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Sustainable Biodiesel Production from a New Oleaginous Fungus, *Aspergillus carneus* Strain OQ275240: Biomass and Lipid Production Optimization Using Box–Behnken Design

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Abstract: Due to their low cost and ability to synthesize lipids for sustainable biodiesel production, oleaginous fungus has recently gained more prominence than other microorganisms. The new oleaginous fungus *Aspergillus carneus* OQ275240's dry biomass, lipid content, and lipid yield were all optimized in this work, using the response surface methodology-based Box–Behnken design. Analysis of variance (ANOVA) was also used to examine the experimental data, and multiple regression analysis was used to fit the data to a second-order polynomial equation. Three independent variables, such as the concentration of yeast, glucose, and phosphorus, were examined for their mutual impacts. Maximum dry biomass (0.024 g/50 mL), lipid content (36.20%), and lipid yield (8.70 mg/50 mL) were achieved at optimal concentrations of 2.68 g/L of yeast, 20.82 g/L of glucose, and 0.10 g/L of phosphorus, respectively, showing that the actual data and predictions of the models were in good agreement. *A. carneus* OQ275240 has a favorable fatty acid profile that can be used to successfully create biodiesel, as shown by the presence of palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1) in its fatty acid methyl esters (FAMEs) profile. Furthermore, the qualities of the biodiesel were investigated, and it was found that they fell within the parameters established by the international specifications EN 14214 (Europe) and ASTM D6751-08 (United States). These findings point to the newly evaluated filamentous fungal strain as a potential feedstock for the production of high-quality biodiesel.

Keywords: *Aspergillus carneus*; biodiesel; Box–Behnken design; lipids; oleaginous fungi; optimization

1. Introduction

The widespread use of nonrenewable fossil fuels and environmental pollution are currently driving interest in sustainable renewable energy research [1]. Alternative fossil fuel sources should be ecofriendly, cost-effective, renewable, sustainable, and widely available [2].

Biodiesel is a promising fuel with potential to substitute traditional fossil fuel because it is a low-carbon, toxin-free fuel with enhanced lubricity [3]. Nowadays, biodiesel is produced by transesterifying fatty acids derived from a variety of feedstocks, including microalgae, cyanobacteria, yeasts, fungi, bacteria, and plant-seed oils [4].

Oleaginous filamentous fungi are attractive sources for the industry of sustainable biodiesel due to their high growth rate, lack of reliance on light energy, capacity to grow and produce large quantities of lipids using a variety of carbon sources, and good fatty acid fractions for high-quality biodiesel production [5].

Oleaginous fungi can produce more than 20% lipids when grown on high carbon level and limited-nitrogen media, and thus are regarded as rich sources of oils for the production of biodiesel [6].

Cultivation requirements, including carbon, phosphorus, and nitrogen; environmental conditions such as pH, temperature, and incubation time; as well as inorganic salts and the type of microorganisms used, all have different impacts on the production of the lipid and fatty acid compositions of oleaginous microorganisms [7].

Carbon supply in the culture medium affects triacylglycerol (TAG) synthesis in fungi. Oleaginous fungi first use the available carbon for fungal growth and maintenance, then provide functional lipids, and eventually accumulate storage lipids if carbon is still available [8]. Nitrogen is necessary for fungal cell proliferation and growth, and its deficiency promotes the initiation of lipogenesis [9]. When nitrogen is depleted, oil begins to accumulate in the fungal cells, causing fungal growth to cease. Along with nitrogen, phosphorus is involved in lipid droplets formation because it is the component of cell membrane phospholipids [10].

Numerous studies have shown the importance of these factors in lipid production by oleaginous fungi. Amaretti et al. [11] reported that both glucose and temperature affected the composition and degree of unsaturation of fatty acids in *Rhodotorula glacialis*. Furthermore, limiting phosphorus sources under nitrogen-free conditions promotes the accumulation of lipid in oleaginous yeast [12]. Growth of *Mortierella isabellina* ATHUM 2935 and *Mortierella alpina* on glucose increased lipid production to about 50.4% and 40%, respectively [13,14]. Abdellah et al. [15] stated that *Aspergillus* sp. strain EM2018 produced 53.3% lipids during growth on potato dextrose supplemented with 0.05% yeast extract at a pH of 5.0 and an incubation temperature of 30 °C.

Due to the different accumulation of lipids in oleaginous fungi, not all oleaginous fungal cells can be utilized as a source for the production of biodiesel. So, careful choice of fungal strains and identification of fatty acid composition are required to determine their suitability for the production of biodiesel and its uses in various industries. Furthermore, biodiesel derived from oleaginous fungi lipids must meet biodiesel criteria, including cetane number, which relates to better ignition characteristics and lower carbon content, as well as appropriate density and viscosity [16].

Response surface methodology (RSM) has recently been widely used to optimize media composition and culture conditions [17]. RSM is a combination of statistical and mathematical tools for assessing problems whose response depends on numerous factors, with the goal of optimizing independent variables for maximum response [18]. Response surface methodology is a very useful technique that reduces the number of tests when compared to conventional approaches, ultimately saving time and chemicals, and provides accurate response predictions, making it an advantageous selection for experimental design [19]. Therefore, there is a need to combine media optimization with statistical design methods to understand the impact of different independent variables and their interactions on lipid production.

This study used RSM to optimize the fungal biomass, lipid content, and lipid yield of a novel oleaginous *Aspergillus carneus* strain OQ275240 in order to reduce the cost of biodiesel production. Correlations between the three independent variables (yeast, glucose, and phosphorus), their interactions, and their effects on the responses were studied. The

biodiesel's fatty acid composition and some physicochemical properties were also examined to determine its possible appropriateness as a fuel.

2. Materials and Methods

2.1. Strain and Growth Conditions

Based on preliminary experiments, *Aspergillus carneus* strain OQ275240 was used in this study for its high lipid content. The fungal strain was isolated from soil samples from the Second Industrial City in Jeddah, Saudi Arabia. *Aspergillus carneus* strain OQ275240 was maintained on potato dextrose agar (PDA) slants and stored at 4 °C [15]. Spores of a 5-day-old fungal culture were collected by adding 5 ml of sterile saline solution to the slant, and the obtained suspension (1 mL; 1×10^6) was transferred to 100 mL of Czapek Dox's medium to inoculate a 250 mL Erlenmeyer flask. Typical formula is (g/L): 0.4 KH_2PO_4; 0.2 $MgSO_4$, 0.4 NaCL, 0.001 $ZnSO_4.7H_2O$; 4 yeast extract; and 30 glucose; with an initial pH of 6.0 (±1).

2.2. Morphological and Molecular Characterizations of the Tested Isolate

The fungal isolate was characterized by its morphological features (color, textural appearance, and colony diameter), microscopic features (microscopical slide analysis of spores and mycelium), and molecular characteristics utilizing 28S-rRNA.

