector was a split system with a set temperature of 250 °C and a sample volume of 1 μL. The sample was prepared at a concentration of 5 mg biodiesel per mL toluene. The detectable minimum concentration by GC-FID analysis was 1 %.

2.2. Transesterification reaction

2.2.1. Design of Experiments (DOE)

The MBO transesterification reaction was optimized using RSM. Design Expert 13 software was employed with a two-level factorial

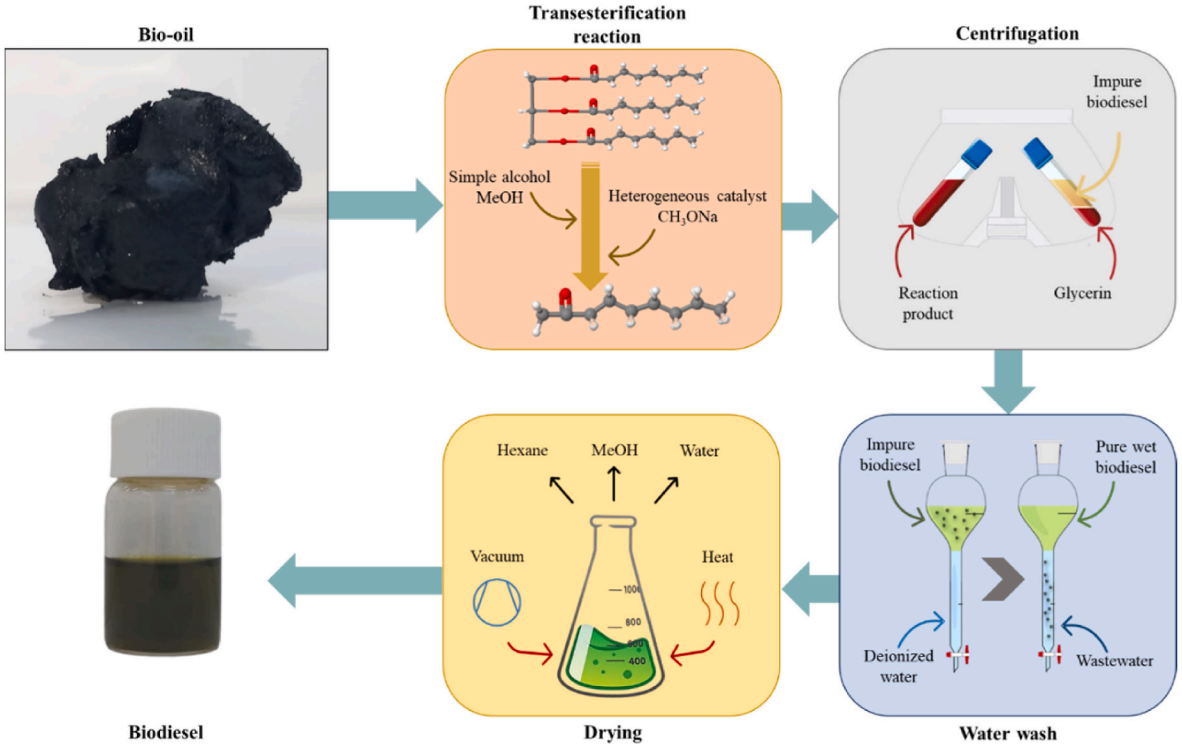

Fig. 3. Biodiesel purification process after transesterification.

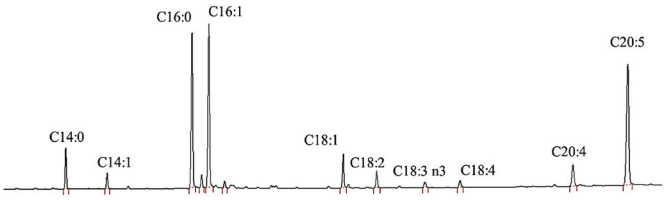

Fig. 4. Lipid profile chromatogram.

