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ORIGINAL ARTICLE

Olive mill wastewater biodegradation for bacterial lipase production using a response surface methodology

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Abstract
Olive mill wastewater (OMW) represents the effluent generated during the olive oil extraction process. OMW, as an oil-rich residue, provides a source of lipase-producing microorganisms and a source of complex nutrients that is potentially suitable to be applied in bacterial lipase production. This study focused on isolating actinobacteria able to produce extracellular lipases with OMW as the sole carbon source. Thirteen isolates of actinobacteria have been obtained and screened for their lipase activity using rapid plate detection methods. Then, the extracellular lipolytic activity was monitored under shaking flask conditions, and the titrimetric method was applied for lipase activity determination. Maximum lipase activity was detected for the SC1 strain (5.33 U/mL), which was then identified as a *Streptomyces* species. To enhance lipase production using the SC1 strain, Box-Behnken design (BBD)-based response surface methodology (RSM) has been adopted, by varying incubation time (days), pH, temperature (°C), inoculum size (spores/mL), and initial OMW concentration (%, v/v). An accompanying analysis of variance (ANOVA) was carried out, and the production of lipase was reported by a mathematical equation according to the factors. Non linear regression equations with significant R^2 and *p*-values were used to depict individual term, interaction, and square effects on lipase production. Individual term, interaction, and square effects on lipase production were shown using nonlinear regression equations with significant R^2 and *p*-values. The optimization results obtained showed that the optimal lipase activity was achieved after a 9 days incubation time at pH 4, with an inoculum size of 1.7×10^7 (spores/mL), incubated at a temperature of 30°C using 6% (v/v) OMW as the sole carbon source.

Keywords Olive mill wastewater · Lipase activity · Actinobacteria · Response surface methodology · Biodegradation

1 Introduction

Olive mill wastewater (OMW) is produced annually in large quantities around the Mediterranean basin. Olive mill wastewater is considered a toxic pollutant, especially when discharged into aquatic environments [1]. This toxicity is mostly because it contains fats and oils that have previously undergone physical and chemical treatments and are now in a dispersed form. Recently, the biological treatment process is a more efficient method to eliminate fats and oils by degradation to miscible molecules. Oil mill wastewater can be considered a resource that contains simple and complex carbohydrates, proteins, and minerals and therefore can be used for fermentation processes. Furthermore, the wastewater contains lipids and residual oil, a fact that makes the effluent the ideal medium for the growth of lipase-producing microorganisms [2].

Lipases (triacylglycerol acyl hydrolase; E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis and synthesis of esters from long-chain glycerol and fatty acids. For decades lipases have been employed in various industries to hydrolyze fats and catalyze several useful reactions including esterification and transesterification—applications typically applied in leather industries [3].

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Through their catalytic action, lipases help to minimize the environmental impact of OMW. In addition, lipases can be used in oleochemical processing. The application of lipases in oleochemical processing conserves energy and reduces thermal degradation in alcoholysis, acidolysis, hydrolysis, and glycerolysis. Promising applications of lipases include organic chemical processing, detergents formulation, synthesis of biosurfactants, the dairy industry, the agrochemical industry, papermaking, nutrition, pharmaceuticals, and cosmetics processin [4].

The lipases have been discovered in many different species of plants, animals and microorganisms [5]. Microbial lipases, however, have drawn the industry's attention for their high levels for substrate specificity, selectivity, and stability, placing them among the leading groups of biocatalysts for biotechnology applications [6]. However, the popularity of lipases in various industries has led to a request for a new lipase source with novel catalytic characteristics, which necessitates the isolation and selection of new strains of lipase-producing microorganisms. Microorganisms producing lipases were found in different habitats like plants processing vegetable oil, processing dairy plants, and contaminated soils by oils and industrial wastes, inter alia [7].

These ecosystems all share a sizable amount of lipid-based residual nutrients that can be used as a growth medium for microorganisms that produce lipase. Although significant work has been made in recent years toward creating cost-effective methods for lipases, the high cost of this enzyme's production still represents a significant barrier to widespread industrial use. Solid leftovers from the agriculture sector have been employed as inexpensive culture media and substrates to increase the economic viability of lipase production [8]. However, liquid effluents from palm oil mills and OMW have the potential to be utilized in the manufacturing of lipase [9].

