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Thermostable $CaCO_3$-Immobilized *Bacillus subtilis* Lipase for Sustainable Biodiesel Production from Waste Cooking Oil

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Abstract: Due to the increasing demand for green processes in renewable energy production, the extracellular *Bacillus subtilis* B-1-4 lipase was used as a biocatalyst for producing biodiesel from waste cooking oil. Response surface methodology was employed for the optimization of enzyme production. Lipase activity was modeled with a quadratic function of four factors that primarily influence the culture medium. Thanks to this model, an optimal lipase activity of 1.7 ± 0.082 U/mL was achieved with the best culture medium composition: 16 g/L of tryptone, 15 g/L of yeast extract, 15 g/L of NaCl, and a 0.15 initial optical density at 600 nm (OD600 nm). The maximal lipase activity was measured at 45 °C and pH 8, using para-nitrophenyl palmitate as a substrate. The enzyme maintained above 94% and 99% of its initial activity at temperatures ranging from 40 to 50 °C and at pH 8, respectively. Moreover, it exhibited a higher residual activity than other *Bacillus* lipases in the presence of organic solvents. Residual activities of 86.7% and 90.2% were measured in the presence of isopropanol and ethanol, respectively. The lipase was immobilized by adsorption onto $CaCO_3$ powder. FT-IR and SEM were used to characterize the surface-modified support. After immobilization, a lipase activity of 7.1 U/mg of $CaCO_3$ was obtained. Under the optimized conditions, the highest biodiesel yield of 71% was obtained through the transesterification of waste cooking oil using the $CaCO_3$-immobilized *Bacillus subtilis* lipase. This research reveals a method for the utilization of waste cooking oil for biodiesel production using an efficient immobilized thermostable lipase, providing environmental and economic security.

Keywords: biodiesel; $CaCO_3$ immobilization; response surface methodology; thermostable *Bacillus subtilis* lipase; transesterification; waste cooking oil

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1. Introduction

Energy consumption and environmental problems need to be solved using alternative energy sources. Energy sources that use fossil fuels produce gases that contribute to the aggravation of the greenhouse effect [1]. To achieve sustainable development, society is being encouraged to find new fuels with fewer environmental impacts [2]. The eco-friendly process of transesterification generates a renewable and nontoxic biodegradable fuel that replaces fossil fuels, with lower exhaust emissions and greenhouse gases [3,4]. Chemically, triglycerides are converted into fatty acid alkyl esters in the presence of methanol or ethanol and a chemical catalyst, generating glycerol as a by-product [5].

In recent years, chemical catalysts have been replaced by enzymes as an environmentally friendly alternative [6–8]. Also, when compared with chemical methods, lipase has

great potential as a catalyst for biodiesel fuel production [9]. It has become increasingly attractive for biodiesel producers to utilize lipase-catalyzed methanolysis over traditional chemical methods because conventional biodiesel production involves chemical catalysts that are used at high temperatures near the boiling point of alcohol, producing soap and other unwanted by-products [8]. Furthermore, using this chemical method is costly and complicated due to the complex separation of biodiesel from these by-products and catalysts [10]. Unlike chemical methods, biocatalysis, which is considered an eco-friendly process [3,4], provides significant advantages, such as mild reaction conditions, the regio- and stereo-selectivity of enzymes, a high substrate specificity, and the absence of by-product production [11–15]. For these reasons, researchers have sought to identify new lipolytic enzymes that would be more suitable. The most suitable enzyme in this regard must possess not only the ability to produce biodiesel efficiently using oil but also the ability to utilize all mono-, di-, and triglycerides, as well as offering a high yield of free fatty acids, low product inhibition, high activity and yield in nonaqueous media, low reaction time, temperature and alcohol stability, reusability of the immobilized enzyme, and so forth [16]. Although industrial biodiesel production plants prefer chemical transesterification with alkaline catalysts, lipase-based methods have already been implemented on an industrial scale. Also, several companies have designed modified immobilized enzymes capable of converting any oils or fats into biodiesel, even cheap oils rich in free fatty acids and polar lipids [17].

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) catalyze the hydrolysis of the ester bounds of triglycerides at the lipid–water interface. They are also able to synthesize esters in organic media through direct esterification or via transesterification [18–21]. Compositional differences in thermophilic lipases contribute to thermal adaptation by fine-tuning stability and flexibility, which increases their resistance to high temperatures. This lends immediate support to the hypothesis that thermostability is associated with an enhanced structural rigidity of the folded native state [22]. Microbial lipases have enormous application potential because they are stable and active in organic solvents. They are highly enantio- and regioselective and possess a wide range of substrate specificity for converting unnatural substrates [23,24].

The main industrial application of lipases is in the hydrolysis of fats and oils; although, their use in the transesterification of oils for biodiesel synthesis is increasing [25,26]. In waste cooking oil, residual fats from frying foods, such as vegetables and meats, are mixed with cooking oil [27]. Waste cooking oil also contains glycerol esters, which can be dissolved in organic solvents [28]. Biodiesel can be produced from waste cooking oil as a feedstock by employing and recycling cooking oil. In addition to being environmentally friendly, biodiesel can be made from these waste materials without causing competition with food sources [29]. Furthermore, it is more economical than fresh vegetable oil, costing two to three times less [27].

This paper deals with screening lipolytic bacteria from Saudi Arabia's extreme biotopes. Response surface methodology was used for the optimization of growth conditions and enzyme production. The isolated lipase was characterized and immobilized onto $CaCO_3$. The enzyme was used in biodiesel production by using cooking oil to reduce waste that harms the environment, which supports the goal of using clean and renewable fuel for future greening opportunities.

2. Results and Discussion

2.1. Screening and Molecular Identification of the Lipase-Producing Strain

Isolates from different Saudi Arabian biotopes were examined for their ability to produce lipases. Among the positive strains, only one was retained based on its attractive positive zone obtained in the presence of both rhodamine B and phenol red indicators. The biochemical and morphological properties of this bacterium isolate correspond to a Bacillus genus.

The DNA sequence of the 16S rRNA gene of the retained strain was amplified, sequenced, and subjected to NCBI BLAST analysis. The strain was identified as *Bacillus subtilis* strain B-1-4, with an identity of 99.78%.

2.2. Growth and Lipase Activity Characteristics of Bacillus subtilis

The effects of cultural conditions were studied to maximize the production of the enzyme. The highest growth of *B. subtilis* was at alkaline pH 8 with an optimum temperature of 45 °C and agitation at 230 rpm. With increases in the incubation period, enzyme production increased and then decreased. The cells' dynamic growth condition and lipase activity in the fermentation medium were reflected in the absorbance value, as seen in Figure 1. This figure shows that the growth phase of *B. subtilis* started quickly after culturing in a fermentation medium for 3 h and lasted for approximately 20 h. A slow growth from 16 to 20 h was observed, in which the strain entered a stationary phase and a dead phase from 22 to 24 h. In addition, Figure 1 shows changes in the lipase activity, with an increase from the beginning of inoculation, and then the values continued to fluctuate until 10 h. The growth rate declined from 16 to 24 h, which may have resulted from the depletion of nutrients in the fermentation medium and a low metabolism level under the aging phase of the growth. The time of the peak (14 h) lipase activity (0.593 U/mL) in the culture period was retained as the optimal enzyme production time.

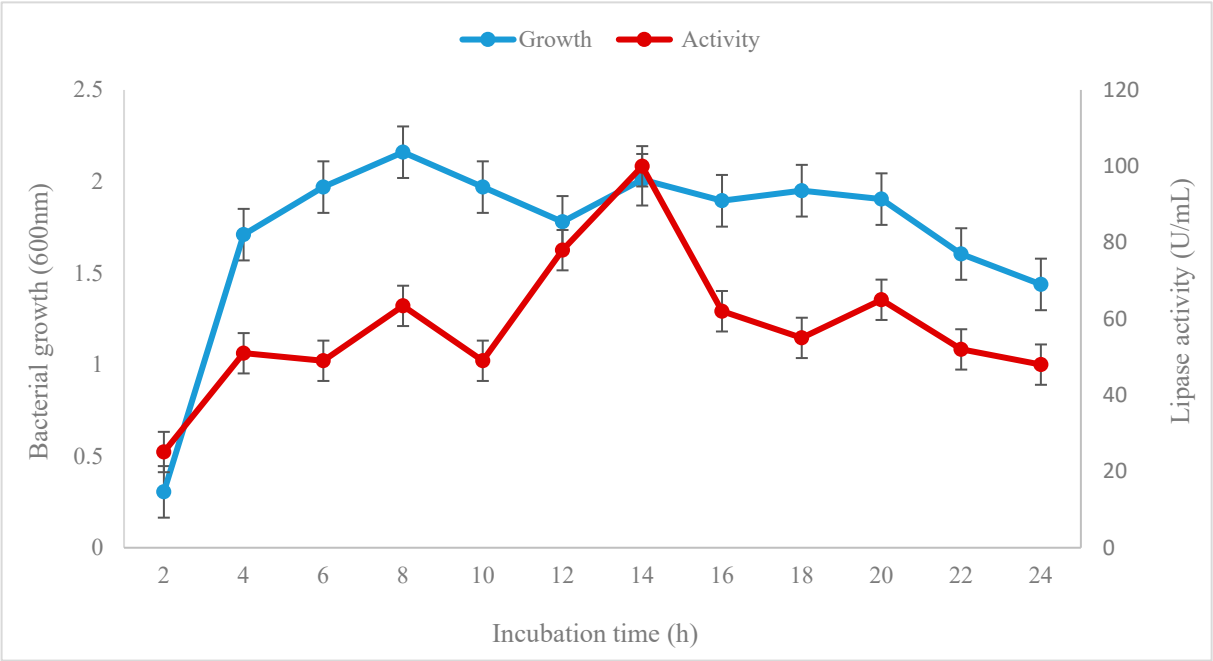

Figure 1. The time course of *Bacillus subtilis* cell growth and lipase production. The culture was carried out at 45 °C with agitation at 230 rpm. Cell growth was monitored every 2 h by measuring the absorbance at 600 nm. Lipase activity was measured at 45 °C and pH 8 using para-nitrophenyl as substrate.