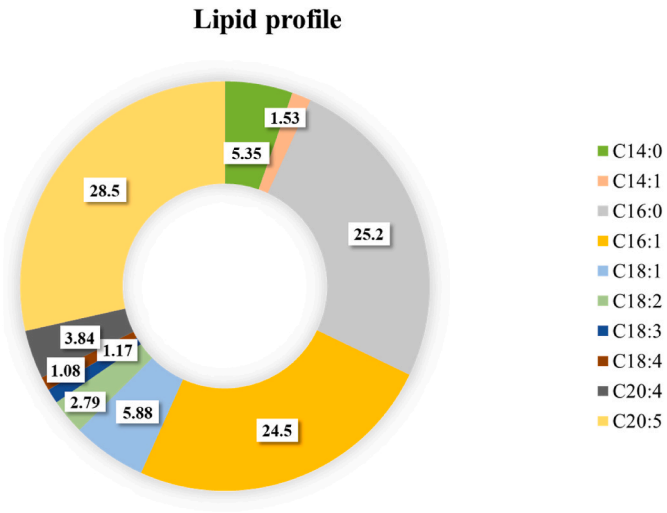

Fig. 5. Distribution of fatty acids in *N. gaditana*.

Table 4
Lipid content and fatty acids distribution of different of *N. gaditana* cultures.

SFA (%)	MUFA (%)	PUFA (%)	Total lipid content (%)	Ref.
30.55	31.91	35.6	35	Current
19	20.5	26.5	10.8	[67]
31.17	30.27	20.79	14	[20]
36.49	18.34	43.41	24.11	[66]
52.47	40.36	7.16	29.73	[68]
24.59	34.41	41	17.82	[69]
29.78	20.49	25.31	–	[70]

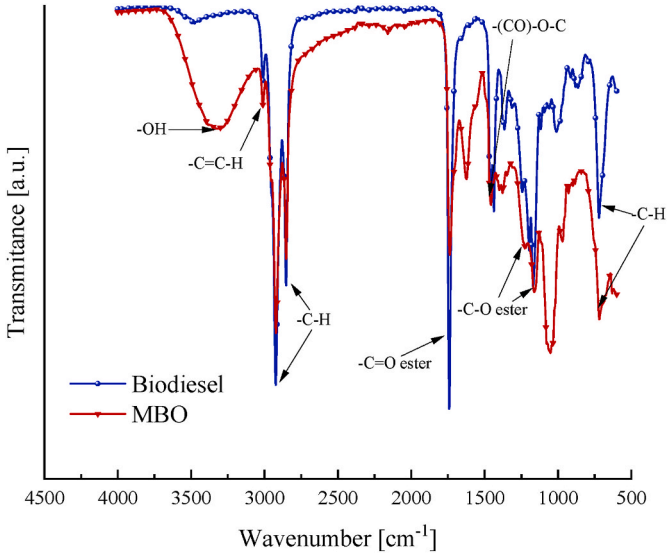

Fig. 6. FTIR spectra of bio-oil and biodiesel.

Table 5
ANOVA analysis of the fitting model for Y.

Source	*df*	*F* value	*p* value	Remark
Model	9	24.28	<0.0001	significant
A - Bio-oil:methanol ratio	1	61.52	<0.0001	
B - Reaction temperature	1	51.75	<0.0001	
C - Reaction time	1	1.29	0.2891	
AB	1	0.3973	0.5461	
AC	1	21.44	0.0017	
BC	1	9.02	0.0170	
A^2	1	50.70	<0.0001	
B^2	1	7.35	0.0266	
C^2	1	45.03	0.0002	
Residual	8			
Lack of Fit	5	105	0.0014	significant
Model summary	R^2	R^2 (adj.)	R^2 (pred.)	Adeq. Precision
	0.9647	0.9250	0.7366	17.3292

approach resulting in CCD [62]. Three numerical factors (input variables) were selected: bio-oil:methanol ratio (A), reaction temperature (B), and reaction time (C). Table 2 shows the values chosen for each input variable. They were chosen after a thorough evaluation of previous studies on biodiesel production from vegetable sources (Table 1). The parameter levels are coded as −1 (minimum), 0 (center), +1 (maximum), and ± α (extreme star points), as shown in Table 2. The distance between α and the center point was set at 2. The study consisted of eighteen runs in total, including eight factorial design runs, six star points, and four replications of the center point, as detailed in Table 3 [63]. The FAME conversion (Y) was evaluated based on to Eq. (1). Optimization runs were conducted using 1 g of MBO as a simple size and 2 mL of hexane in the presence of the basic catalyst CH_3ONa at 1.5 %.