Gram-positive soil bacteria called *Streptomycetes* have a remarkable ability to synthesize secondary metabolites and produce extracellular hydrolytic enzymes, including lipases, to break down organic matter in their natural environment [10]. However, only a small number of the lipases made by *Streptomyces* species have been identified. Two extremely similar lipases derived from *Streptomyces exfoliatus* and *Streptomyces albus* G have been genetically identified [11], and the *S. exfoliatus* enzyme's three-dimensional structure has been revealed [12].

Recently, different statistical schemes for fermentation condition optimization for lipase production were introduced to increase the enzyme yield. The RSM (response surface methodology) is a combination of a mathematical and a statistical approach used for analyzing the effects of several independent variables, permitting the interaction effects between the different input parameters to be identified [13]. In this study, lipase-producing actinobacteria were isolated from soil samples taken from olive oil mills in Bejaia, Algeria, and the conditions for lipase production by *Streptomyces* sp. strain SC 1 were adjusted using RSM based on the Box-Behnken design (BBD). OMW was used as the only carbon source for lipase production because it is a cheap resource that is accessible in Algeria. It was one of the variables examined in the RSM application together with the impact of incubation time, temperature, the initial pH of the production medium, and the impact of inoculum size on lipase application.

2 Materials and methods

2.1 Sampling, soil pretreatment, and preparation of spore suspensions

Samples of soil were collected from different olive oil mills in Bejaia, located in northeastern Algeria (Fig. 1). After removing the soil from the top 5 cm, an adequate amount of soil for each sample was collected, transferred into sterile plastic storage bags and directly transported to the laboratory. To promote actinobacterial growth, samples were pretreated by adding 0.1g of calcium carbonate ($CaCO_3$) to 1g of each sample and incubating them over 7–9 days at 37 °C. After incubation, samples were diluted serially to 10^{-5} and spread on International *Streptomyces* Project 2 (ISP-2) medium with the following composition per liter of yeast extract (4 g), the malt extract (10 g), the glucose (4 g), and the agar (18 g), at pH 7.2. For the isolation of individual colonies (for screening them for their capacity for lipase production), plates of agar were incubated for 5 to 7 days at 37 °C [14].

Actinobacterial colonies were inoculated onto International *Streptomyces* Project medium (ISP-3) containing oat meal (20 g), trace salts (1mL) (trace salts composition: 1.0 g $FeSO_4$, 0.9 g $ZnSO_4$, 0.2 g $MnSO_4$, $7H_2O$ in 100 mL H_2O), and agar (20 g) (pH 7 ± 0.02), and incubated for 7 days at 37 °C. Spores were carefully collected to avoid detachment of the mycelium with the addition in sterile distilled water (5 mL), and the suspension was collected into sterile bottles to be used as inoculum for the production and optimization of lipase under submerged fermentation (SMF). The suspension of spores was then diluted 1:100 (v/v), and counting of spores was accomplished with a counting chamber (Malassez REF 06 106 10 MARIENFELD, Germany) [15, 16].

2.2 Screening for lipase-producing actinobacteria

a. Qualitative screening Isolates of actinobacteria were screened for lipase production capacity on Phenol Red agar medium that contains the carbon source olive oil.