2.3. Optimization of B. subtilis Lipase Production

In this study, the Box–Behnken design was utilized to select the factors most influential (Table 1) on lipase production by *Bacillus subtilis*. Table 2 presents the experimental results for the lipase activity obtained in this work by testing the following factors: tryptone, yeast extract, and NaCl concentrations, as well as the initial optical density used. These experimental lipase activities were from 0.598 ± 0.007 U/mL (run 21) to 1.587 ± 0.047 U/mL (run 10). The best experimental value was obtained when we used 16 g/L of tryptone, 10 g/L of the yeast extract, 10 g/L of NaCl, and a 0.10 initial OD.

Table 1. The various factors of the media studied to determine the best culture conditions for optimal enzyme production and their corresponding concentration levels: high (+1), medium (0), and low (−1).

Variable	Low Level (−1)	Central Level (0)	High Level (+1)
Tryptone (g/L)	8	12	16
Yeast extract (g/L)	5	10	15
NaCl (g/L)	5	10	15
Initial OD	0.10	0.15	0.20

Table 2. Box–Behnken design of experiments with the corresponding enzymatic activity.

Run	Tryptone (g/L)	Yeast Extract (g/L)	NaCl (g/L)	Initial OD	Lipase Activity (U/mL)
1	8	5	10	0.15	0.549 ± 0.006
2	16	5	10	0.15	0.691 ± 0.006
3	8	15	10	0.15	0.767 ± 0.018
4	16	15	10	0.15	1.526 ± 0.018
5	12	10	5	0.10	0.752 ± 0.029
6	12	10	15	0.10	0.695 ± 0.005
7	12	10	5	0.20	0.650 ± 0.003
8	12	10	15	0.20	0.781± 0.029
9	8	10	10	0.10	0.722 ± 0.010
10	16	10	10	0.10	1.587 ± 0.047
11	8	10	10	0.20	0.735 ± 0.006
12	16	10	10	0.20	0.623 ± 0.006
13	12	5	5	0.15	0.775 ± 0.050
14	12	15	5	0.15	0.721 ± 0.007
15	12	5	15	0.15	0.704 ± 0.001
16	12	15	15	0.15	1.508 ± 0.005
17	8	10	5	0.15	0.699 ± 0.003
18	16	10	5	0.15	0.725 ± 0.016
19	8	10	15	0.15	0.731 ± 0.005
20	16	10	15	0.15	0.741 ± 0.003
21	12	5	10	0.10	0.598 ± 0.007
22	12	15	10	0.10	1.497 ± 0.006
23	12	5	10	0.20	0.758 ± 0.024
24	12	15	10	0.20	0.796 ± 0.005
25	12	10	10	0.15	0.748 ± 0.031

All experiments were performed in triplicate and the results are presented as the mean ± standard deviation.

Moreover, the observed accuracy (presented with the calculation of the standard deviation (SD)) of the experimental values presents significantly fewer values, and the coefficient of variation (CV = standard deviation/average value) is very low (less than 6.4%). This is a very interesting accuracy, displaying the experimental results' stability and strength.

Levene's test of the homogeneity of variance was carried out before applying the protocol of determining the coefficients of the quadratic model (See Section 3). The *p*-value was found to be less than 0.001, which shows that the studied response (lipase activity) could be considered non-homogeneous, and it could be influenced by the factors studied with a higher probability. As a second step, a linearity test was applied. This test showed that the studied response could depend linearly on the tested factors, with a very low regression fitting quality (R^2 = 47.64%, R^2adj = 44.65, RMSE = 0.229 U/mL, and $p < 0.001$), confirming that the quadratic model could be considered for the studied response (lipase activity).

After fitting and determining the corresponding coefficients of the model and using the least-squares method, the model is presented as follows (Equation (1)):

$$\begin{aligned}\hat{y}_k = 0.880 + \quad & 0.141 \cdot x_{1k} + 0.228 \cdot x_{2k} + 0.070 \cdot x_{3k} - 0.126 \cdot x_{4k} + 0.154 \cdot x_{1k} \cdot x_{2k} \\ & -0.004 \cdot x_{1k} \cdot x_{3k} - 0.244 \cdot x_{1k} \cdot x_{4k} + 0.215 \cdot x_{2k} \cdot x_{3k} - 0.216 \cdot x_{2k} \cdot x_{4k} \\ & +0.047 \cdot x_{3k} \cdot x_{4k} - 0.021 \cdot {x_{1k}}^2 - 0.070 \cdot {x_{2k}}^2 + 0.018 \cdot {x_{3k}}^2 - 0.027 \cdot {x_{4k}}^2\end{aligned} \quad (1)$$

where x_{1k}, x_{2k}, x_{3k}, and x_{4k} are the centered reduced levels (−1, 0 or +1) of the tryptone (in g/L), yeast extract (in g/L), NaCl (in g/L), and initial OD, respectively, of the kth experiment.

The fitting quality of this model was determined by using four statistical criteria: (1) the coefficient of determination (R^2 = 83.95%), (2) the adjusted coefficient of determination (R^2_{Adj} = 80.21%), (3) the root-mean-square error (RMSE = 0.137 U/mL), and (4) the degree of significance with the *p*-value ($p < 0.001$). These criteria show an interesting fitting quality of the identified model (Equation (1)) describing the studied dependent response: namely, lipase activity as a function of four dependent variables: tryptone, yeast extract, NaCl, and initial OD. This confirms that this quadratic model is better than the multilinear one and can be adopted for the studied response, i.e., lipase activity.

Tables 3 and 4 present the Student's *t*-test and ANOVA test results, respectively. Both tables show the degree of significance for each factor's influence according to two different types of logic: Student's logic and analysis of variance (ANOVA) logic [30]. First, we can remark that all tested factors have a very significant effect in linear terms (yeast extract (Coeff. $A_2 = 0.228$) < tryptone (Coeff. $A_1 = 0.141$) < initial OD (Coeff. $|a_4| = |-0.126|$) < NaCl (Coeff. $A_3 = 0.070$), with $p < 0.01$) on the lipase activity as a response. For the quadratic relationships between the response and the different factors, only the yeast extract (Coeff. $|a_{22}| = |-0.070|$) has a highly significant effect on the lipase activity ($p = 0.004 < 0.01$). Furthermore, four interactions among six are very highly significant ($p < 0.001$): tryptone × yeast extract (Coeff. $a_{12} = 0.154$), tryptone × initial OD (Coeff. $a_{14} = -0.244$), yeast extract × NaCl (Coeff. $a_{23} = 0.215$), yeast extract × initial OD (Coeff. $a_{24} = -0.216$). Response surface methodology (RSM) was developed by Box and Wilson [31] to improve production processes in chemical industries. Their main objective was to optimize chemical reactions to achieve high yields and purity at a low cost. This was realized by conducting experiments involving many factors such as the pH, initial OD, temperature, and carbon or nitrogen source. The same methodology can be used to model or optimize any response affected by the levels of one or more quantitative factors. The statistical basis of this method is polynomial regression modeling.

Table 3. Statistical Student tests on the determined coefficients of the model, presenting the *Bacillus subtilis* production dependence as the function of four factors.

Factor	Coeff.	Std. Err.	t (60)	*p*	−95% Cnf. Limt	+95% Cnf. Limt
Mean/Interc.	0.880	0.034	25.898	<0.001	0.812	0.948
(1) Tryptone (g/L) (L)	0.141	0.023	6.171	<0.001	0.095	0.186
Tryptone (g/L) (Q)	−0.021	0.024	−0.873	0.386	−0.068	0.027
(2) Yeast extract (g/L) (L)	0.228	0.023	10.016	<0.001	0.183	0.274
Yeast extract (g/L) (Q)	−0.070	0.024	−2.983	0.004	−0.117	−0.023
(3) NaCl (g/L) (L)	0.070	0.023	3.062	0.003	0.024	0.115
NaCl (g/L) (Q)	0.018	0.024	0.760	0.450	−0.029	0.065
(4) Initial OD (L)	−0.126	0.023	−5.508	<0.001	−0.171	−0.080
Initial OD (Q)	−0.027	0.024	−1.135	0.261	−0.074	0.020

Table 3. *Cont.*

Factor	Coeff.	Std. Err.	t (60)	*p*	−95% Cnf. Limt	+95% Cnf. Limt
1 L by 2 L	0.154	0.039	3.910	<0.001	0.075	0.233
1 L by 3 L	−0.004	0.039	−0.097	0.923	−0.083	0.075
1 L by 4 L	−0.244	0.039	−6.183	<0.001	−0.323	−0.165
2 L by 3 L	0.215	0.039	5.434	<0.001	0.136	0.294
2 L by 4 L	−0.216	0.039	−5.459	<0.001	−0.295	−0.137
3 L by 4 L	0.047	0.039	1.186	0.240	−0.032	0.126

Coeff.: coefficient; Std. Err.: standard error; t (60): Student factor for a degree of freedom equaling 60 (Coeff./Std. Err.); *p*: probability value; −95% Cnf. Limt: minimum limit of a 95% confidence interval for the corresponding coefficient; +95% Cnf. Limt: maximum value limit of a 95% confidence interval.

Table 4. Analysis of variance (ANOVA) for the production of the *Bacillus subtilis* lipase.