$$Y(FAME\ conversion\ \%) = \frac{Amount\ transformed\ to\ FAME\ (g)}{Total\ SL\ amount\ convertible\ to\ FAME\ (g)} \times 100 \quad \text{Eq.1}$$

2.2.2. Purification process

After completing the transesterification reaction is complete, a purification process is necessary to remove any impurities mixed with the biodiesel. The purification steps used in this study are illustrated in Fig. 3.

After the transesterification reaction, the resulting product was centrifuged at 7000 rpm for 10 min using a Microcen 24 - CE 202 to recover the biodiesel and remove the glycerol formed during the reaction. The liquid was then washed with deionized water until it reached a neutral pH. This step is essential to remove non-reacting MeOH, catalyst residues, and other impurities present in the MBO. Finally, the solution was transferred to a rotary evaporator (Hei-Vap Core, Heidolph) and evaporated at 60 °C and 20 mbar for 20 min to remove any remaining hexane, moisture, and MeOH.

2.3. Biodiesel physicochemical characterization

The GC-FID equipment was used to evaluate the FAME content for all runs. The quantitative analysis was performed according to UNE-EN 14103 using the procedure previously described for the lipid profile, with methyl nonadecanoate as an internal standard.

The density and dynamic viscosity of the samples were determined using a high-precision rotational viscometer (Stabinger SVM 3001) in accordance with the ASTM D7042 guidelines under atmospheric pressure conditions. The density was determined at a temperature of 20 °C, while the viscosity was determined at a temperature of 40 °C.

To determine the pour point (PP), Differential Scanning Calorimetry (DSC Mettler 822e 700) analysis was used, which has a heat flow accuracy of better than ± 2 % and a temperature accuracy of ±1 °C. This