2.3. Box–Behnken Design (BBD) and Response Surface Analysis

RSM-based BBD was used to optimize the media composition for improving the fungal biomass, lipid content, and lipid yield in *A. carneus*. The experimental design included three independent variables, such as yeast extract concentration (nitrogen source), glucose concentration (carbon source), and potassium dihydrogen phosphate concentration (phosphorous source) at three different levels: low (−1), medium (0), and high (+1) as shown in Table 1.

Table 1. Levels of three independent variables in Box–Behnken design.

	Range and Levels		
Factors (mg/L)	**Low (−1)**	**Medium (0)**	**High (+1)**
Yeast	1.0	2.5	4.0
Glucose	0.0	15.0	30.0
Phosphorus	0.1	0.25	0.4

The Box–Behnken model was used to design seventeen experiments with five replicates, which were carried out at 30 °C for seven days. Correlation between factors and responses was assessed using a second order polynomial equation, and multiple regressions were conducted on the experimental results to obtain an experimental model related to the most significant variables. The second-order polynomial equation is expressed as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2{}_i + \sum \beta_{ij} X_i X_j + \mathcal{E} \quad (1)$$

where Y is the predicted response; X_i and X_j are the independent variables; β_0 is the intercept; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction coefficient, respectively; and $\mathcal{E}$ is the error.

Statistical software Design Expert 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA) was employed for experimental design and data regression analysis. Statistical parameters were evaluated using analysis of variance (ANOVA).

2.4. Analytical Methods

2.4.1. Estimation of Dry Weight (Fungal Biomass)

After 7 days of incubation at 30 °C and 200 rpm, the culture was filtered (Whatman No. 1) and the biomass was collected and washed three times with distilled water, and

then the exact weight of the biomass was determined. The fungal biomass was dried in an oven at 60 °C until it reached a constant weight. Fungal growth was measured as the dry weight of the biomass per 50 mL of culture medium [20].

2.4.2. Estimation of Lipids

The colorimetric sulfo-phospho-vanillin (SPV) method is a quick technique for the direct quantification of lipids [21]. In order to make the SPV reagent, 0.6 g of vanillin was dissolved in 10 mL of 100% ethanol. Subsequently, 90 mL of deionized water was then added, and the mixture was continuously agitated. The mixture was then mixed with 400 mL of concentrated phosphoric acid and stored in the dark until use. The extracted samples (0.1 mL) were mixed with 2 mL of concentrated sulfuric acid (98%) and heated in a boiling water bath to 100 °C, then cooled in an ice bath for five minutes to determine the amount of lipids present. The samples were incubated for 15 min in an incubator shaker at 200 rpm with the addition of five mL of SPV reagent. The intensity of the characteristic pink color produced by the SPV's reaction with lipids was measured at 530 nm with a UV-Vis Spectrophotometer. The total lipid content was given as % dry wt., and the lipid yield was expressed as mg lipid per 50 ml of culture medium.

2.4.3. Profiling of Fatty Acid Methyl Esters (FAMEs)

The dried fungal biomass was exposed to direct acid transesterification to convert lipids to fatty acid methyl esters [22]. In 40 mL of methanol, HCl:chloroform (10:1:1) and 0.5 g of dried biomass were added. After 6 h at 120 rpm and 60 °C, FAMEs were extracted with n-hexane and analyzed by GC/MS (Agilent Technologies) equipped with a gas chromatograph (7890B) and mass spectrometer detector (5977A) in a Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was outfitted with a DB-WAX column (30 m × 250 µm internal diameter and 0.25 µm film thickness). Hydrogen was used as the carrier gas in the analyses, with a flow rate of 1.9 mL/min at a split of 50:1, an injection volume of 1 µl, and the temperature program was as follows: 50 °C for 1 min; 25 °C/min to 200 °C and hold for 5 min; 3 °C/min to 220 °C and hold for 10 min; 5 °C/min to 240 °C and hold for 3 min. The detector and injector were kept at 250 and 260 °C, respectively. Electron ionization (EI) at 70 eV was used to generate mass spectra with a spectral range of m/z 50–550 and a solvent delay of 3 min. Fatty acids were identified by comparing mass spectrum data of each peak in the chromatogram with data stored in Willey 9 and NIST library data. The area normalization method was used to calculate the relative percentages of fatty acids.

2.4.4. Estimation of Physicochemical Characteristics of Biodiesel

Various physicochemical properties of biodiesel, such as kinematic viscosity (*kv*), density (*p*), iodine value (*IV*), oxidation stability (*OS*), cetane number (*CN*), saponification value (*SV*), high heating value (*HHV*), pour point (*PP*) and cloud point (*CP*), were calculated from the FAMEs profile of the fungal strain Ras101 according to the following equations [23]:

$$\text{Ln}\,(\upsilon) = -12.503 + 2.496 \times \ln MW_i - 0.178 \times A_i \quad (2)$$

$$p = 0.8463 + (4.9/MW_i) + 0.0118 \times A_i \quad (3)$$

$$IV = \Sigma\,[(254 \times DB \times A_i)/MW_i] \quad (4)$$

$$OS = -0.0384 \times \Sigma\,\text{MUFA} + (2 \times \text{PUFA}) + 7.77 \quad (5)$$

$$CN = 46.3 + (5458/SV) - (0.225/IV) \quad (6)$$

$$SV = \Sigma\,[(560 \times A_i)/MW_i] \quad (7)$$

$$HHV = 46.19 - (1794/MW_i) - (0.21 \times A_i) \quad (8)$$

$$PP = (0.571 \times \mathrm{C16}) - 12.24 \quad (9)$$

$$CP = (0.526 \times \mathrm{C16}) - 4.992 \quad (10)$$

where MW_i is the molecular weight of each fatty acid methyl ester; A_i is the percentage of the ith component in the mixture; and DB is the number of double bonds.

3. Results and Discussion

3.1. Molecular Identification of the Fungal Strain

On the basis of previous studies [24,25] that show the ability of the *Aspergillus* sp. to produce lipids and fatty acids, the tested fungal strain with the capacity to synthesize lipids was used. Molecular phylogenetic tree analysis indicated that this isolate was a new species of *Aspergillus* and was confirmed as *Aspergillus carneus* (Figure 1). The 28S-rRNA genes of *Aspergillus carneus* have been identified and entered into GenBank with accession number OQ275240. The studied strain belongs to the fungal class (Ascomycetes).