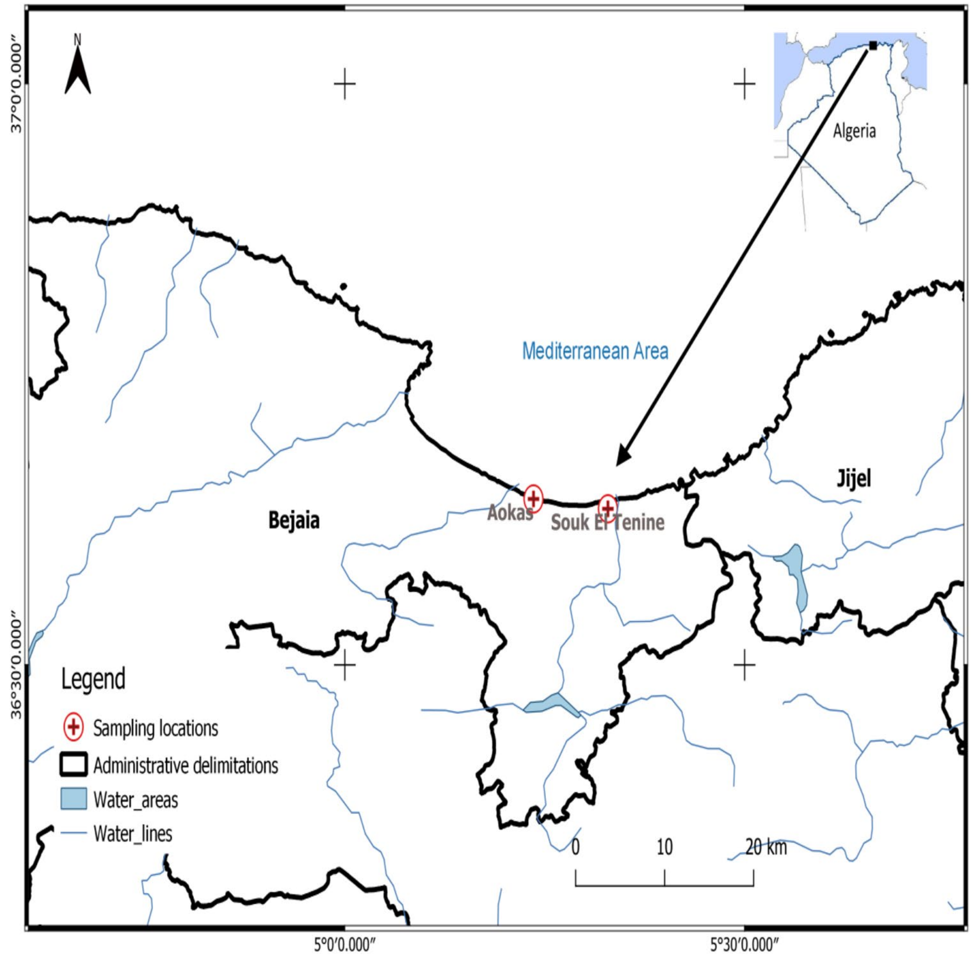


Fig. 1 The geographic map showing the locations of the sampling sites for this study

The agar was prepared by dissolving (g/L) 5 peptone, 3 yeast extracts, 1 $CaCl_2$, and 15 agar, in distilled water. An adjustment of pH to 7.4 was made using 0.1 M NaOH solution. After autoclaving and cooling to 60 °C, 10 mL of olive oil (Ithri Olive® Algeria) and 10 mL of phenol red (1 mg/mL) were added. Actinobacterial isolates plated onto the phenol red agar medium were incubated at 37 °C for up to 5 days [17].

b. Quantitative screening in agitated submerged fermentation For culture preparation, 10^7 spores/mL of each isolate selected were separately transferred to 20 mL of lipase production medium [0.4g KH_2PO_4, 2.1 g K_2HPO_4, 0.2 g $MgSO_4$, 0.002 g $CaCl_2$, 0.002 g $FeCl_3$, 0.5 g NH_4NO_3, distilled water to 1 L, 2% (v/v) olive oil; pH 7± 0.2 in shake flasks. The flasks were then incubated in a rotating shaker with 150 rpm for 5 days at 37 °C. After fermentation, 5-mL samples were taken for each isolate and then centrifuged at 4000 rpm during 30 min time at 4 °C, and the corresponding supernatants have been tested for the lipase activity [18].

2.3 Enzyme activity assay

The fatty acids release from olive oil was estimated by the titrimetric method [19]. The enzyme assay was performed with the culture supernatants (enzyme extracts) obtained from submerged fermentation (see section above). Reaction mixtures contained 0.5 mL of enzyme extracts and 5 mL of 10 % (v/v) olive oil and 10 % (w/v) gum are dissolved in 0.1 M phosphate buffer (pH 7.0). A 150-rpm orbital shaker was used to stir the enzyme-substrate combination for 30 min at 37 °C. An additional 1 mL of ethanol/acetone (1:1, v/v) was added to the reaction mixture to stop the reaction. The blank tests were carried out by adding distilled water instead of the enzyme extract. The fatty acids released have been titrated with 0.2 M NaOH by use of phenolphthalein as pH index, the end point was obtained by the appearance of a pink color [20].