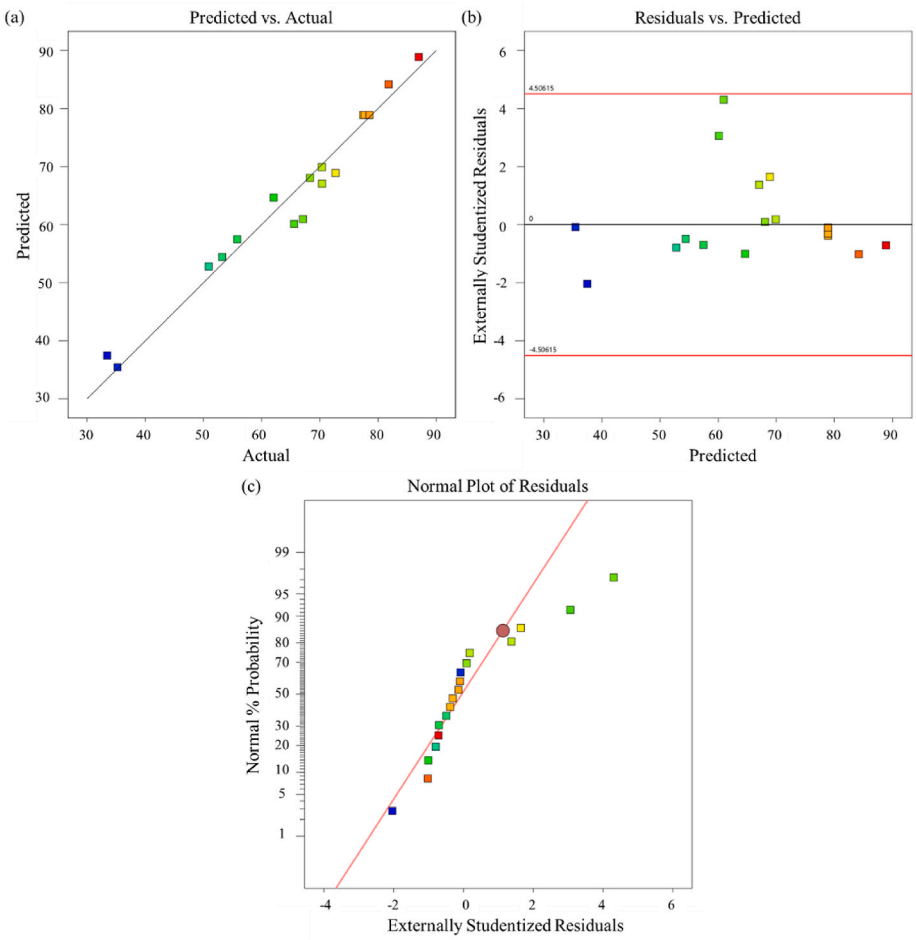

Fig. 7. Statistical analysis of the quadratic model generated for the response Y: (a) Predicted vs. Actuals; (b) Residuals vs. Predicted; (c) Normal plot of Residuals.

technique can provide a direct measurement of the change in enthalpy for a system during cooling, which can be approximated by the sample PP [64]. The procedure involves heating the sample to 50 °C at a steady rate of 10 °C/min and then holding it under isothermal conditions for 10 min. The system is then cooled to −50 °C at a steady rate of 10 °C/min under a nitrogen atmosphere. The PP value can be obtained at the maximum point of the curve by using Heat flow (W/g) versus temperature plots [65]. Additionally, the composition was analyzed using a Fourier Transform Infrared (FTIR) spectrometer (Varian 670-IR) with an accuracy of better than 0.07 cm^{-1}.

Thermal stability (TS) was evaluated using thermogravimetric analysis (TGA). The TA Instruments DSC SDT Q600 TGA & DSC was used for the analysis, which has a temperature accuracy of 0.001 °C (200–1300). The results were analyzed using TA Instruments Universal Analysis 2000 version software. The sample, approximately 6 mg, underwent dynamic scans at a constant heating rate of 20 °C/min from 25 to 600 °C under a nitrogen atmosphere at a flow rate of 50 mL/min. The sample's weight loss was plotted against time to obtain the onset temperature of decomposition.

The modified Cleveland Open Cup Tester, following EN ISO 2592 and ASTM D92 standards, was used to determine the flash point (FP). To determine the FP, 15 mL of biodiesel was gradually heated from room temperature in 5 °C increments until the FP was reached.

3. Results and discussion

3.1. Lipid profile and molecular structure

Fig. 4 displays the chromatogram of the lipid profile of the MBO, while Fig. 5 shows the distribution of FAs. The FAs are evenly balanced between saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA). The GC-FID analysis of the biodiesel sample revealed three major peaks: C16:0 (25.2 %), C16:1 (24.5 %), and C20:5 (28.5 %). Additionally, minor peaks were found between C14:0 to C20:4, which accounted for 21.64 % of the sample. Microalgae oils often contain long-chain fatty acids and numerous double bonds, which are not typical of crop oils. However, a high concentration of double bonds in the molecular structure of fatty acids negatively impacts the material's oxidative stability. The presence of 70 % unsaturated FAs indicates poor resistance to oxidation.

The results are consistent with other cultures of the same species. Callejón et al. [39] and Tang et al. [66] reported a similar lipid profile, as shown in Fig. 4. However, the lipid content of the studied oil is higher than that reported in the literature (Table 4), reaching a lipid content of 35 %. This difference could be attributed to variations in culture and growth stage. Optimizing triglyceride production is essential for biodiesel conversion during these stages.