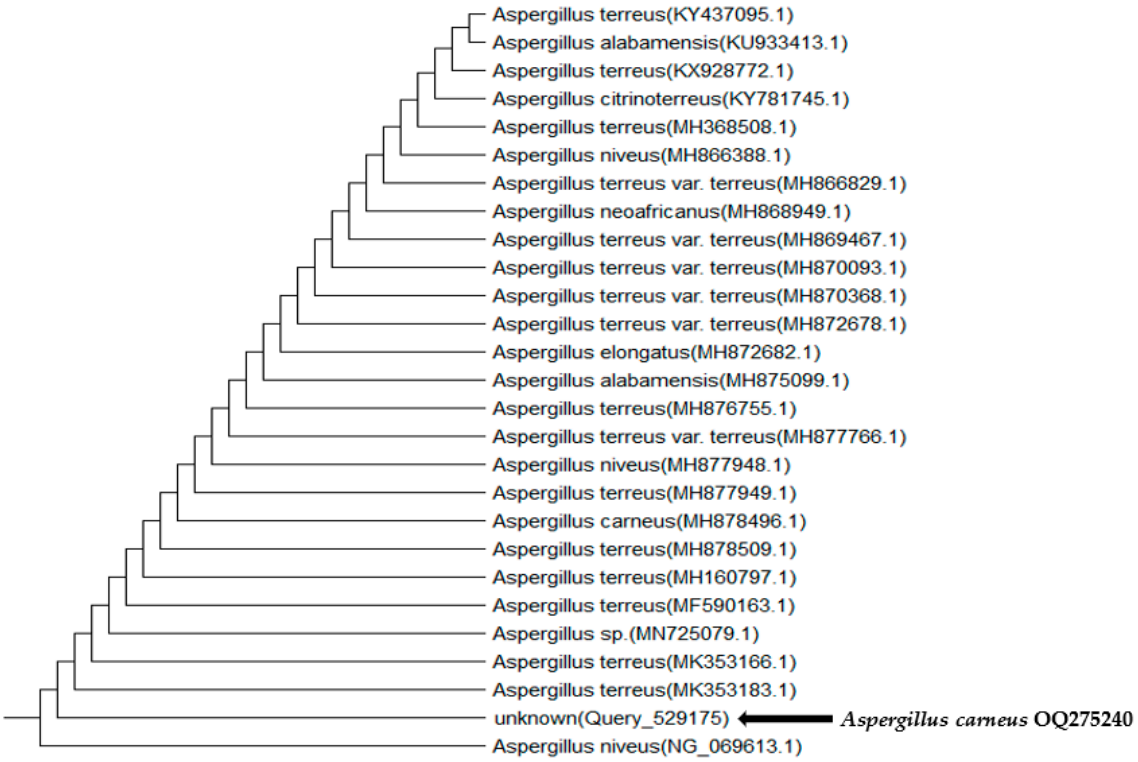

Figure 1. Phylogenetic study of the *Aspergillus carneus* OQ275240 based on the outcomes of PCR amplification of the 28S-rRNA gene.

3.2. Optimization of the Culture Conditions for the Dry Biomass, Lipid Content, and Lipid Yield from Aspergillus carneus

A Box–Behnken experimental design (BBD) was carried out for selecting the optimum concentrations of medium constituents (yeast, glucose, and phosphorus) that supported the maximum dry biomass, lipid content, and lipid yield in *A. carneus*. A total of 17 runs with various combinations of independent variables were achieved to investigate the impact of these variables on the dependent responses.

Table 2 displays the BBD matrix and independent variables, as well as the predicted and actual responses. The maximum fungal biomass (0.16 g/50 mL) was observed at concentrations of 2.5 g/L of yeast, 15 g/L of glucose, and 0.25 g/L of phosphorus, as shown in runs 13–17. On the other hand, the concentration of 2.5 g/L of yeast, 30 g/L of glucose, and 0.1 g/L of phosphorus yielded the highest lipid content in *A. carneus* (53.55%) (run 10). While the condition of 1 g/L of yeast, 15 g/L of glucose, and 0.4 g/L of phosphorus produced the highest lipid yield (12.65 mg/50 mL), as shown in experimental run 7 (Table 2).

Table 2. Box–Behnken experimental design matrix with experimental and predicted responses of dry biomass (g/50 mL), lipid content (%), and lipid yield (mg/50 mL) of *A. carneus*.

Run	Yeast (g/L)	Glucose (g/L)	Phosphorus (g/L)	Experimental Responses			Predicted Responses		
				Dry Biomass	Lipid Content	Lipid Yield	Dry Biomass	Lipid Content	Lipid Yield
1	1	0	0.25	0.020 ± 0.002	1.630 ± 0.278	0.330 ± 0.061	0.033	1.410	0.550
2	4	0	0.25	0.010 ± 0.002	2.020 ± 0.095	0.200 ± 0.040	0.002	1.220	0.190
3	1	30	0.25	0.050 ± 0.004	9.740 ± 0.238	4.870 ± 0.725	0.053	12.660	5.070
4	4	30	0.25	0.030 ± 0.003	5.790 ± 0.564	1.740 ± 0.325	0.022	14.290	2.420
5	1	15	0.1	0.123 ± 0.020	7.560 ± 0.867	9.300 ± 0.851	0.106	14.060	7.080
6	4	15	0.1	0.040 ± 0.004	29.190 ± 0.884	11.670 ± 0.785	0.039	32.270	10.470
7	1	15	0.4	0.081 ± 0.005	14.850 ± 1.340	12.030 ± 0.446	0.082	15.070	12.650
8	4	15	0.4	0.070 ± 0.007	3.320 ± 0.412	2.330 ± 0.229	0.087	2.120	3.960
9	2.5	0	0.1	0.015 ± 0.005	18.190 ± 0.923	2.730 ± 0.420	0.012	17.470	3.770
10	2.5	30	0.1	0.011 ± 0.003	53.550 ± 1.480	5.890 ± 0.628	0.032	44.700	8.280
11	2.5	0	0.4	0.024 ± 0.003	9.500 ± 1.100	2.280 ± 0.950	0.023	15.060	3.300
12	2.5	30	0.4	0.060 ± 0.005	18.550 ± 1.560	11.100 ± 0.592	0.044	15.980	7.820
13–17 [a]	2.5	15	0.25	0.160 ± 0.035	2.620 ± 0.541	4.190 ± 0.685	0.155	1.480	4.230

[a] mean value of five center-point assays. The data are given as averages of five replicates ± standard deviation.

After the backward removal of insignificant variables, analysis of multiple regressions of the results yielded the following second-order equations for dry biomass, lipid content, and lipid yield of *A. carneus* (Equations (11)–(13)):

$$\text{Dry biomass (g/50 mL)} = 0.155 - 0.016\,A + 0.0103\,B + 0.006\,C + 0.018\,AC - 0.038\,A^2 - 0.089\,B^2 - 0.038\,C^2 \quad (11)$$

$$\text{Lipid content (\%)} = -1.48 + 0.82\,A + 7.04\,B - 7.78\,C - 8.29\,AC - 6.58\,BC + 7.92B^2 + 16.86\,C^2 \quad (12)$$

$$\text{Lipid yield (mg/50 mL)} = 4.23 - 1.32\,A + 2.26\,B - 0.23\,C - 3.02\,AC - 2.74\,B^2 + 4.31\,C^2 \quad (13)$$

where A is yeast (g/L); B is glucose (g/L); and C is phosphorus (g/L).