Factor	SS	df	MS	F	*p*
(1) Tryptone (g/L) (L)	0.713	1	0.713	38.079	<0.001
Tryptone (g/L) (Q)	0.014	1	0.014	0.762	0.386
(2) Yeast extract (g/L) (L)	1.877	1	1.877	100.317	<0.001
Yeast extract (g/L) (Q)	0.166	1	0.166	8.896	0.004
(3) NaCl (g/L) (L)	0.175	1	0.175	9.374	0.003
NaCl (g/L) (Q)	0.011	1	0.011	0.578	0.450
(4) Initial OD (L)	0.568	1	0.568	30.338	<0.001
Initial OD (Q)	0.024	1	0.024	1.289	0.261
1 L by 2 L	0.286	1	0.286	15.290	<0.001
1 L by 3 L	0.0002	1	0.0002	0.009	0.923
1 L by 4 L	0.715	1	0.715	38.228	<0.001
2 L by 3 L	0.553	1	0.553	29.526	<0.001
2 L by 4 L	0.558	1	0.558	29.802	<0.001
3 L by 4 L	0.026	1	0.026	1.406	0.240
Error	1.123	60	0.019		
Total	6.997	74			

SS: sum of square; df: degree of freedom; MS: mean square (SS/df); F: Fisher value (MS of factor/MS of error); *p*: probability value.

Also, response surface methods may be employed to find factor settings that produce the desired (maximum, minimum, or optimum) response, to find factor settings that satisfy management specifications, and to model the relationship between the quantitative factors and the response [32].

From Figure 2, we can verify all interpretations highlighted above. Each of the tested factors influences the lipase activity linearly. The value of the response in the lower level of the factor is significantly different from the one shown in the higher level of the same factor: tryptone concentration (Figure 2a–c); yeast extract concentration (Figure 2a,d,e); NaCl concentration (Figure 2b,d,f); and initial OD (Figure 2c,e,f). Moreover, only the yeast extract influences the lipase activity quadratically, as shown by the parabolic form of the 3D surface response of the lipase activity when the yeast extract changes the values (Figure 2a,d,e). In addition, the influence of the significant interactions can be observed clearly: tryptone × yeast extract (Figure 2a), tryptone × initial OD (Figure 2c), yeast extract × NaCl (Figure 2d), and yeast extract × initial OD (Figure 2e).

After adopting the model in Equation (1), since its fitting quality is very high, we can use it to determine better conditions, maximizing the response (lipase activity). With the STATISTICA 12 Software optimization tool (Figure 3), we find that the best conditions to maximize the lipase activity (1.7 ± 0.082 U/mL) are 16 g/L of tryptone, 15 g/L of the yeast extract, 15 g/L of NaCl, and a 0.15 initial OD.

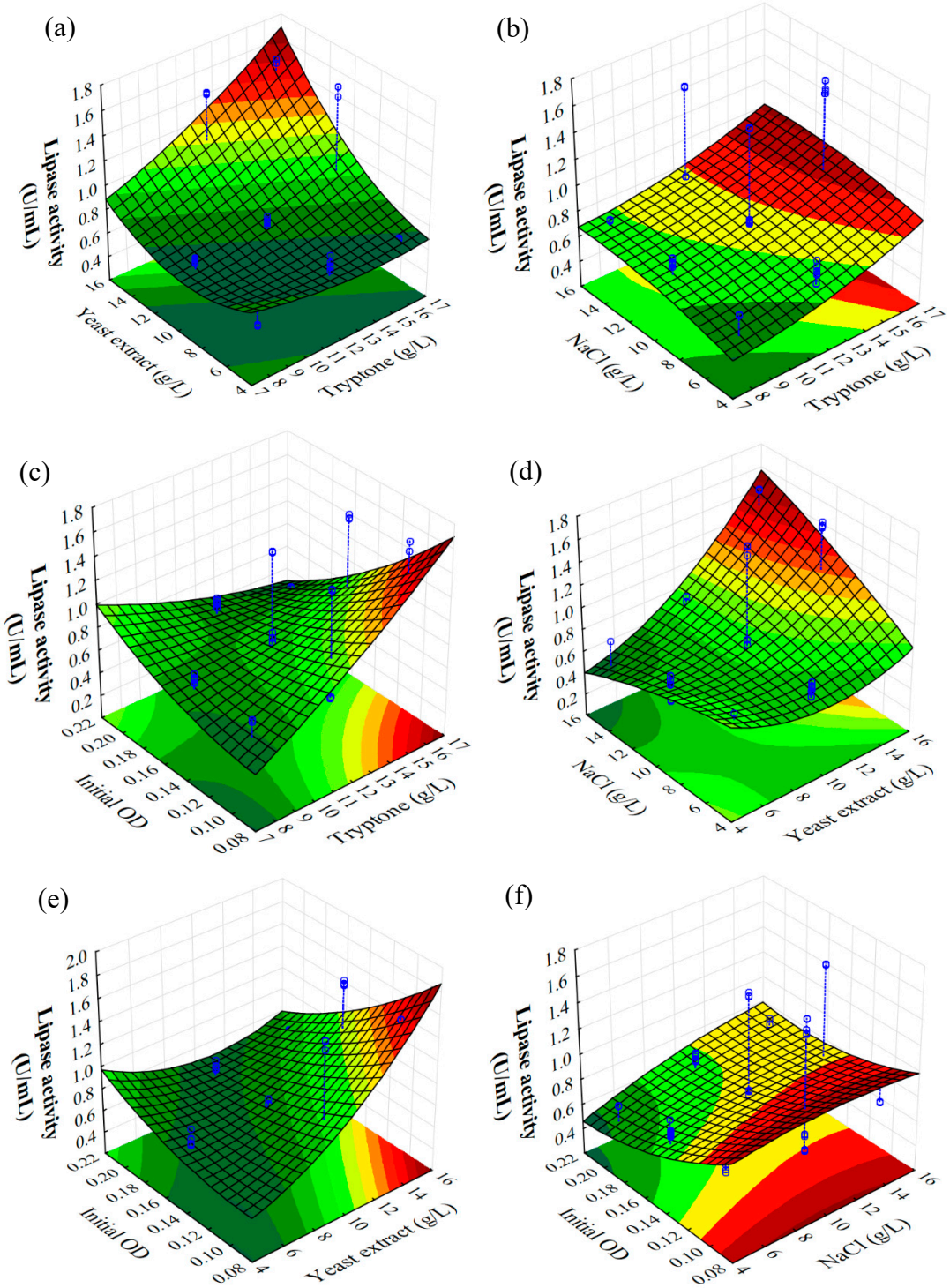

Figure 2. Three-dimensional surface response graphs of the lipase activity as a function of (**a**) the tryptone and yeast extract concentrations, (**b**) the tryptone and NaCl concentrations, (**c**) the tryptone concentration and initial OD, (**d**) the yeast extract and NaCl concentrations, (**e**) the yeast extract concentration and initial OD, and (**f**) the NaCl concentration and initial OD. For each test, the other factors were fixed to their central levels. Bleu points present the tested experimental values.

2.4. Effects of pH and Temperature on B. subtilis Lipase Activity and Stability

The effect of the pH on lipase activity and stability was investigated in this study. The lipase exhibited optimum activity at pH 8 (Figure 4a). Similarly, Akhter et al. [33] reported an optimal lipase activity at pH 8 for *Bacillus cereus* NC7401.

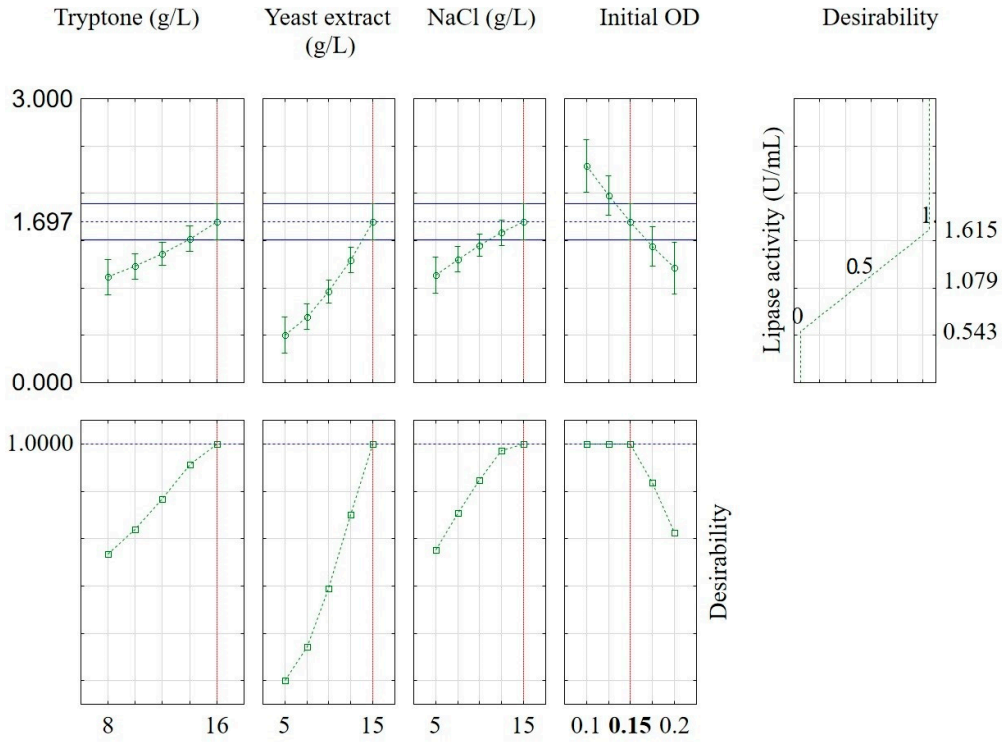

Figure 3. Profiles for the predicted values and desirability. Green curves represent the lipase activity evolution as function of each factor. Vertical green lines represent the error bars (−95% to +95% range). Red lines represent the level of the factor giving the optimum of lipase activity. Dash blue lines represent the predicted optimum value of lipase activity and its desirability. Continuous blue lines represent the range of lipase activity.