Fig. 6 shows the FTIR spectrum, highlighting the structural differences between the original bio-oil and the biodiesel. A band at 3342.03 cm^{-1} indicates the presence of alcohol compounds (-OH) used in the bio-oil extraction process. The increase of the characteristic bands at 2921.63 cm^{-1} and 2852.2 cm^{-1} is due to the increase in ($-CH_3$) alkanes, which result from the breaking of the triglyceride structure. The decrease in bands between 715 and 725 cm^{-1} is due to the loss of alkenes ($-CH_2$). The constant band at 3012.27 cm^{-1} is attributed to the (-C═C-) bonds. Additionally, esters (-C═O) at 1739.48 cm^{-1}, methyl acetate compounds ((CO)–O–C) at 1434.78 cm^{-1}, and other methyl compounds (-C–C(O)–C) at 1166.72 cm^{-1} are consistently present. The

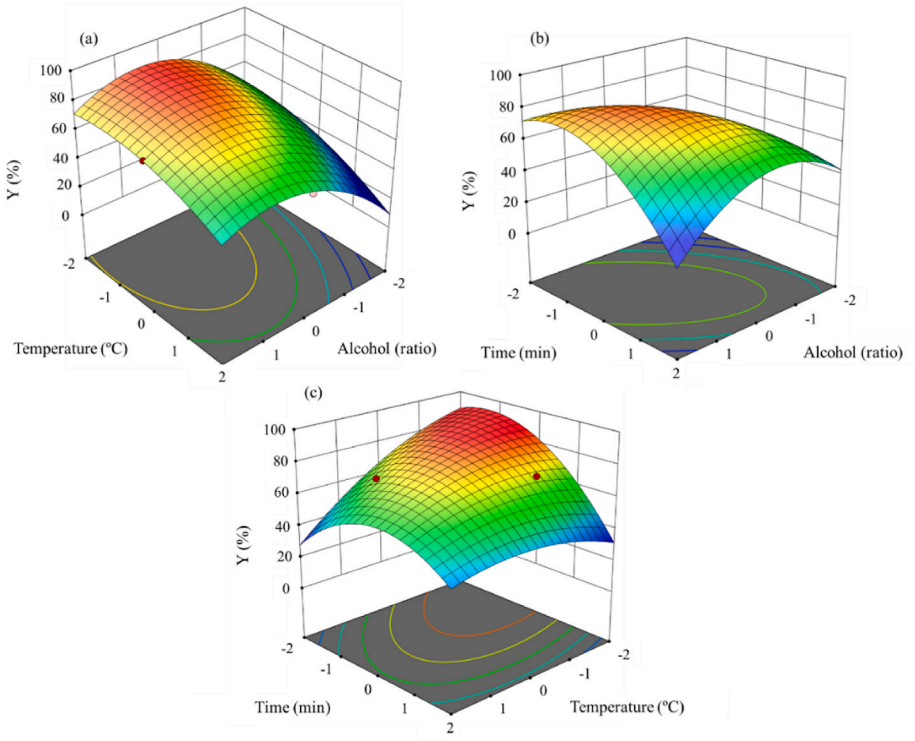

Fig. 8. Interaction between independent variables for response Y. (a) Interaction between temperature and alcohol ratio; (b) Interaction between reaction time and alcohol ratio; (c) Interaction between reaction time and temperature.

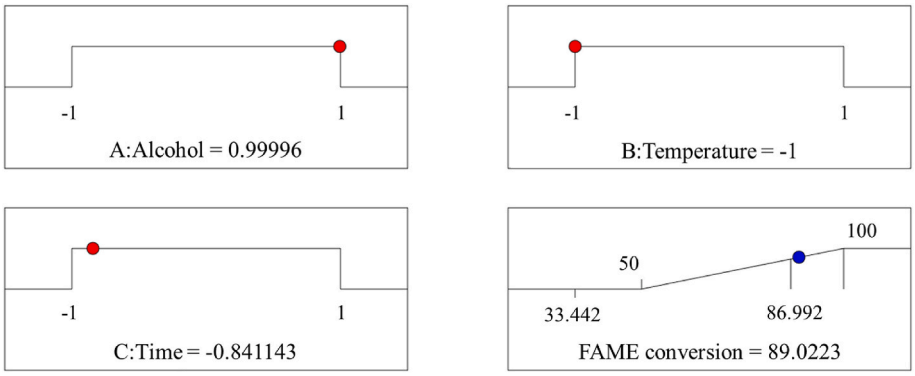

Fig. 9. Optimal conditions designed to produce desired results from independent variables using RSM-CCD.

spectrum of biodiesel obtained from *N. gaditana* agrees with those reported by other authors for the characterization of various vegetable biodiesels [34,51,57].