Table S1 shows the ANOVA data of the quadratic regression models of dry biomass, lipid content, and lipid yield for *A. carneus*. Based on the ANOVA analysis, the *F*-values for dry biomass, lipid content, and lipid yield were 16.82, 6.90, and 6.45, and the *p*-values were 0.0015, 0.016, and 0.013, respectively. *F*- and *p*-values showed that the second-order polynomial models were significant [26]. Additionally, the lack of fit test was nonsignificant, indicating that the quadratic models adequately fit the experimental data ($p > 0.05$).

The coefficient of determination (R^2) was used to assess the models' goodness of fit. The R^2 value quantified the variability in values of experimental responses that could be clarified by the independent variables and their interactions. The R^2 value of one indicated the ideal case in which the model can explain 100% of the variation in actual values [27]. In this study, all models had R^2 values greater than 0.84, indicating that the quadratic models were strong and well predicted the dependent responses. In addition, the values of the adjusted R^2 for all the regression models were high and close to the values of the predicted R^2, representing that the actual results were in full agreement with the predicted results. The adequate precision was used to check the ratio of signal-to-noise, and a ratio greater than four was considered favorable [28]. In this study, adequate precision values for all responses resulted in ratios higher than four that indicated a suitable signal (Table S1).

3.3. Impact of Process Variables on Dry Biomass, Lipid Content, and Lipid Yield of A. carneus

The interaction of the different factors, including yeast, glucose, and phosphorus, was described by 3D response surface plots. These plots were also used to assess the optimal concentration of each factor for obtaining the maximum fungal biomass, lipid content, and lipid yield from *A. carneus*. Figures 2–4 depict the interaction of two independent variables, with the other factors set to zero.

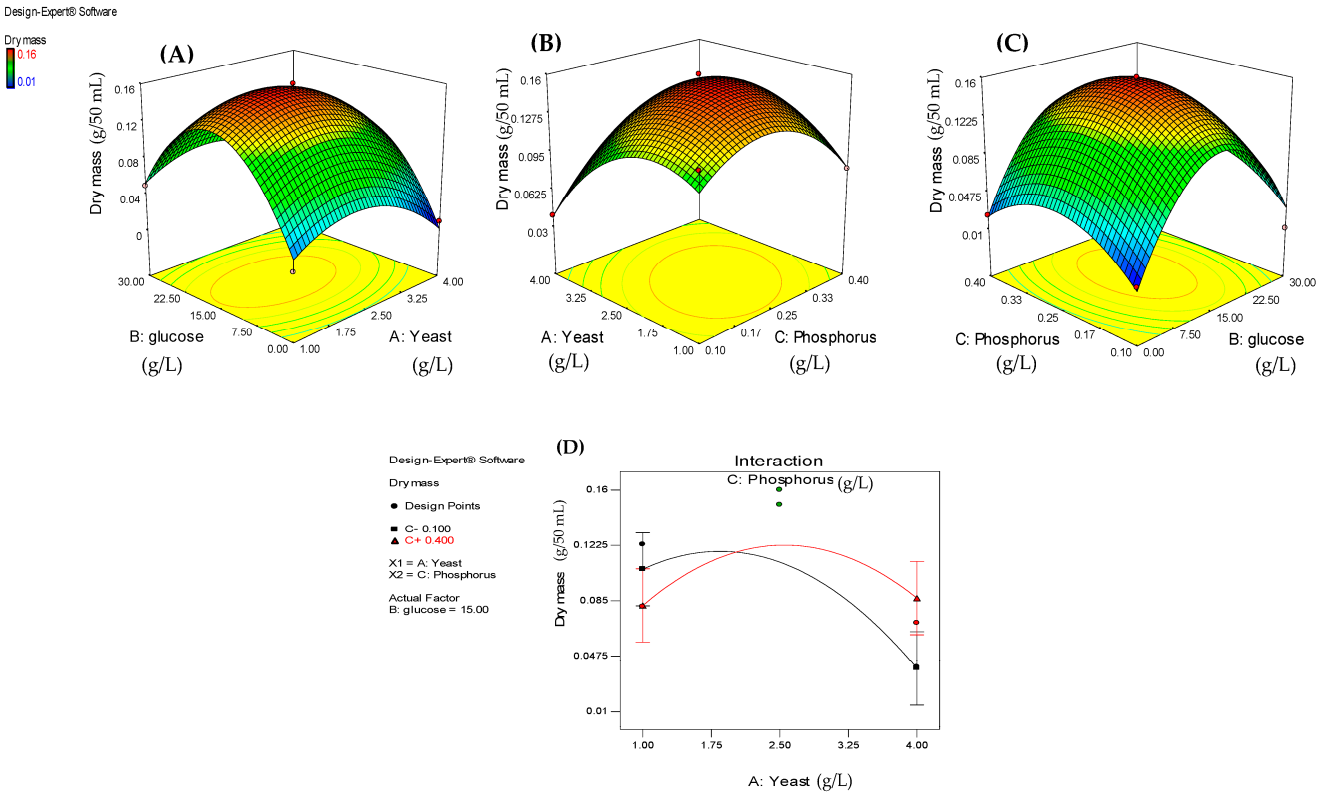

Figure 2. Impact of process variables on dry mass (g/50 mL) of *A. carneus*. (**A**–**C**) 3-D response surface plots and (**D**) interaction plot.