The pH stability of the lipase was examined by incubating the enzyme for 1 h at room temperature over a wide range of pH levels, ranging from 3 to 12. Our results show that the *B. subtilis* lipase was very stable over an extensive pH range and retained 99% of its activity at pH 8 and 70 to 80% at pH values ranging between 4 and 11 (Figure 4a).

Like most bacterial lipases, those from the Bacillus genus are primarily active and stable at neutral or alkaline pH values. Zhao et al. [34] reported that the NCU S6 lipase from *Staphylococcus caprae* exhibited optimal activity at pH 9, and the enzyme was stable at pH values ranging from 6 to 9. Other researchers found that the maximum lipase activity from *Bacillus subtilis* was obtained at pH 8. Moreover, Akhter et al. [33] proved that a *Bacillus cereus* NC7401 lipase was stable over a pH range of 5 to 10. Their results suggested that the lipase was suitable for applications under alkaline conditions.

It is well known that thermal stability is advantageous for applications and industrial processes. In this study, we report the effect of temperature on lipase activity and stability. The lipase activity was investigated at different temperatures from 25 to 60 °C (Figure 4b) under standard assay conditions at pH 8 and using *p*-NP palmitate as a substrate. Our results showed that the lipase is significantly active at this interval of temperature values, except at 25 and 60 °C. The maximal activity was recorded at 45 °C (Figure 4b). The optimum temperature for lipase activity is in accordance with previous findings corresponding to *B. subtilis* and *Lysinibacillus macroides* lipases [35,36]. Meanwhile, Zhao et al. [34] found that the highest lipase activity from *Staphylococcus caprae* NCU S6 was obtained at 40 °C.

Regarding the thermal stability profile of the lipase, our results, represented in Figure 4b, show that the enzyme is thermostable. The lipase could maintain approximately 94% and 99% of its initial activity at 40 °C and 50 °C, respectively, after 15 min of incubation using *p*-NP palmitate as the substrate. These results conform to others showing that the lipase of *Lysinibacillus macroides* was stable at high temperatures [36]. This remarkable stability makes our *B. subtilis* lipase a potential candidate for industrial applications.

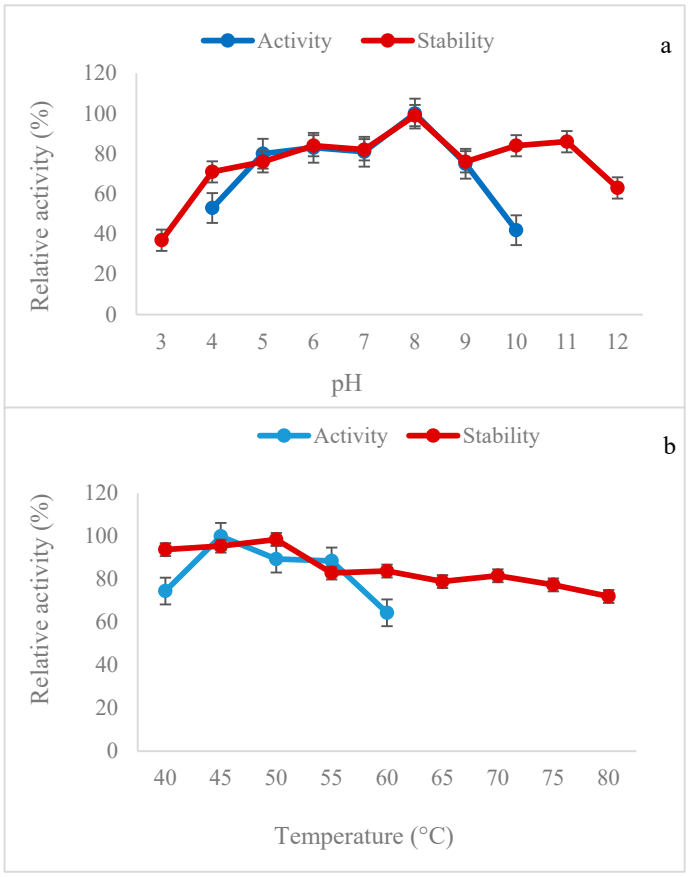

Figure 4. (**a**): Effect of pH on lipase activity and stability. The lipase activity was measured using para-nitrophenyl palmitate as a substrate at 45 °C and at various pH levels, ranging from 5 to 12. The pH stability was examined through incubation of the enzyme for 1 h at 25 °C in various media, namely citrate buffer (pH 3.0–6.0), phosphate buffer (pH 6.0–8.0), Tris–HCl (pH 7.5–9.0), and carbonate buffer (pH 9.0–12.0). (**b**): Effect of temperature on lipase activity and stability. The lipase activity was investigated using para-nitrophenyl palmitate as a substrate at pH 8 and different temperatures ranging from 25 to 60 °C. The thermostability was studied by incubating the enzyme for 15 min at temperatures ranging from 20 to 90 °C. The residual activity was measured under standard conditions (45 °C and pH 8) using para-nitrophenyl palmitate as the substrate.

2.5. Effects of Incubation Time and Substrate Concentration on Lipase Activity

Our results showed that 15 min of incubation for the mixture of the enzyme and substrate in the water bath exerted the maximum activity (0.593 U/mL). The lipase enzyme remained active until 20 min, and then its activity declined. The decline in enzyme activity after 20 min of incubation could be due to either a decrease in the substrate availability or the enzyme's catabolizing repression. This result is in line with those obtained previously [37]. Other lipases showed a curve with an upward trend even after 20 min of incubation time, which continued to increase as the incubation period increased [38].

Lipase activity is highly affected by physicochemical conditions, such as the enzyme–substrate molar ratio, by playing a notable role in the variation in enzymatic activity. The reaction rate is relatively slow in the range of low substrate concentrations, whereas it gradually increases with the substrate concentration until the substrate saturation point is reached [39]. To investigate the effects of the substrate concentration, lipase activity was measured using different initial concentrations of *p*-NPP (0.5–1.5 mM) under optimum assay conditions (pH 8 and 45 °C) for 15 min. The results show that the lipase activity was 50% up to a concentration of 0.5 mM of the substrate, where the enzyme activity gradually increased. The optimum lipase activity (100%) was reached at a substrate concentration of 1 mM. Beyond this value, the lipase activity decreased. The detailed catalytic characteristics

of the studied lipase differ; however, our results indicate that the present range of *p*-NPP concentrations is enough to find various lipases with adequate catalytic activity in a small-scale reaction. Notably, the lipase activity was reduced at higher substrate concentrations; this phenomenon is similar to the results reported by Park et al. [39], in which the *Chromobacterium viscosum* and *Pseudomonas fluorescens* lipases showed a reaction that started slow in a range of low substrate concentrations, then gradually increased with increases in the substrate concentration. The optimum substrate concentrations for the lipase activity of *C. viscosum* and *P. fluorescens* were 3 mM and 2 mM, respectively. Other reports also show that lipase activity increases as the substrate concentration increases, as seen in the results reported by Martínez et al. [40].

2.6. Effects of Metal Ions on Lipase Activity

The effects of various metal ions on lipase activity were also assessed. The lipase was mixed with various metal ions and incubated at 45 °C for 1 h. Its residual activity was determined with *p*-nitrophenyl palmitate as the substrate. Most metal ions did not significantly affect the lipase activity (Table 5). In fact, residual activities of 87.2 and 77.2% were measured in the presence of $MgSO_4$ and $NaCl_2$, respectively. However, only 13.3, 10, and 5.1% residual activities were obtained in the presence of $CoCl_2$, KCl, and $FeSO_4$. Similar results were obtained by Sugihara et al. [41], who found that some metal ions did not affect the residual activity of a lipase from *Bacillus* sp. According to Hasanah et al. [42], $FeSO_4$ and $MgSO_4$ inhibited the lipase, while Ghori et al. [43] showed that $FeSO_4$ and $MgSO_4$ enhanced *Bacillus* sp. lipase activity, indicating that these ions do not compete with the enzyme. The inhibitory nature of transition metals has been thought to be due to the interaction of ions with charged side-chain groups of surface amino acids, thus influencing the conformation and stability of the enzyme [41]. This indicates that the effects of metal ions on different lipases are unequable.

Table 5. Effects of metal ions, inhibitors, and surfactants on the lipase activity.

Metal Ions	Relative Activity (%)	Inhibitors and Surfactants	Relative Activity (%)
	5 mM		0.1%
Control	100.0	Control	100.0
$NaCl_2$	77.2	EDTA	32.4
$CaCl_2$	68.6	DTT	11.5
$NiCl_2$	72.3	BME	37.4
KCl	10.0	SDS	14.2
$MnCl_2$	67.5	H_2O_2	59.2
$CoCl_2$	13.3	Tween 20	17.0
$MgSO_4$	87.2	Tween 80	16.4
$FeSO_4$	5.1		

2.7. Effects of Enzyme Inhibitors and Surfactants on the Stability of the Lipase

Various enzyme inhibitors and surfactants concentrated at 0.1% were also selected to study their effects on the lipase enzyme activity (Table 5). The results showed that the extracellular *B. subtilis* lipase activity was negatively affected by all of the studied inhibitors, especially by DTT and SDS, which blocked the lipolytic action by approximately 88.5% and 85.8%, respectively. In the presence of the non-ionic surfactants Tween 80 and Tween 20, residual activities of 17% and 16% were obtained, respectively. However, a relative activity of 59% was observed in the presence of H_2O_2. These results are in line with previous findings showing that *B. subtilis* PCSIRNL-39 lipase activity was inhibited by SDS, Tween 80, and Tween 20 [35].