2.4 Morphological characterization and molecular identification of lipase-producing strain

Sizes, colors, shapes, and other characteristics of the colonies were determined. Gram staining and cell and spore morphologies of the isolate were examined under an optical microscope [21]. The highest lipase-producing strain was identified at the molecular level using the GF-1 Nucleic Acid Extraction Kit (Vivant is Technologies SdnBhd, Selangor DE, Malaysia). PCR was performed on DNA extracted at 4 °C until required. PCR amplification was performed using universal 16S rRNA primers (27 F: 5′–AGAGTTTGATCCTGGCTCAG–3′, and 1492 R: 5′–CCGTCAATTCCTTTGAGTTT-3′) [22].

The PCR reaction mixture (25 µL) contained 1.25 U Taq DNA polymerase (Solis Biodyne, Estonia), an additional 3 µL of DNA template, 5 µL of each primer, and was completed to a 50µL reaction volume with distilled water. Polymerase chain reaction conditions consisted of 94 °C initial denaturation (2 minutes), 55°C annealing (1 mi), and extension at 72 °C (1 min). The amplification was replicated in 30 cycles with a final extension at 72 °C (7 min). PCR was performed using a thermal cycler (Bio-Rad bicycler, USA), and amplicon concentrations were determined using a Nanodrop spectrophotometer (NanoDropTM 2000, USA). These sequences were submitted to BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information) and identified using the GenBank database. The phylogeny of the lipase-producing strain was determined using MEGA 11, by the neighbor-joining method [23].

2.5 Box-Behnken design for determining optimal physicochemical parameters

Based on the previous results obtained for the optimization of the production of microbial lipase in OMW [24–26]. The Box-Behnken design (BBD) was employed for optimization purposes, as it estimates the principal effects of the selected variables simultaneously [27]. Metrics and levels for this optimization (Table 1) were chosen based on results reported in the literature [28–31]. To achieve the maximal production of lipase, five factors (Table 1) were selected as independent variables including temperature: X_1, the initial pH: X_2, the incubation time: X_3, inoculum size: X_4, and OMW: X_5.

Based on 43 experiments with center point triplicates, a polynomial model was constructed (Table 2) using Design Expert 11® software (version 11.0.5.0., Statease, Minneapolis, MN, USA). The parameters have been analyzed at 3 levels: low (−1), medium (0), and high (+1), while the production of lipase has been defined as the response.

A three-level, five-factor BBD experiment was carried out, and the number of tests (N) was determined according to equation (1):

$$N = 2k \cdot (k - 1) + C_0 \tag{1}$$

where k is the number of factors studied and C_0 is the number of center points.

The experimental data is fit by the response surface model to detect the interaction between the factors which can be tested and the response [32]. A second-order polynomial equation (quadratic model) was fitted to the data by regression analysis. This was conducted based on the proposed general eq. 2, which has been used to predict the optimal conditions for lipase production.

$$R = \beta_0 + \sum_{i=1}^{n} \beta iXi + \sum_{i=1}^{n} \beta iiX_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta ijXiXj + \varepsilon \tag{2}$$

where the R is lipase activity, β_0 is intercept derivative, β_i is linear derivative, β_{ii} is squared derivative, β_{ij} is interaction derivative, and X_i and X_j were the independent variables. ε is the associated residual of the experiments, the error of prediction, which represents the deviation between measured and predicted R values and quantifies the random variability in this experimental design. R and ANOVA were used to perform regression analysis.

Table 1 Independent factors and levels of variation in the Box-Behnken (BBD) model used in this study

Study type			Response surface			
Design type		Box-Behnken		Runs		43
Design mode		Quadratic				
Factor	Name	Units	Coded low	Coded high	Mean	Standard deviation
X_1	Temperature	°C	30.00	44.00	37.00	4.32
X_2	pH		4.00	10.00	7.00	1.85
X_3	Incubation time	Days	4.00	10.00	7.00	1.85
X_4	Inoculum size	Spores/mL	10^5	$1.99.10^7$	10^7	$6.11.10^6$
X_5	Olive wastewater	%	5.00	45.00	25.00	12.34
Response	Name	Unit	Observed	Analysis		
R	Lipase activity	U/mL	43	Polynomial		

Table 2 Design matrix of Box-Behnken and the lipase production results of strain SC1