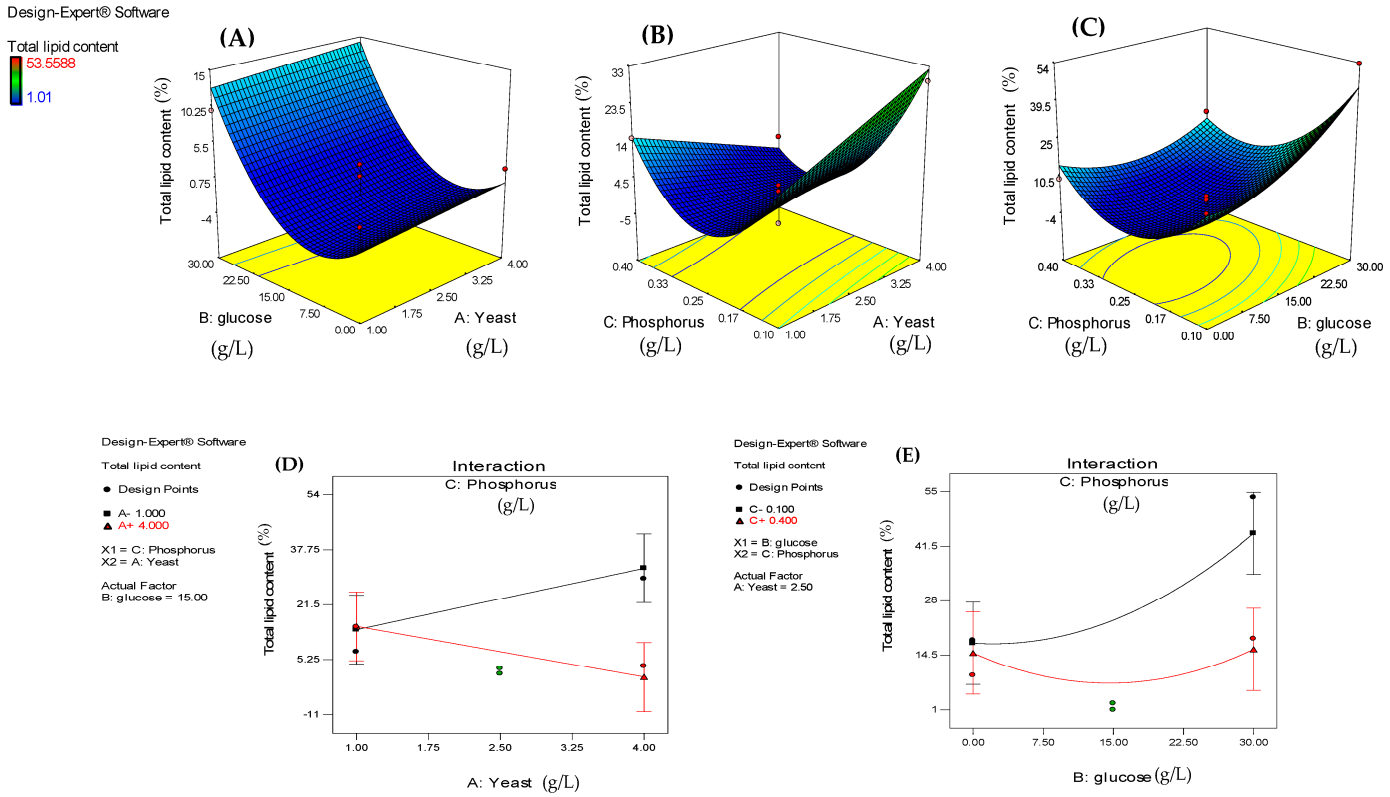

Figure 3. Impact of process variables on total lipid content (%) of *A. carneus*. (**A**–**C**) 3-D response surface plots and (**D**,**E**) interaction plots.

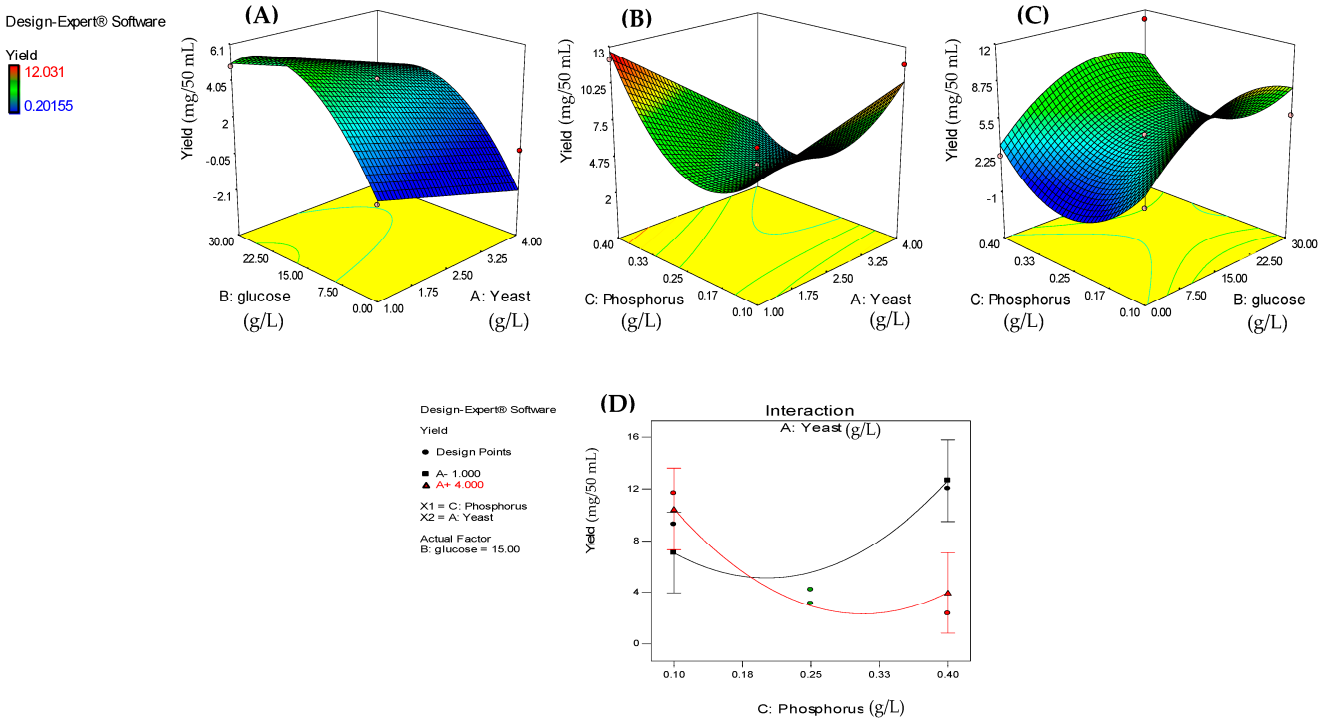

Figure 4. Impact of process variables on total lipid yield (mg/50 mL) of *A. carneus*. (**A**–**C**) 3-D response surface plots and (**D**) interaction plot.

The interaction between yeast extract and glucose and their impact on the dry biomass of *A. carneus* is shown in Figure 2A, whereas phosphorus amount was fixed considering its central value (0.25 g/L). It can be noticed that increasing the concentrations of yeast and glucose first increased the dry biomass of *A. carneus*, but then decreased it by further increasing their concentrations.

The value of dry biomass peaked at a 2.5 g/L yeast concentration and 20 g/L glucose concentration. According to the ANOVA, yeast was the most significant factor ($p = 0.038$; Table 3), and it has a negative significant effects on the dry biomass of *A. carneus* in linear and quadratic terms. In this regard, Dzurendova et al. [29] found that when all oleaginous *Mucoromycota* fungi were grown at high yeast levels, biomass and lipid yield decreased. For glucose concentration, it had a nonsignificant positive effect on the dry biomass in linear terms ($p > 0.05$), but a significant negative impact in quadratic terms ($p < 0.0001$; Table 3).

Table 3. Analysis of variance results for the regression coefficients of the models of dry biomass, lipid content and lipid yield of *A. carneus*.