2.8. Effects of Organic Solvents on the Stability of Lipase

The utilization of enzymes, especially lipases, in organic solvents is gaining much industrial importance as the process leads to the development of products of high added

value [44,45]. Many enzymes are easily inactivated or denatured in organic solvents. We tested the stability of the lipase in four different solvents: ethanol, isopropanol, acetone, and chloroform (Table 6). Compared with the control, organic solvents did not stimulate lipolytic activity. The maximum lipolytic residual activity was observed in ethanol, followed by isopropanol and chloroform, respectively; the exception was acetone, with which the residual enzyme activity was decreased. Our results show that in the presence of 0.1% of both ethanol and isopropanol, a residual activity of approximately 90% was obtained. Other concentrations did not stimulate the lipolytic activity; however, decreased lipolytic residual activity was observed at higher concentrations. Contradictory reports regarding the effects of organic solvents on lipase activity are found in the literature: A lipase from *Bacillus* sp. was stimulated in the presence of acetone [41]. On the other hand, the lipase activity from *Streptomyces bambergiensis* OC 25-4 was stable in the presence of ethanol [10].

Table 6. Effects of organic solvents on lipase activity.

Solvent	Concentration (%)	Relative Activity (%)
Control	0	100.0
Isopropanol	0.1	86.7
	0.3	75.7
	0.5	23.4
Ethanol	0.1	90.2
	0.3	63.2
	0.5	20.0
Acetone	0.1	39.1
	0.3	35.9
	0.5	7.0
Chloroform	0.1	59.5
	0.3	60.5
	0.5	11.5

2.9. Optimization of the Transesterification Reaction with the Immobilized Lipase

In order to determine the optimal conditions for biodiesel synthesis with the $CaCo_3$-immobilized lipase, the transesterification reaction was performed with different temperatures, reaction times, and speeds.

First, SEM analysis at various magnifications was used to confirm the adsorption of the *Bacillus subtilis* lipase on $CaCO_3$. Our results show clearly the change in the morphology of $CaCO_3$ after immobilization (Figure 5). The surface of the free $CaCO_3$ particles presents a smooth and well-defined structure (Figure 5A–C). After lipase adsorption (Figure 5D–F), the support changes into an irregular structure, indicating the modification of the surface by enzyme adsorption, resulting in the formation of enzyme-supporting aggregates (Figure 5D–F).

Temperature is a critical variable that must be optimized during transesterification. As a result of these considerations, the optimal temperature for transesterification was set to 45 °C, contributing the highest fatty acid ethyl ester (FAEE) yield of 71% (Figure 6a). Despite this, the highest biodiesel yields were obtained at temperatures far lower than the boiling point of ethanol or methanol. Moreover, this result is important financially, since it decreases manufacturing costs. As a result, the optimum temperature for the reaction was determined to be 45 °C. This result confirms previous findings [44]. The initial decrease in the curve suggests that the reaction did not reach the required temperature; after that, it was increased at the optimum temperature. Binhayeeding et al. [45] reported that 45 °C was the optimum temperature for biodiesel production using a mix of PHB-immobilized *C. rugosa* and *R. miehei* lipases. Similarly, Tran et al. and Ben Bacha et al. [46,47] found that the curve of biodiesel production using the *C. vulgaris* ESP-31 lipase declined after increasing the temperature, because exposing a lipase to overheating leads to protein denaturation and, therefore, reduced catalytic activity.

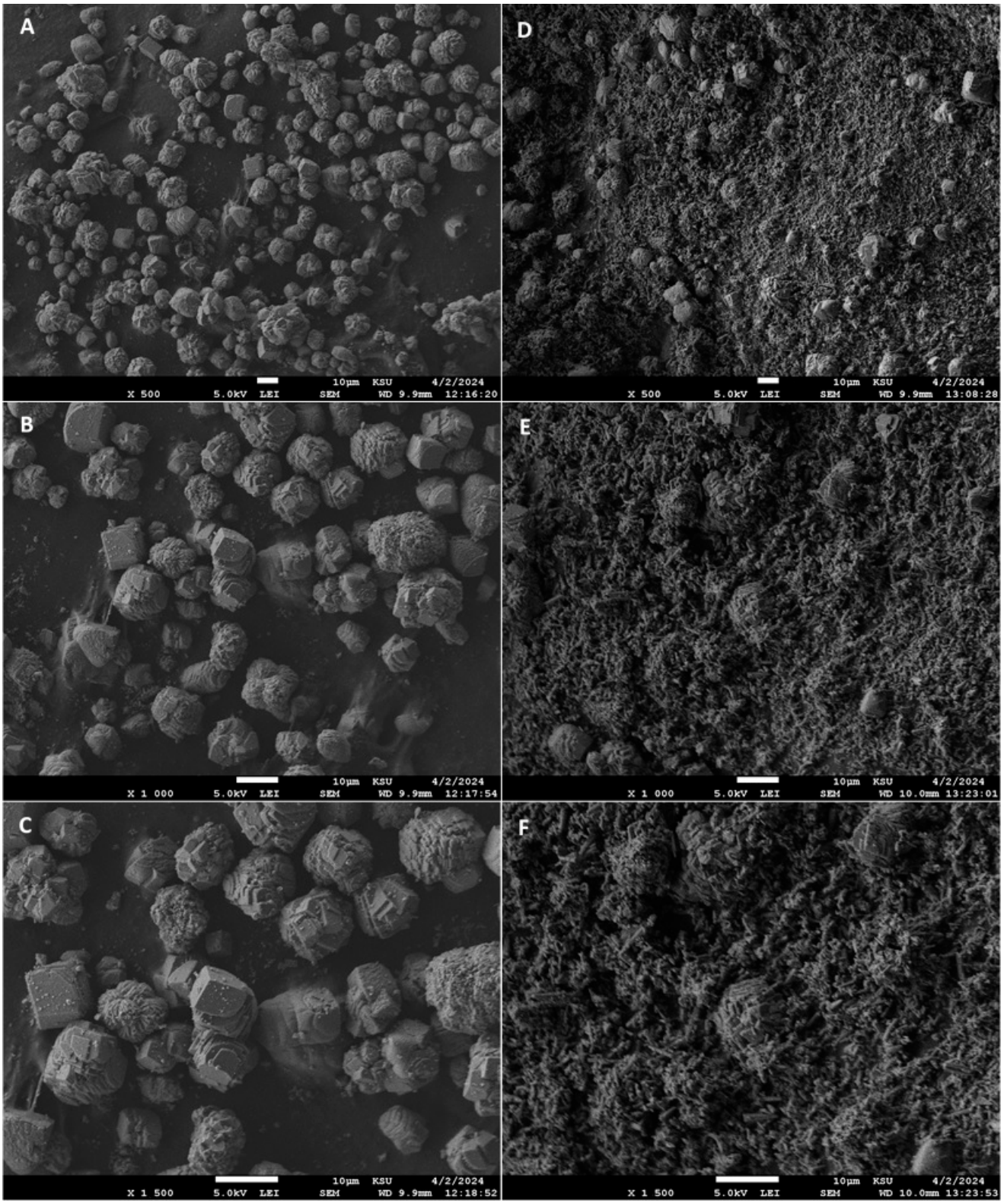

Figure 5. Field-emission scanning electron microscopy (FE-SEM) images of calcium carbonate before and after lipase immobilization. (**A**–**C**) Different magnifications of the $CaCO_3$ before immobilization ((**A**), 500×; (**B**), 1000×; (**C**), 1500×). (**D**–**F**) Various magnifications of the $CaCO_3$ after lipase adsorption ((**D**), 500×; (**E**), 1000×; (**F**), 1500×).

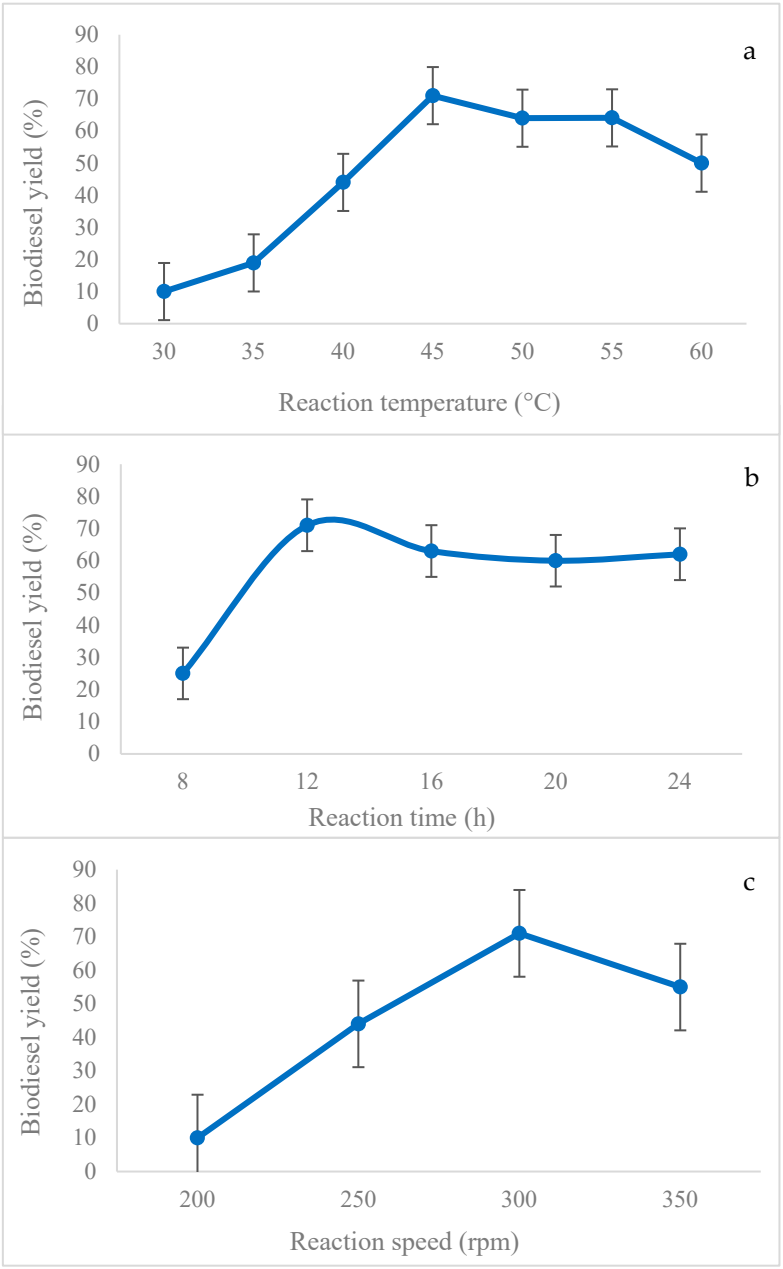

Figure 6. Effects of temperature (**a**), time (**b**), and agitation (**c**) on the transesterification reaction catalyzed by the $CaCO_3$-immobilized *Bacillus subtilis* lipase. The final yield of fatty acid ethyl esters (FAEEs) was calculated as described in Section 3.