3.2. Optimization of transesterification reaction by RSM

3.2.1. Model adequacy check

After conducting the 18 tests, the empirical model correlating the input variables (coded) with Y (FAME conversion in %) was generated using a quadratic regression model fitted to Eq. (2):

$$Y = 78.90 + 8.11A - 7.44B + 1.17C + 0.9217AB - 6.77AC + 4.39BC - 6.31A^2 - 2.40B^2 - 5.94C^2 \quad \text{Eq.2}$$

The analysis of variance (ANOVA) was used to determine the interaction between each input variable and the response Y. The final results of the ANOVA analysis are summarized in Table 5.

The *F* and *p* values indicates the significance of the model [48]. An *F*-value of 24.28 and a *p*-value less than 0.05 indicate that the model terms are significant. A value of R^2 of 0.9647 means that this quadratic regression model can explain over 96.47 % of the changes in the output response. The adjusted R^2 is also consistent with this value at 0.9250. Adequate precision (Adeq. Precision) measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Therefore, a ratio of 17.3293 indicates sufficient signal to allow the model to be used to navigate the design space.

Fig. 7 shows the statistical analysis for the model. In particular, Fig. 7 (a) compares the values predicted by Eq. (2) with the experimental values. The predicted values closely match the actual data, indicating a reasonable correlation between them. The differences between the experimental and predicted values are less than 10 % for all the runs, except for one point (run 7). This result is consistent with the reported R^2, R^2_{adj} and R^2_{pred}. The plot of the studentized residuals against the predicted values is shown in Fig. 7 (b). A random distribution of points is observed within the limit of ±5, indicating a constant discrepancy. This suggests that the models are suitable for their intended application

Table 6
Physicochemical characterization of bio-oil and biodiesel.

		Parameter					Ref.
		TAN	Humidity	PP	FP	TS (T_{onset})	
		mg KOH/g	ppm	°C	°C	°C	
Microalgae bio-oil (MBO)		1.11	1583.7	–	–	208.82	Current
Biodiesel	Microalgae	0.42	850	−7.2,−8.7	140–150	244.8	Current
	Soybean	0.34	–	−2	160	–	[72]
	Sunflower	0.41	–	−6	155	–	
	Corn	0.24	–	−5	140	–	
	Rice bran	0.71	–	−2	180	–	
	Olive	0.60	–	−6	155	–	
	Grape seed	–	–	−9	185	–	
	Edible waste	–	–	15	176	–	[73]
	Palm kernel	–	–	6	171	–	
	Chlorella vulgaris			−4.33	98.67		[74]
	Microalgae (not specified)	0.39		−10	165		[75]
	Chlorella protothecoides	0.224	183		183.9		[76]
	Spirulina platensis	0.75	39	−9	189	330	[77]

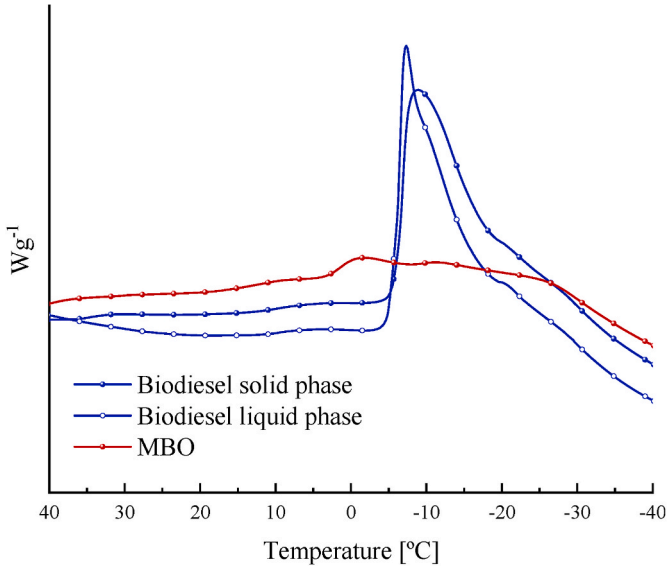

Fig. 10. DSC curve to study the PP of bio-oil and biodiesel.