	Dry Biomass					Lipid Content					Lipid Yield				
Model Term	**CE**	**df**	**SE**	**F Value**	***p*-Value Prob. > F**	**CE**	**df**	**SE**	**F Value**	***p*-Value Prob. > F**	**CE**	**Df**	**SE**	**F Value**	***p*-Value Prob. > F**
Intercept	0.155	1	0.012	-	-	−1.480	1	3.480	-	-	4.230	1	1.230	-	-
A-Yeast (g/L)	−0.015	1	0.006	7.030	0.0379	0.820	1	2.480	0.110	0.753	−1.320	1	0.790	2.790	0.139
B-Glucose (g/L)	0.0103	1	0.006	3.080	0.1300	7.040	1	2.480	8.060	0.030	2.260	1	0.790	8.110	0.025
C-Phos. (g/L)	0.0058	1	0.006	0.970	0.3632	−7.780	1	2.480	9.870	0.020	−0.230	1	0.790	0.090	0.778
AC	0.0180	1	0.008	4.740	0.0401	−8.290	1	3.500	5.590	0.056	−3.020	1	1.120	7.250	0.031
BC	-	-	-	-	-	−6.580	1	3.500	3.520	0.110	-	-	-	-	-
A^2	−0.038	1	0.009	17.130	0.0061	-	-	-	-	-	-	-	-	-	-
B^2	−0.089	1	0.009	93.260	<0.0001	7.920	1	3.840	4.260	0.085	−2.740	-	1.230	4.990	0.061
C^2	−0.038	1	0.009	17.130	0.0061	16.860	1	3.840	19.290	0.005	4.310	-	1.230	12.290	0.010

Phos.: phosphorus; df: degree of freedom; CE: coefficient estimate; SE: standard error.

The interactive impacts of yeast and phosphorus concentrations on the dry biomass of *A. carneus* at a constant glucose concentration (15 g/L) are shown in Figure 2B. The dry biomass of *A. carneus* decreased significantly ($p = 0.038$) with a decreasing yeast concentration, but a nonsignificant increase in dry biomass ($p > 0.05$) was observed with increasing phosphorus concentration.

ANOVA data confirmed these findings and showed that while variations in phosphorus concentration had a positive impact on the dry biomass; variations in the yeast concentration had a negative impact (Table 3). Additionally, the mutual interaction between yeast and phosphorus concentrations had a significant negative impact on the dry biomass of *A. carneus* (Table 3, Figure 2D).

The highest response values were obtained at a yeast concentration of 2.5 g/L and a phosphorus concentration of 0.25 g/L (Figure 2B). High phosphorus concentrations in the growth media resulted in a slight reduction in the growth and lipid production of *Mucoromycota* fungi [29].

Figure 2C depicted the mutual impact of glucose and phosphorus concentrations on dry biomass at a constant yeast concentration of 2.5 g/L. Initially, it was observed that the dry biomass of *A. carneus* increased with increasing glucose and phosphorus concentrations. When glucose levels reached a certain concentration, the trend was reversed. Gong et al. [30] reported that additional concentrations of glucose in the culture medium as a carbon source resulted in the partitioning of available glucose to two metabolic pathways: biomass production and accumulation of lipids.

Figure 3A–D illustrates the effect of process variables on the lipid content of *A. carneus*. Regardless of the yeast concentration, the lipid content increased by up to 14.50% when the glucose concentration was increased from 0 to 30 g/L (Figure 3A). The lipid content value peaked at a glucose concentration of 30 g/L. According to the ANOVA data, glucose concentration had a significant positive effect on lipid content ($p = 0.03$). However, at a glucose concentration of 30 g/L, yeast concentrations had no effect on the lipid content of *A. carneus* ($p > 0.05$, Table 3). In this regard, Srinivasan et al. [31] reported that *Aspergillus caespitosus* ASEF14 exhibited its maximum lipid content (54.60%) when grown in carbon-enriched and nitrogen-limited media for up to seven days. Glucose is the most commonly used carbon source to promote growth and lipid production by oleaginous fungi [32]. Under nitrogen-limiting conditions, high levels of glucose are suggested to improve carbon flux towards triacylglycerol (TAG) synthesis, which is then retained in liposomes, thereby enhancing the accumulation of lipids in numerous microorganisms [33]. According to Shoaib et al. [34], increasing the concentration of glucose increased the lipid content of *Aspergillus wentii* Ras101 until it attained its highest value (38.02%) at around 50 g/L of glucose. Additionally, Al-Hawash et al. [35] stated that the favored level of carbon source for maximum lipid production by oleaginous fungi ranged from 20 to 80 g/L.

The interaction impacts of yeast and phosphorus concentration on the lipid content of *A. carneus* are depicted in Figure 3B. The lipid content of the *A. carneus* strain increased when phosphorus concentrations reduced. This was supported by the ANOVA data, indicating that the phosphorus concentration had a significant negative effect on the lipid content of *A. carneus* ($p = 0.02$; Table 3). Nevertheless, the nonsignificant negative value for the mutual independent variables (yeast with phosphorus) indicated that there was no correlation between these variables, implying that changing one factor had no effect on the other ($p > 0.05$; Table 3, Figure 3D). In some cases, the deficiency of the phosphorus concentration in the growth medium increases the lipid content [36], whereas in other cases it produced the opposite result [37]. In nitrogen-limited media, phosphorus limitation is useful for lipid accumulation in oleaginous yeasts [12].

Figure 3C shows the effect of glucose and phosphorus concentrations on the lipid content of the *A. carneus* strain at a constant yeast concentration (2.5 g/L). According to the ANOVA results, glucose had a nonsignificant positive impact in the quadratic term, but phosphorus had a significant positive effect. As a result, at glucose concentrations up to 30 g/L and 0.1 g/L of phosphorus, lipid content was promoted (Table 3, Figure 3E). In addition, phosphorus and glucose showed a negative mutual interaction. In this regard, Dourou et al. [38] reported that limiting phosphorus or nitrogen in the culture medium promoted lipid production by oleaginous microorganisms.

Variations in lipid yield in response to independent variables are shown in Figure 4A–D. Lipid yield by fungal strain increased exponentially with increasing glucose concentrations, whereas increasing yeast or phosphorus produced the opposite results. Maximum lipid yield (5 mg/50 mL) occurred at a glucose concentration of 30 g/L and low nitrogen or phosphorus concentrations. Similar findings were obtained for *Aspergillus terreus* IBB M1, where lipid yield was found to be highest at a 30 g/L glucose concentration [39].

According to the ANOVA results, glucose concentration was the most statistically significant variable affecting lipid yield in *A. carneus* ($p = 0.025$; Table 3), whereas yeast and phosphorus had negative, nonsignificant influences on lipid yield. In terms of quadratic terms, glucose had a negative, nonsignificant impact ($p > 0.05$), whereas phosphorus had a positive, statistically significant effect on the lipid yield of the fungal isolate ($p = 0.01$, Table 3). Regarding the interactive impacts, a combined interaction between yeast and phosphorus was found to have a significant negative impact (Table 3, Figure 4D). Therefore, decreasing the yeast concentration increased the lipid yield in *A. carneus* in the presence of phosphorus concentration.