The effect of the reaction time on the immobilized enzyme was examined throughout a range of reaction periods (8, 12, 16, 20, and 24 h). As illustrated in Figure 6b, as the reaction time grew from 8 to 12 h, and biodiesel production climbed from 25% to 71%, with the most significant yield being recorded at 12 h. On the other hand, increasing the reaction time at 45 °C, particularly after 12 h, resulted in a slight reduction in yield. Other groups have used immobilized lipases to produce biodiesel after 24 h [44,47]. In contrast, our immobilized lipase of *B. subtilis* was used for a catalyst reaction to produce biodiesel at 12 h. As a result, our subsequent analyses utilized a 12 h reaction time to improve the other parameters. It is worth noting that short reaction times offer several advantages, such as lower energy consumption, thereby facilitating large-scale production.

Several studies have demonstrated that high biodiesel production yields might be reached with immobilized enzymes using an optimized agitation speed [48]. In this investi-

gation, agitation speeds in the range of 200 to 350 rpm were considered. Figure 6c shows the effects of the agitation speed on converting WCO to biodiesel. The results revealed that the 300 rpm mixing speed, for this particular reaction, was sufficient for the biodiesel conversion to succeed at 71%. Poor agitation may lead to a lower reaction rate, thus affecting the conversion rate, as shown in the range of 200 to 250 rpm. Therefore, 300 rpm was selected as a suitable mixing speed for the transesterification of WCO. Moreover, the conversion at 300 rpm was higher than that at 350 rpm, which, when increasing the mixing rate decreases the conversion rate, clearly indicates that diffusion-controlling steps have an effect at higher mixing rates [47]. Similarly, according to Tran et al. [46], when the speed is increased beyond the optimum, it could likely cause agitation-associated mechanical damage to the immobilized enzyme.

2.10. Transesterification Reaction

The immobilized B-1-4 lipase produced by *Bacillus subtilis* was used to produce fatty acid ethyl esters. After the transesterification reaction, two layers were formed: the top layer was the fatty acid ethyl esters, and the bottom was glycerol (Figure 7). The immobilized lipase of *B. subtilis* showed good performance for biodiesel production from waste cooking oil. A 71% yield of fatty acid ethyl esters (FAEEs) was confirmed using GC-MS, which indicates that the immobilized lipase exhibited catalytic activity in the transesterification reaction. The GC-MS analysis of the obtained biodiesel showed that each peak represented individual FAEE components. As shown in Figure 8, the most abundant esters were eluted at retention times of 18.57, 20.764, and 23.669 min, corresponding to an oleic acid ester, linoleic acid ester, and arachidic acid ester, respectively. These results are in line with those obtained by Shi et al. [49]. Moreira et al. (2020) obtained a 78.9% yield of fatty acids ethyl esters from free fatty acids of babassu oil by using an immobilized lipase from *Rhizomucor miehei* [50].

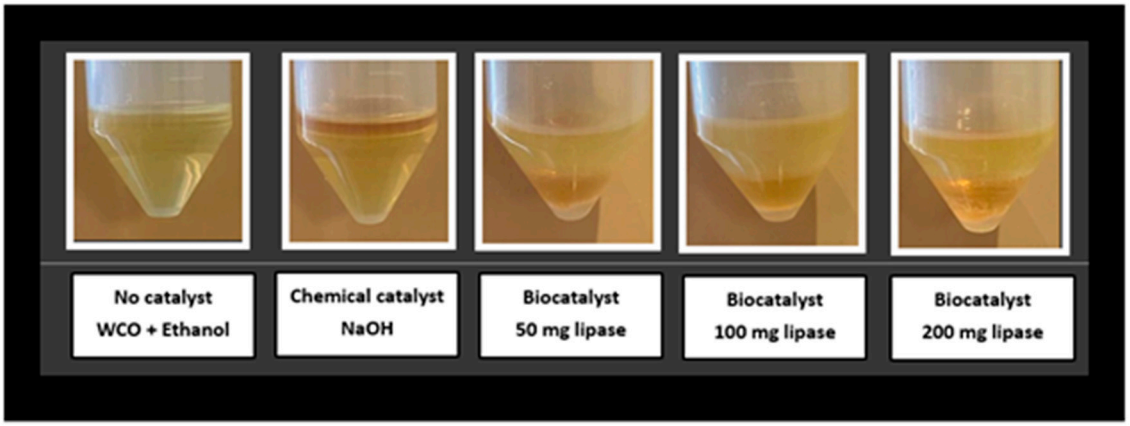

Figure 7. Effects of enzyme quantity on the transesterification reaction. The reaction was set at 45 °C and 300 rpm for 12 h using different quantities of a $CaCO_3$-immobilized lipase (50, 100, and 200 mg). The upper and lower phases represent the synthesized biodiesel and the aqueous phase (excess of ethanol + glycerol), respectively. A control was prepared using 3 mL of waste cooking oil and 0.875 mL of 99% ethanol. The final yield of fatty acid ethyl esters (FAEEs) was calculated as described in Section 3.

2.11. Reusability of the Immobilized Lipase

The reusability of the $CaCO_3$-immobilized lipase was also tested. After the first reaction, 80% of the original lipase activity was maintained. A 68.3% residual activity was measured after the second reaction, indicating potential reusability under the optimized experimental conditions, probably due to the enzyme's interaction with the support, enhancing its stability. Nevertheless, after three cycles, the activity decreased drastically to 23.9% of the initial lipase activity. After numerous uses of the ethanol and reaction

cycles, the conformational properties of the lipase were remarkably altered, and this caused inactivation; also, this decrease in activity could be explained by the leaching of the enzyme from the support [47]. The results found by Hernández-Martín and Otero [51] showed a 90% loss of activity after the first recycling. However, lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei* lost more than 75% of their initial activity after their first reuse, and almost 90% after the second cycle [52].

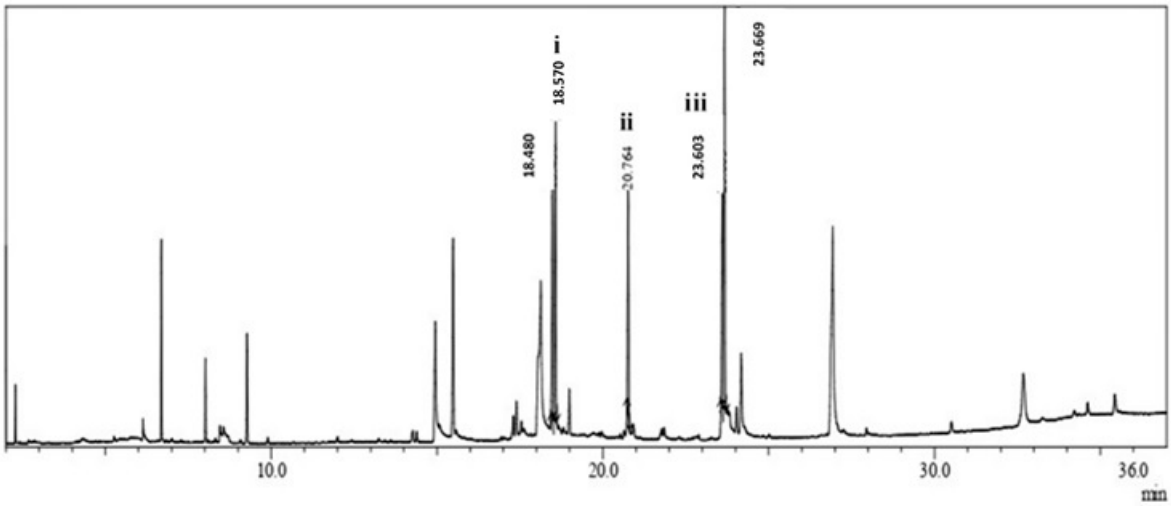

Figure 8. Gas chromatography–mass spectrometry (GC-MS) runs of the fatty acid ethyl esters produced through the enzymatic transesterification of waste cooking oil using $CaCO_3$-immobilized *Bacillus subtilis* B-1-4 lipase. (i) Oleic acid ester, (ii) linoleic acid ester, and (iii) arachidic acid ester.

2.12. FTIR Analysis

The FTIR spectra of $CaCO_3$ and the $CaCO_3$-immobilized lipase before and after the reuse of the immobilized biocatalyst are shown in Figure 9. Figure 9a shows the typical absorption peaks at 1427 cm^{-1}, 856 cm^{-1}, and 711 cm^{-1} associated with $CaCO_3$. The last two peaks are often associated with calcite [53]. After lipase immobilization (Figure 9b), new bands were observed at 1153 cm^{-1}, 3404 cm^{-1}, and 3545 cm^{-1}, corresponding to the presence of C-O, N-H, and O-H stretching, respectively [53,54]. Moreover, after three consecutive cycles and repetitive washes (Figure 9c), two major peaks appeared at 2920 cm^{-1} and 2850 cm^{-1}, corresponding to C-H stretching of the alkane due to the possible adsorption of the synthesized esters to $CaCO_3$. The presence of the peak at 3412 cm^{-1} in Figure 9c confirms that the decrease in activity after three uses could be due to the structural modification and denaturation of the enzyme after numerous washes with ethanol and reaction cycles. The shifts observed in other peaks may imply conformational changes caused by the adsorption process on the surface of the $CaCO_3$ [53].