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Response
Run	X_1	X_2	X_3	X_4	X_5	R
	Temperature	pH	Incubation time	Inoculum size	Olive wastewater	Lipase activity
	°C		day	Spores/mL	%	U/mL
1	44	7.00	7	10^7	5	1.8±0.12
2	44	7.00	7	10^5	25	6.7±0.47
3	37	7.00	10	10^7	5	2.7±0.59
4	37	4.00	7	10^7	5	3.6±0.08
5	37	7.00	7	$1.99*10^7$	45	4.0±0.42
6	37	7.00	7	10^7	25	2.7±0.01
7	44	7.00	7	$1.99*10^7$	25	1.3±0.29
8	37	4.00	7	10^5	25	4.0±0.56
9	30	7.00	7	10^5	25	1.3±0.54
10	37	4.00	7	$1.99*10^7$	25	2.7±0.28
11	30	10.00	7	10^7	25	3.1±0.22
12	37	7.00	4	10^5	25	4.0±0.59
13	37	7.00	10	$1.99*10^7$	25	2.2±0.20
14	44	7.00	10	10^7	25	1.3±0.07
15	30	7.00	7	$1.99*10^7$	25	8.4±0.36
16	37	7.00	7	10^7	25	4.0±1.35
17	37	7.00	7	10^5	5	4.0±0.18
18	37	4.00	7	10^7	45	3.1±0.31
19	37	10.00	7	$1.99*10^7$	25	5.3±0.42
20	44	7.00	4	10^7	25	4.7±0.42
21	30	7.00	10	10^7	25	4.0±0.15
22	44	4.00	7	10^7	25	2.7±0.25
23	30	7.00	7	10^7	45	1.3±0.70
24	37	7.00	7	$1.99*10^7$	5	4.0±0.46
25	37	4.00	4	10^7	25	4.0±0.08
26	37	4.00	10	10^7	25	2.6±0.07
27	37	7.00	4	$1.99*10^7$	25	6.2±0.21
28	37	7.00	7	10^5	45	1.3±0.70
29	37	10.00	7	10^5	25	1.3±0.70
30	30	7.00	4	10^7	25	4.0±0.21
31	30	7.00	7	10^7	5	5.3±0.26
32	37	10.00	7	10^7	5	4.0±0.63
33	30	4.00	7	10^7	25	5.3±0.34
34	37	10.00	7	10^7	45	4.0±0.87
35	37	7.00	10	10^7	45	1.3±0.50
36	37	7.00	4	10^7	5	2.7±0.62
37	37	7.00	7	10^7	25	1.3±1.35
38	37	10.00	10	10^7	25	2.2±0.08
39	37	7.00	4	10^7	45	5.3±0.46
40	37	7.00	10	10^5	25	3.1±0.19
41	44	7.00	7	10^7	45	4.0±0.33
42	44	10.00	7	10^7	25	4.0±0.30
43	37	10.00	4	10^7	25	4.9±0.07

Design Expert10 software was used to create the corresponding variable coefficients, interaction variables, and response surface plots. Two of the factors' values were varied to create the surface plots, while the other factors values were kept at zero. The Student's (*t*) test was used in the statistical analysis to determine the significance of the coefficient estimates. Retests were conducted to validate the proposed model after reanalyzing the regression equation and response surface plots to identify the ideal values of the selected variables [15, 33]. A second-order polynomial model that has been developed by the RSM is composed of linear, square, and interaction models (Eq. 2) [34].

3 Results and discussion

3.1 Isolation and screening for lipase-producing isolates

When serial dilution of the samples was performed and placed on nutrient-rich medium enriched with actinobacteria, 13 strains from the different soil samples were isolated. Figure 2 shows the proportions of isolates obtained from the different sampling sites. However, 46 % of the strains have been isolated from the contaminated soil collected in an olive oil mill in Souk El Tenin, while 39 % of the isolates have been isolated from contaminated soil obtained from another mill in Aokas, with the remaining 15 % being recovered from olive brine generated by each of these oil mills (Fig. 2). This low number of isolates obtained may be due to the composition of OMW, which is rich in polyphenols, which does not favor the growth of actinobacteria. Olive oil wastewater is considered to be a potential source of lipolytic bacteria. The oil environment has been indicated as a good source of nutrients for the development of lipolytic microorganisms [35]. Therefore, the strains of actinobacteria were not surprisingly isolated from the various soil samples.