Comparative literature studies estimating lipid yield by oleaginous fungi grown on medium supplemented with glucose, *Aspergillus* sp. produced 13.6 g/L in 48 h with a lipid

content of 23.3%, while *Mucor circinelloides* produced a 4.17 g/L lipid yield in 96 h with a lipid content of 23% [40]. Kraisintu et al. [41] reported that in nitrogen-limited medium (0.75 g/L yeast) containing a 70 g/L glucose concentration, *Rhodosporidium toruloides* DMKU3-TK16 produced about 8.11 g/L in lipid yield. Additionally, Hashem et al. [42] stated that *Syncephalastrum racemosum* had a lipid yield of 0.52 g/L and a lipid content of 21.26% when cultivated in a culture medium that contained glucose and yeast extract as carbon and nitrogen sources, respectively. Dean et al. [43] also found that nitrogen and phosphorus limitations in a fermentation medium increased the lipid accumulation and lipid productivity of oleaginous microorganisms.

3.4. Validation of the Proposed Models

Models were validated in triplicate under optimal medium situations predicted by the quadratic models. Validation was performed to confirm optimal values and maximize the dry biomass, lipid content, and lipid yield of the *A. carneus* strain. The data showed that *A. carneus* reached the maximum dry biomass, lipid content, and lipid yield of 0.024 g/50 mL, 36.2%, and 8.7 mg/50 mL, respectively, when grown at 30 °C for 7 days in a culture medium containing yeast, glucose, and phosphorus at optimal levels of 2.68 g/L, 20.82 g/L, and 0.1 g/L, respectively. It can be noticed that the data were close to the expected results (0.045 g/50 mL, 30.72%, and 9.44 mg/50 mL, for dry biomass, lipid content, and lipid yield, respectively), proving the validity of the proposed polynomial models.

3.5. Fatty Acid Methyl Esters (FAMEs) Analysis

Analysis of FAMEs composition and biodiesel characteristics is critical for the selection of fungal species for the production of biodiesel. Fawzy [44] reported that the quality and quantity of fatty acids varied with culture conditions. The main fatty acids in *A. carneus* lipids in this study were stearic acid (C18:0, 43.81%), ethyl stearate, 9, 12-diepoxy (C19:0, 27.51%), palmitic acid (C16:0, 16.16%), and oleic acid (C18:1, 9.53%; Figure 5), which were similar to the fatty acids of most oleaginous fungi [15]. According to Chuppa-Tostain et al. [45], *Aspergillus niger* grown on sugarcane effluent contained high levels of methyl linolenate (42.66%) and methyl palmitate (24.95%).

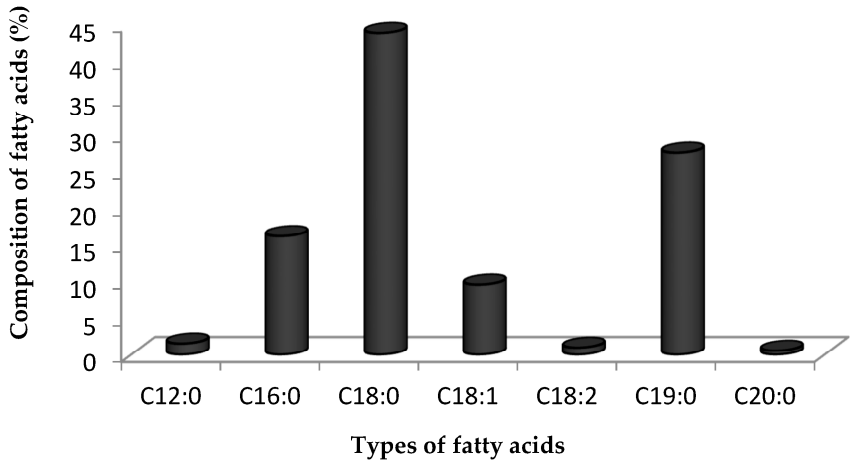

Figure 5. Fatty acid profile of oleaginous fungus.

On the other hand, lauric acid (C12:0), linoleic acid (C18:2), and arachidic acid (C20:0) were found in low quantities, accounting for 1.52%, 0.95%, and 0.53%, respectively (Figure 5). Palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1), specifically, are possible targets for biodiesel production, as they exhibit higher-quality oil characteristics that can increase oxidative stability and have greater capacity flexibility for industrial biodiesel production [46]. In general, C16 and C18 carbon chains dominate the fungal profile of fatty acids, which are important for the production of biodiesel. This composition is very close to plant oils, the most commonly used feedstock for biodiesel.

3.6. Properties of Fungal Biodiesel

The fatty acid profile of the oils used as feedstock for biodiesel production is directly related to their quality. The properties of fatty acids, such as unsaturation degree and carbon chain length, have a significant impact on biodiesel characteristics and therefore on diesel engine operation. FAMEs derived from *A. carneus* had favorable profiles; they were suggested to have better fuel characteristics, indicating their possible viability for biodiesel. In the current investigation, the saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated (PUFAs) fatty acid contents were 89.5, 9.5, and 1.0%, respectively, which were favorable for biodiesel production (Table 4). In this respect, Fawzy et al. [47] reported that high levels of SFAs and MUFAs are needed for biodiesel quality, with low amounts of PUFAs.

On the other hand, shorter and more abundant UFAs raise the viscosity and flow properties at low temperatures, which are unfavorable characteristics. As a result, the *A. carneus* strain can be used as a source for the production of high-quality biodiesel because of its high level of SFAs and low level of PUFAs.

The transformation of a microbial biomass into biodiesel fuel is not the only responsibility as the appropriateness of the produced biodiesel as an alternative to petrodiesel must be evaluated. This is verified by evaluating the main physicochemical characteristics of biodiesel and comparing them with international standards, including EN14214 and ASTM D6751-08 [48], and waste vegetable oil (WVO) [49,50]. The goal of these criteria is to guarantee the ecofriendly and cost-effective use of a biodiesel derived from an isolated fungal strain as a transportation fuel.

Table 4. Properties of the produced biodiesel compared to international standards and waste vegetable oil (WVO).

Biodiesel Properties	EN14214	ASTM D6751-08	WVO [49,50]	*A. carneus*
Saturated fatty acids (SFAs) (%)	-	-	-	89.50
Monounsaturated fatty acids (MUFAs) (%)	-	-	-	9.50
Polyunsaturated fatty acids (PUFAs) (%)	-	-	-	1.00
kinematic viscosity (ln(*kv*)); mm^2/s	3.5–5.0	1.9–6.0	4.54	4.48
Density (ρ; g/cm^3)	-	0.86–0.9	0.88	0.87
Iodine Number (IN; gI_2100/g oil)	≤120	-	-	10.29
Oxidation Stability (OS; h)	≥8	≥3	5.80	7.33
Cetane Number (CN)	≥51	≥47	58.30	71.81
Saponification Value (SV; mg KOH/g)	-	-	-	196.15
High Heating Value (HHV; MJ/kg)	-	-	40.11	39.57
Pour Point (PP; °C)	-	-	−11.00	−3.01
Cloud Point (CP; °C)	-	-	−8.00	3.51

Standard biodiesel parameters, including kinematic viscosity (*kv*), density (*p*), iodine value (*IV*), oxidation stability (*OS*), cetane number (*CN*), saponification value (*SV*), high heating value (*HHV*), pour point (*PP*), and cloud point (*CP*) were assessed in this investigation for determining the biodiesel quality of *A. carneus* (Table 4).