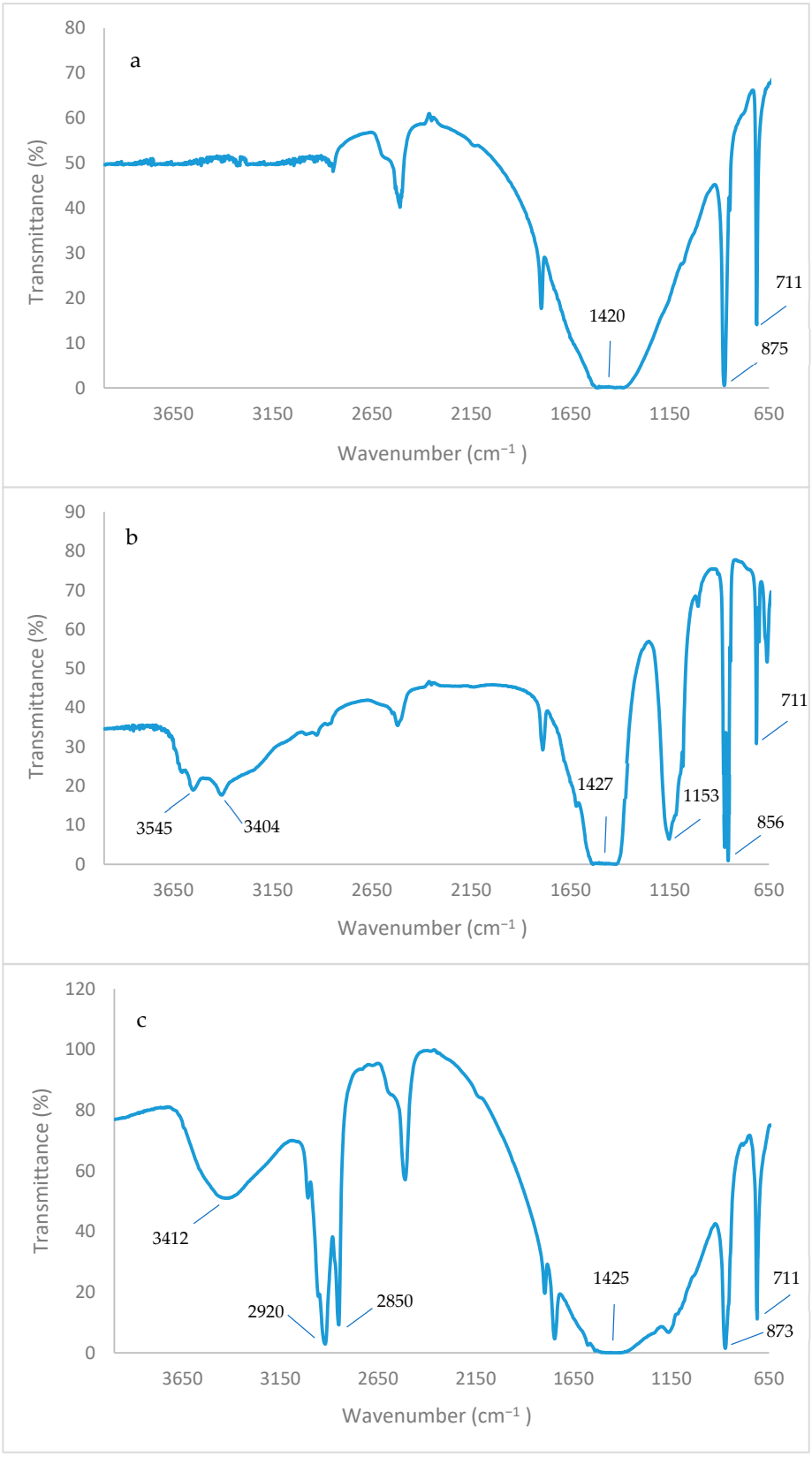

Figure 9. FTIR spectra of $CaCO_3$ before and after lipase immobilization. (**a**) FTIR spectra of $CaCO_3$. (**b**) FTIR spectra of the $CaCO_3$-immobilized lipase. (**c**) FTIR spectra of the $CaCO_3$-immobilized lipase after three consecutive cycles and repetitive washes.

3. Material and Methods

3.1. Chemicals

Rhodamine B, phenol red, and olive oil were purchased from JADCO (Sakakah, Saudi Arabia); the yeast extract, agar, para-nitrophenyl palmitate, and all other chemicals were from Sigma Chemical (St. Louis, MO, USA).

3.2. Screening of Lipase-Producing Bacteria

Bacteria were isolated from oil-contaminated soil and olive pomace–soil mixtures from the Al-Jouf region, Saudi Arabia. Initial lipolytic bacteria screening of 80 strains was performed using rhodamine–olive oil agar and phenol red–olive oil agar media [55]. The rhodamine–olive oil agar medium contained 2.5% olive oil, 0.5% peptone, 0.3% yeast extract, 0.4% NaCl, 1.5% agar, and 1% rhodamine B (pH 7). The phenol red–olive oil agar was prepared with 1% olive oil, 0.5%, 0.3% yeast extract, 0.1% $CaCl_2$, 1.5% agar, and 1% phenol red (pH 7.4). Pure bacterial strains were streaked separately onto plates and incubated at 45 °C for 12 h. Colonies with orange-fluorescent halos or giving rise to a widespread pink clearing around them, respectively, were considered putative lipase producers.

3.3. Identification of the Selected Strain

The extraction of genomic DNA from the selected strain was carried out by centrifuging the bacterial culture at 6000 rpm for 15 min. DNA was extracted using a Gene JET Genomic DNA Purification kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

For the species identification, 16S rDNA universal oligonucleotide primers were used as described previously [56]. An internal part of the 16S rRNA gene was amplified using genomic DNA as a template and universal forward-flowing (5′GCAACGAAGTATGGAACTGC3′) and reverse-flowing (5′TTAATAAATCGCCTTATTAAAGG3′) primers.

The DNA sequence of the 16S rRNA gene was amplified using a thermal cycler PCR machine (Bibby Scientific, Stone, UK) in a reaction mixture (25 µL) containing 2 µL of DNA, 1 µL of each primer (10 pmol), 12.5 µL of the master mix (2×), and 9.5 µL H_2O.

Sanger sequencing was carried out at the Beijing Genomic Institute (BGI), Hong Kong, China, for the 16S rRNA gene. The sequence was compared to the NCBI database using the BLAST software (BLAST+ 2.15.0). The obtained sequences were aligned in Ugene [57] using the T-Coffee algorithm (https://www.ebi.ac.uk/, EMBL-EBI, Cambridgeshire, UK), accessed on 1 July 2022.

3.4. Growth of Lipase-Producing Bacteria

The selected lipase-producing bacteria were precultured in a 250 mL Erlenmeyer flask containing 25 mL of unstimulated nutrient broth medium at pH 8. The 12 h overnight preculture was inoculated in another Erlenmeyer flask containing 25 mL of the same production medium with a 0.1 initial optical density at 600 nm and incubated on a shaker at 45 °C and 230 rpm. Growth was followed by determining the culture OD at 600 nm every 2 h. The tested culture was centrifuged at 6000 rpm for 15 min, and the supernatant was used for lipase activity assays.

3.5. Lipase Activity Measurement

Measurements of lipase activity were examined using a spectrophotometric method with slight modifications, using para-nitrophenyl palmitate as a substrate. The reaction mixture consisted of 0.1 mL lipase solution, 0.8 mL 0.5 M Tris–HCl buffer (pH 8), and 0.1 mL of 0.01 M para-nitrophenyl palmitate dissolved in isopropanol [58]. The reaction mixture was incubated at 45 °C for 15 min. Then, 0.25 mL of 0.1 M Na_2CO_3 was added to stop the reaction. The reaction mixture's final volume (1.25 mL) was centrifuged at 11,000 rpm for 15 min, and then the supernatant's optical density (OD) was determined at 410 nm. Finally, the lipase activity of both crude and partially purified enzymes was determined using the standard curve of para-nitrophenol (Equation (2)). One unit of lipase

activity was defined as the ability of several enzymes to free 1 µmol of para-nitrophenol from the para-nitrophenyl palmitate per minute under assay conditions [58].

$$\text{unit/mL } enzyme = \frac{(\mu mole\ para-nitrophenol\ equivalents\ released) \times (11)}{(1) \times (10) \times (2)} \quad (2)$$

where 11 = total volume (in milliliters) of the assay; 10 = time of the assay (in minutes), as per the unit definition; 1 = volume (in milliliters) of the enzyme used; 2 = volume (in milliliters) used in colorimetric determination.

3.6. Media Optimization for Bacillus subtilis Lipase Production

The one-factor-at-a-time method was used to select the best carbon and nitrogen sources and to study the effects of temperature, pH, agitation, and incubation time on lipase production. In this context, cultural media were tested, and the lipase activity was measured every 2 h to follow the enzyme production. The nutrient broth culture medium (50 mL) was inoculated from a 12 h preculture. The culture medium was calibrated at pH 8 and then incubated aerobically on a rotary shaker set at 45 °C and 230 rpm for 14 h.

The experimental design using response surface methodology was set up to determine the best culture conditions for optimal enzyme production. The effects of four factors (tryptone concentration, yeast extract concentration, NaCl concentration, and initial OD) were studied.