Screening for lipase-producing isolates on solid medium is shown in Fig. 3. Lipase-producing isolates have been identified by the color change from pink to yellow around the colony growth when the plates containing olive oil and phenol red have been incubated at 37°C. Phenol red is a pH indicator; the presence of fatty acids in a medium causes a slight decrease in pH, turning the phenol red color from pink to yellow. The titrimetric assay was performed for quantification of lipase activity for four promising isolates and is presented in Fig. 4. Among them, strain SC1 exhibited the highest enzyme activity of 5.33 U/mL. This was followed by strain GO2 which showed 4.66 U/mL of lipase activity. Microorganisms reported to produce lipase, including *Bacillus* species, *Pseudomonas* species, *Proteus* species, *Staphylococcus* species, *Geotricum candidium*, *Candida* species, and *Mucor* species have been referred to in various publications for lipase production in OMW and other oil-based media [36, 37]. The use of actinobacteria as lipolytic agents has been described in a large number of publications [38]. However, based on the best of knowledge, prior to this study, actinobacterial species have not been used for the degradation of OMW.

3.2 Morphological characterization

The characterization of the morphology of SC1 isolate was found to be important for determining the microorganism's classification. According to morphological observations, this isolate's colony morphology appeared distinct

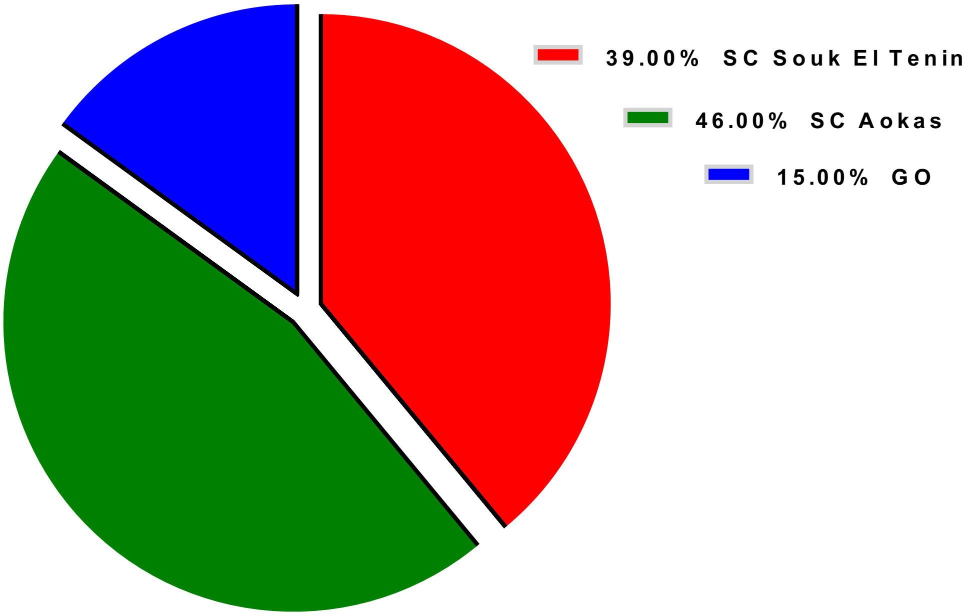


Fig. 2 The proportion of isolates from different sampling sites

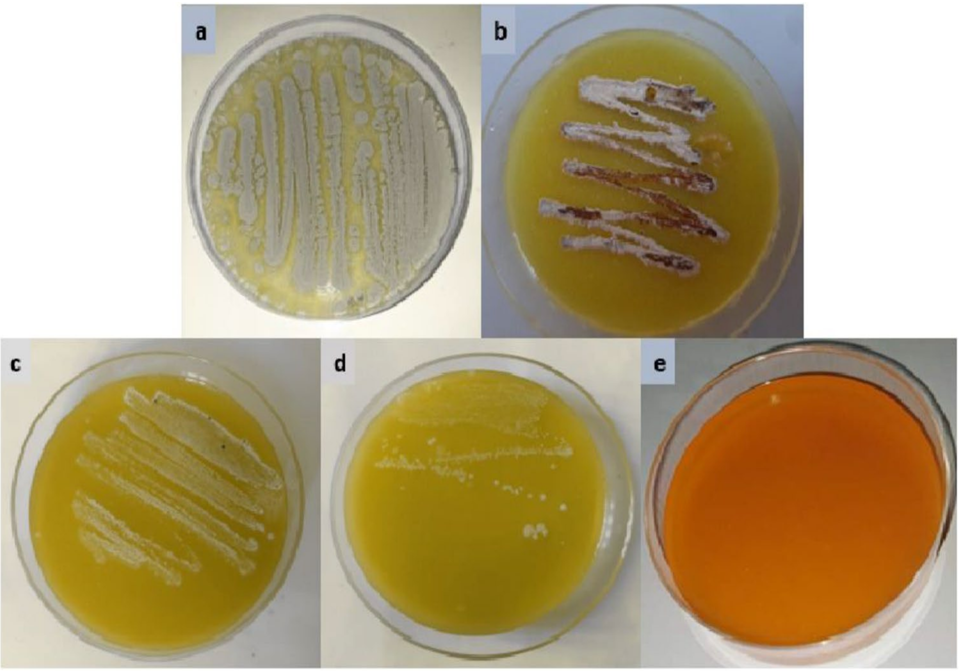


Fig. 3 Lipase activity on phenol red agar plate. Plates **a** (SC1) and **b** (GO2) indicate the isolates from the Aokas oil mill, **c** (ES3) and **d** (ES4) show the isolates from the Souk El Tenin oil mill and **e** is plaque without isolates as the control

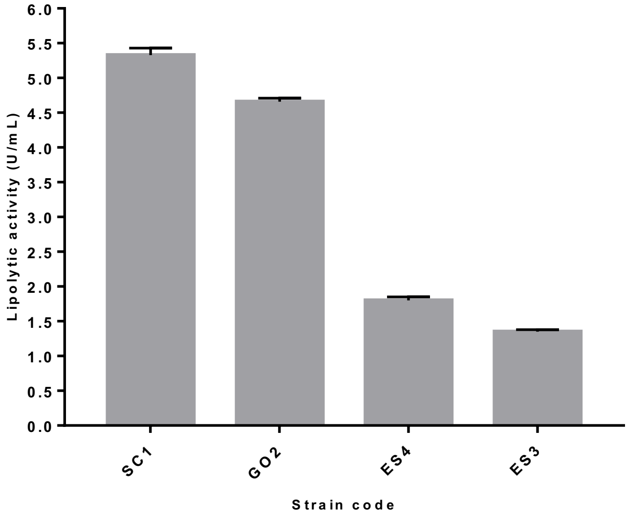


Fig.4 Lipase activity of the four isolates produced under submerged fermentation in the presence of olive oil