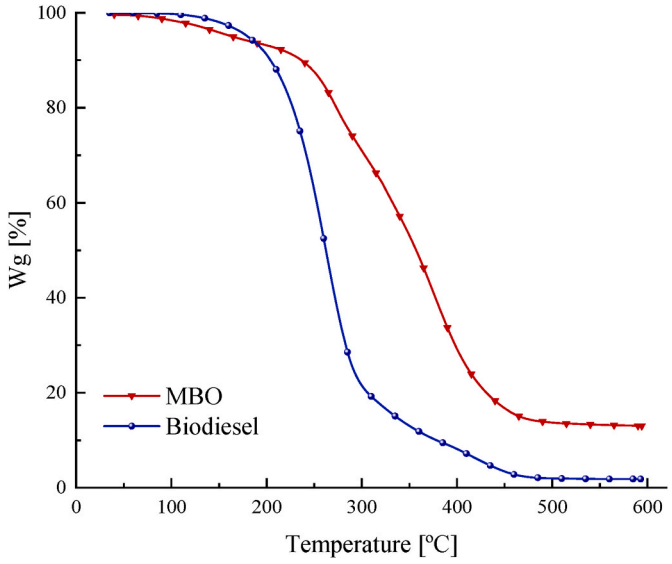

Fig. 11. TGA curve to study the thermal stability of bio-oil and biodiesel.

without requiring modifications to reduce the scatter [71]. Finally, Fig. 7 (c) shows the normalized residuals of Y. The studentized residuals follow a linear distribution that predicts the accuracy of the quadratic model, generally with an S-shape generated by an additional transformation of the response [57]. The lack of precision between the actual and predicted values may be due to the low homogeneity of the sample, as the lipid content is only 35 % of the total of the sample. Additionally, the interference of foreign compounds from the microalgae oils such as liposoluble pigments (astaxanthin or zeaxanthin), may interfere and are difficult to remove during the biodiesel purification stage [67].

The study examines how the independent variables interact with the response using a 3D surface plot (see Fig. 8). The impact of the alcohol ratio is evident in Fig. 8 (a) and (b), where low alcohol ratios consistently lead to low reaction yields. However, an optimal point is reached in relation to the other parameters at a coded value of 1 (ratio 1:12). Fig. 8 (a) and (c) illustrate the relationship between temperature and other variables. Extreme temperatures, both high and low, can negatively affect reaction performance, as can reaction time. However, these variables tend to balance each other out, resulting in optimal performance between 50 and 70 °C and 67.5–105 min.

3.2.2. *Optimized conditions of the RSM analysis*

The ANOVA-validated model was used to determine the best conditions for producing biodiesel from the chosen microalgae, using Eq. (2). The input variables were limited to the bio-oil:MeOH ratio, reaction temperature, and reaction time within the study range (−1, 1), and the responses were maximized at 100 %. The software provided options with the highest FAME conversion, and the one that required the least amount of time and temperature was chosen. The model suggests that the experimental conditions shown in Fig. 9 are the best for achieving the highest reaction yield.

The transesterification reaction's optimal conditions were determined by decoding the independent variable set values. The reaction time was 75 min, and the temperature was 50 °C, with a bio-oil to alcohol ratio of 1:12. These test conditions resulted in an 87.2549 % FAME conversion, validating the generated model with a 2.20 % error between actual and predicted values.

3.3. *Biodiesel physicochemical characterization*

Table 6 presents the physicochemical properties of the current biodiesel, MBO, and vegetable and microalgae biodiesels from other

studies. Moreover, the fluid was found to have a density of 885 kg/m^3 and a viscosity of 6.54 mPa s.

The low TAN values of the bio-oil simplified the preparation of the material before the transesterification reaction. A TAN of less than 2 mg KOH/g eliminated the need for a prior esterification due to the low levels of FFAs. The TAN slightly decreases after the reaction, possibly due to FFAs removal during purification or neutralization. Normal moisture levels for both compounds range from 850 to 1590 ppm, depending on ambient humidity. No drying was observed for the of bio-oil.