Each factor was studied at three levels: (−1) low, (0) medium, and (+1) high (Table 1). The minimum and maximum ranges of variables were obtained from preliminary studies. A matrix of 25 experiments with 4 factors was generated (Table 2). All tests were carried out in triplicate, and the lipase activity values, which were taken as a dependent response (Y), are presented as the mean ± standard error (Table 2).

The mathematical model design could be sufficient to fit a multivariable polynomial quadratic model, which includes the square effects and interaction effects between factors (Equations (3) and (4)):

$$y_k = \hat{y}_k + e_k \quad (3)$$

$$\hat{y}_k = \beta_0 + \sum_{i=1}^{n} \beta_i \cdot x_{ik} + \sum_{i=1}^{n} \beta_{ii} \cdot {x_{ik}}^2 + \sum_{i=1}^{n} \sum_{j>i}^{n} \beta_{ij} \cdot x_{ik} \cdot x_{j_k} \quad (4)$$

where y_k and $\hat{y}_k$ represent the experimental and calculated kth values of the dependent variable (enzyme production in U/mL), respectively. The calculated values were obtained using a multivariable polynomial quadratic model (Equation (4)), which was determined using the STATISTICA 12 Software, as will be described later; k is the experiment number ($1 \leq k \leq 25$); e_k is the difference between the kth experimental and calculated values; β_0, β_i, β_j, and β_{ij} are the model's intercept, linearity, interaction, and quadratic coefficient; x_{ik} is the centered reduced level (value equals to −1, 0, or +1) of factor i of the kth experiment; n is the number of factors.

3.7. Effects of pH and Temperature on Lipase Activity and Stability

To determine the effects of pH on lipase activity, buffers with various pH levels ranging from 4 to 10 were used. The pH stability of the isolated lipase was examined by incubating it in various buffer solutions at 0.5 M: sodium acetate (pH 3.0–6.0), Tris–HCl (7.0–9.0), and sodium bicarbonate (10.0–12.0). After 1 h incubation at 4 °C, residual lipase activity was measured under standard conditions using *p*-NPP as the substrate.

The optimal temperature for the lipase activity was determined with a spectrophotometric assay using *p*-NPP as the substrate at different temperatures (25–60 °C) and at pH 8. The thermal stability of the enzyme was evaluated by measuring the residual activity after incubating the enzyme solution for 15 min at various temperatures (40–80 °C). The remaining enzyme activity was measured using the standard *p*-NPP method and calculated while considering the initial activity to be 100% [10].

3.8. Effects of Incubation Time and Substrate Concentration on Lipase Activity

The effects of the incubation time on the lipase activity were studied by measuring the enzyme activity under standard conditions (45 °C, pH 8) for different incubation times (5–25 min). *p*-NPP was used as a substrate [37].

In order to determine the optimal substrate concentration, the lipase activity was measured under optimal conditions (45 °C, pH 8, and for 15 min) using different *p*-nitrophenyl palmitate concentrations (0.5, 0.75, 1, 1.25, and 1.5 mM) as substrates.

3.9. Effects of Metal Ions, Enzyme Inhibitors, and Surfactants on Bacillus subtilis Lipase Activity

The effects of metal ions, inhibitors, and surfactants on the crude lipase activity were investigated. Using 5 mM of each one, various metal ions ($NaCl_2$, $CaCl_2$, $NiCl_2$, KCl, $MnCl_2$, $COCl_2$, $MgSO_4$, and $FeSO_4$) were individually incubated with the enzyme for 60 min. The residual activity of the lipase was measured under standard conditions.

Inhibitors concentrated at 0.1% (ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), β-mercaptoethanol (BME), sodium dodecyl sulphate (SDS), and H_2O_2), and surfactants (Tween 20 and Tween 80) [34] were incubated with the enzyme at room temperature for 1 h. The lipase activity without adding metal ions, inhibitors, or surfactants was defined as 100%, and the relative activity was determined compared to the control. Experiments were performed as described above, using *p*-NPP as a substrate.

3.10. Effects of Organic Solvents on the Stability of Lipase

The effects of various organic solvents (ethanol, isopropanol, acetone, and chloroform) on the lipase activity were investigated. The crude enzyme was incubated in different concentrations (0.1, 0.3, and 0.5%) of organic solvents for 1 h at 37 °C. The control was the crude enzyme without any organic solvents and incubated under the same experimental conditions. Residual activity was measured under standard conditions using *p*-NPP as the substrate. Residual activity was expressed by taking the activity without any organic solvents as 100% [10].

3.11. Immobilization of Bacillus subtilis Lipase

A 12 h overnight preculture of the lipolytic strain was used to inoculate a flask containing 350 mL of a nutrient broth medium at a final absorbance of 0.1 at OD600 nm. The culture was grown for 14 h at 45 °C and 230 rpm. The culture was then centrifuged at 6000 rpm for 15 min to eliminate cells. The obtained crude enzyme solution (300 mL), containing 1 U/mL, was brought to 80% saturation with ammonium sulphate powder under stirring conditions and at 4 °C. After centrifugation (10,000 rpm for 10 min and 4 °C), the pellet was dissolved in 2 mL of 0.05 M Tris–HCl buffer (pH 8). The obtained dissolved pellet was immobilized via adsorption with $CaCO_3$ powder [47]. Finally, the suspension was washed with ethanol three times and filtrated using a Buchner funnel, and then the powder was dried at room temperature. The immobilized lipase activity was measured using *p*-NPP as a substrate under standard conditions. The dried immobilized lipase was used in the transesterification reaction.

3.12. FT-IR and FE-SEM Analyses

FTIR analysis was used to examine the enzyme's interactions with $CaCO_3$. The FT-IR spectra of the dried forms of $CaCO_3$ and $CaCO_3$-immobilized lipase (before and after reuse) were recorded using a Fourier Transform Infrared Spectrophotometer (Shimadzu IRPrestige-21, Kyoto, Japan) ranging from 4000 to 650 cm^{-1}. Field-emission scanning electron microscopy (FE-SEM) (JSM-7600F JEOL, Akishima, Japan) was used to examine the morphology of the carrier before and after the immobilization of the *Bacillus subtilis* lipase. Dried samples were positioned on conducting carbon atop aluminum stubs. The SEM images were taken at an accelerating voltage of HV 5.00 kV and a pressure of 9.5×10^{-5} Pa.

3.13. Optimization of the Biodiesel Synthesis Process from Waste Cooking Oil

The effect of the $CaCO_3$-immobilized lipase's quantity (7.1 U/mg) on biodiesel synthesis was first examined by mixing 3 mL of waste cooking oil with different amounts of the immobilized lipase (50, 100, and 200 mg) and 0.875 mL of 99% ethanol. Then, the transesterification reaction was performed with different temperatures (30, 35, 40, 45, 50, 55, and 60 °C), reaction times (8, 12, 16, 20, and 24 h), and speeds (200, 250, 300, and 350 rpm) to define the conditions needed for the best biodiesel production yield. At the end of each batch, the immobilized enzyme was removed, and the reaction media were left for 1 to 2 h until the resulting biodiesel was separated from the aqueous phase. A control was prepared using 3 mL of waste cooking oil and 0.875 mL of 99% ethanol [59].

3.14. Fatty Acid Ethyl Ester Analysis

Separation and identification of the fatty acid ethyl esters (FAEEs) were performed and analyzed using the gas chromatography–mass spectroscopy (GC-MS) technique (Thermo Fisher Scientific, Waltham, MA, USA). The Agilent gas chromatograph system used has a mass spectrometer detector and capillary column (30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness). Helium was used as a carrier gas (1 µL/min) and the mass spectrometers were operated with an injection sample volume of 1.0 µL. The column temperature was set at 250 °C as the maximum temperature. The yield of fatty acid ethyl esters (FAEEs) was calculated using the following formula (Equation (5)) [60]:

$$FAEE\ yield\ \% = \frac{weight\ of\ FAEEs\ in\ grams}{total\ weight\ of\ oil\ in\ grams} \times 100 \quad (5)$$

3.15. Reusability of the Immobilized Lipase

To evaluate its reusability, the immobilized enzyme was recovered after each transesterification reaction and reused for subsequent cycles under similar conditions. After each cycle, the $CaCO_3$-immobilized lipase was filtered using filter paper, washed twice with ethanol, and dried at room temperature before being reused. These experiments were carried out until the lipase exhibited no detectable activity. The residual hydrolytic activity of the immobilized lipase was measured after each reaction under standard conditions, using para-nitrophenyl as the substrate. The initial activity of the immobilized lipase was considered to be 100%.

3.16. Statistical Analyses

The software used for the experimental design, determination of the models' coefficients, statistical analyses (Levene's test, linearity test, ANOVA, Student's *t*-test, statistical coefficients, significance test), the drawing of graphs, and the optimization protocol was STATISTICA 12, Copyright© Stat Soft, Inc., Tulsa, OK, USA, 1984–2014.

The models' coefficients were determined using the least-squares method. Analysis of variance (ANOVA) and Student's *t*-tests were used to identify the level of significance of the studied model and the tested factors and their interactions, with a confidence level of 95% ($p < 0.05$). The coefficient of determination (R^2), the adjusted coefficient of determination (R^2adj), and the root-mean-square error (RMSE) were chosen to quantify principally the model's fitting quality.

4. Conclusions

This study presents a thermostable, unstimulated *Bacillus subtilis* B-1-4 lipase that was biochemically characterized and showed several important industrial characteristics. The activity of the produced lipase was optimized, and better culture conditions were determined. This lipase exhibited high-temperature resistance and great activity and stability at alkaline pH levels. In addition, the lipase was highly active in the presence of a variety of industrial organic solvents. This lipase was immobilized through adsorption on $CaCO_3$. Furthermore, this enzyme could be reused for two cycles, losing only 20%

of its activity after the first reuse and 32% after the second. Our results suggest that this thermostable $CaCO_3$-immobilized lipase is suitable for biodiesel production using waste cooking oil, providing future greening opportunities.

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