It is well known that the kinematic viscosity (*KV*) of biodiesel is important for fuel spraying and burning processes. Fatty acid methyl ester emission and burning issues are caused by their high viscosity, which causes larger droplet sizes and lower engine performance [48]. Viscosity rises as the length of the fatty acid chain rises, while it decreases as unsaturation increases [51]. The viscosity of fungal biodiesel was 4.62 mm^2/s, which was within the range of biodiesel standards EN14214 and ASTM D6751-02.

The density (*p*) of biodiesel is used to assess its homogeneity. It is significant, particularly in airless burning systems since it affects the effectiveness of fuel atomization [47]. In this study, the calculated density of the fungal strain (0.87 g/cm^3) was close to the minimum limit specified by American standards (0.86–0.90 g/cm^3) and waste vegetable oils.

Iodine value (*IV*) measures the number of double bonds in fatty acids. Several authors concluded that a high iodine value is related to low biodiesel stability and the formation of oxidized substances that can produce deposits in the injection system and affect the efficiency of the fuel engine [4,52]. The low *IV* of *A. carneus* (10.29 g $I_2$100 g^{-1}) was in accordance with two-biodiesel criteria, showing that biodiesel has high oxidation stability (Table 4). This value was lower than that found in *Rhodotorula mucilaginosa* KKUSY14 (UDPH) oil (50.53 g $I_2$100 g^{-1}), as stated by Siwina and Leesing [53].

Besides *IV*, oxidation stability (*OS*) is one of the most important quality standards used for biodiesel products in many countries, as it characterizes the stability of the product during long-term storage [54]. Oxidation stability is related to the degree of unsaturation. A low amount of PUFAs (1.0%) and a high amount of SFAs (59.70%) in the *A. carneus* strain enhanced the oxidation stability [55]. The value of the oxidation stability of the tested fungal strain biodiesel was 7.33 h, which is consistent with international standards, indicating its suitability as a biodiesel fuel. The oxidation stability of *Mucor circinelloides* URM 4140 biodiesel was previously reported to be 6.64 h [52].

The cetane number (*CN*) of biodiesel fuel is associated with the combustion delay time and the ignition quality of the fuel. Diesel fuels with a higher cetane number have a shorter ignition delay time than diesel fuels with a lower cetane number.

Higher cetane fuel is a result of higher oxygen content, which can improve engine efficiency and help it start faster [44]. As a result, it is critical to guarantee that the *CN* matches the engine cetane rating [56]. In the current study, the cetane number of *A. carneus* oil was high (72.31), which is in good agreement with the minimum limits of EN14214, ASTM D6751-08 and waste vegetable oil for the optimum properties of biodiesel. According to Srinivasan et al. [31], the cetane number of *Aspergillus caespitosus* ASEF14 grown on synthetic culture medium was 78.44, which was slightly closer to that of the studied strain.

Saponification value (*SV*) is a property that determines the molecular weight of the whole biodiesel-existing FAMEs [57]. The data indicated that the saponification value of biodiesel produced by *A. carneus* was 192.70 mg KOH/g, which was less than that of *Aspergillus niger* biodiesel (206 mg KOH/g) [58]. A high *SV* shows high triacylglycerol (TAG) content, which is consistent with the high value of ester. Canesin et al. [59] stated that higher *SV* would raise the soap content in glycerol, which would complicate biodiesel separation from glycerol.

Higher heating value (*HHV*) refers to heat energy produced when one gram of biodiesel is completely combusted into CO_2 and H_2O at its initial temperature. The *HHV* of *A. carneus* biodiesel (39.65 MJ/kg) was higher than the value of biodiesel from the wild-type strain of *Yarrowia lipolytica* (36.77 MJ/kg) [51]. This indicated that the biodiesel of *A. carneus* has high energy. It reduces as saturation degree increases because of strong intramolecular interactions [60].

The cloud point (*CP*) and pour point (*PP*) of biodiesel are also significant parameters. *CP* is the temperature at which a liquid mixture becomes cloudy due to solid crystals forming [61]. Greater crystals assemble at temperatures below the cloud point producing great aggregates, restricting oil flow across the filters, and creating start-up and engine performance issues the next morning [62]. The PP of biodiesel is the lowest temperature at which it hardens and starts to lose flow ability [63]. These characteristics are associated with the use of biofuel in conditions of cold temperatures. In this study, the *CP* and *PP* were 3.51 and −3.01 °C, respectively (Table 4). As a result, biodiesel produced by the studied fungal strain would be better fit to cold conditions than petroleum diesel. Based on the aforementioned properties, it is possible to conclude that the oleaginous fungus strain *A. carneus* is a potential candidate for the production of biodiesel.

4. Conclusions

Oleaginous filamentous fungi have been identified as renewable feedstocks for biomass and lipid production. The response surface methodology-based Box–Behnken design is a logical statistical and mathematical methodology designed to investigate the interaction between independent variables (yeast, glucose, and phosphorus) and dependent responses to achieve a better understanding with fewer tests for maximum responses. Based on the Box–Behnken design, the optimal concentrations of yeast, glucose, and phosphorus were found to be 2.68 g/L, 20.82 g/L, and 0.10 g/L, respectively, resulting in the highest dry biomass, lipid content, and lipid yield of 0.024 g/50 mL, 36.20%, and 8.70 mg/50 mL, respectively. Furthermore, the quadratic models were statistically significant, as demonstrated by the high *F* and low *p*-values, implying that they can be used to predict the highest biomass, lipid content, and lipid yield in *A. carneus*. The fungal strain's fatty acid profile was dominated by favorable C16 and C18, saturated and monounsaturated fatty acids, with only trace quantities of undesirable polyunsaturated fatty acids, indicating its appropriateness as a high-quality biodiesel. Furthermore, the properties of fungal biodiesel fall within the range imposed by international biodiesel criteria, suggesting that the new oleaginous fungus *A. carneus* strain OQ275240 is a very promising feedstock for the production of biodiesel fuel. The fatty acid profile of the fungal strain was dominated by beneficial C16 and C18 saturated and monounsaturated fatty acids, with only trace amounts of undesired polyunsaturated fatty acids, suggesting its suitability as a high-quality biodiesel.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15086836/s1, Table S1. Analysis of variance for the response surface models for dry biomass, lipid content and lipid yield of *A. carneus*.

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