The working temperature range of the biodiesel was determined through PP and FP measurements. Fig. 10 shows the behavior of both samples during the freezing point test. The bio-oil exhibited a broad exothermic band during the test, and no freezing temperature was defined. In contrast, the biodiesel separated into a liquid and a solid phase. Both phases showed a clear peak. The liquid phase was at −7.2 °C, and the solid phase was at −8.7 °C. These values are slightly lower than those reported for biodiesel from other vegetable sources (see Table 6).

During the biodiesel FP measurements, the sample ignited slightly at 130 °C but remained constant until the temperature range of 140–150 °C was reached. The ASTM D6751 standard [1] states that the FP in biodiesel is never lower than 120 °C, so the sample falls within the acceptable range. However, this value is significantly lower than those reported in Table 6 for biodiesel derived from vegetable sources.

The thermal stability analysis is presented in Fig. 11. The test results indicate that the biodiesel sample degraded completely at the maximum temperature used. The onset of temperature degradation for the biodiesel occurred at 244.8 °C, which was slightly better than the bio-oil that had a T_{onset} at 208.82 °C. Additionally, the biodiesel sample experienced rapid mass loss once it reached 200 °C, while the bio-oil counterpart maintained a constant and lower mass loss until it reached 250 °C. The bio-oil experiences a second phase of degradation at this point, with a steeper slope leading up to 450 °C. The initial loss of mass in the bio-oil may be attributed to the presence of organic solvents or impurities, as its water content was negligible.

Dantas et al. [78] obtained results that agree with ours. They studied the thermal stability variation of corn bio-oil after transesterification for biodiesel production. The degradation curve of the biodiesel showed the same trend as that of the bio-oil, with an onset of mass loss later. Corn oil degradation also proceeded in several phases, similar to our findings. Chand, P. et al. [79] evaluated the variation of the curve obtained from the thermal stability analysis of a soybean bio-oil/biodiesel blend. The trends of the two materials separately were similar to those reported in this study. The authors observed a decrease in thermal stability at higher concentrations of bio-oil. The results showed an improvement in thermal stability when biodiesel (FAME) was used instead of bio-oil (triglycerides).

Mostafa, S. et al. [77] reported higher FP and thermal stability values for a biodiesel derived from S. platensis microalgae compared to the biodiesel studied here (Table 6). This difference can be attributed to the FA distribution, as S. platensis has a higher percentage of PUFAs (2.11 %) which promote better tolerance to biofuel degradation, while the biodiesel studied here has a lower percentage of PUFAs (35.6 %).

4. Conclusions

The study investigated the use of microalgae N. gaditana as a biodiesel feedstock. Optimization of the transesterification reaction by RSM and physicochemical characterization of the biodiesel were carried out. The results showed that RSM is suitable for optimizing the synthesis of biodiesel from low homogeneity materials such as microalgae bio-oil. Using the optimal conditions from the model, the FAME conversion increased to 87.25 %, surpassing previous studies.

However, due to the high presence of double bonds in the fatty acid chains, the final biodiesel's oxidative stability is classified as low and requires their removal. Additionally, the high degree of unsaturation resulted in a lower upper temperature limit, leading to a lower flash point and thermal stability. However, it improved the lower temperature limit by lowering the pour point when compared to first- and second-generation biodiesel.

Future research could explore the removal of double bonds in microalgae fatty acids through epoxidation or the use of antioxidant additives to mitigate their negative effects. Additionally, microalgae biodiesel could be investigated as a pour point depressant additive in diesel.

CRediT authorship contribution statement

C. Sanjurjo: Writing – original draft, Investigation, Conceptualization. **P. Oulego:** Supervision, Conceptualization. **M. Bartolomé:** Formal analysis, Data curation. **E. Rodríguez:** Writing – review & editing, Project administration, Investigation, Funding acquisition. **R. Gonzalez:** Formal analysis, Data curation. **A. Hernández Battez:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

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