when examined on ISP-3 agar medium, where the isolate showed beige, powdery colonies with flat edges (Fig. 5a). The spores appeared to be organized in chains and spore morphology confirmed that aerial and coiled mycelia were observed. The arrangements of the spore chains were observed using a microscope (100×) Gram staining of the strain indicated that it is Gram-positive (Fig. 5b–c). All these properties are consistent with the morphological classification of the isolate in the genus *Streptomyces*.

3.3 Molecular identification of strain SC1

To identify the SC1 strain, the 16S rRNA gene has been amplified and sequenced. Alignment of this sequence with the sequences of the NCBI database from BLASTn program showed that SC1 was identical to the genus *Streptomyces* (87 % identity). The results of this study for the 16S rRNA gene nucleotide sequence (1385 bp) have been submitted to the GenBank database with the name *Streptomyces* sp. strain 22 dz and the accession number ON322956.1.

Figure 6 shows the phylogenetic tree for partial 16S rRNA gene sequence showing the relationship of *Streptomyces* sp. strain 22 dz (SC1) to other type strains of the genus *Streptomyces* and other related actinobacteria [39]. The *Bacillus pumilus* ATCC 7061 sequence was used as an out-group strain for neighborhood matching; the NCBI accession numbers are shown in parentheses (accession number AF478070).

3.4 Optimization of lipase production in olive wastewater

Lipase production from bacterial species has generally been optimized by statistical experimental design. For example, durable lipase production from *Thermomyces lanuginosus* with optimization of the process and enzyme characterization [40]; thermophilic lipase *Serratia rubidaea*was produced, improved, characterized, and covalently immobilized from an Algerian oil waste [41]; optimization of lipase production from a novel strain *Thalassospira permensis* M35-15 using response surface methodology [42], and the

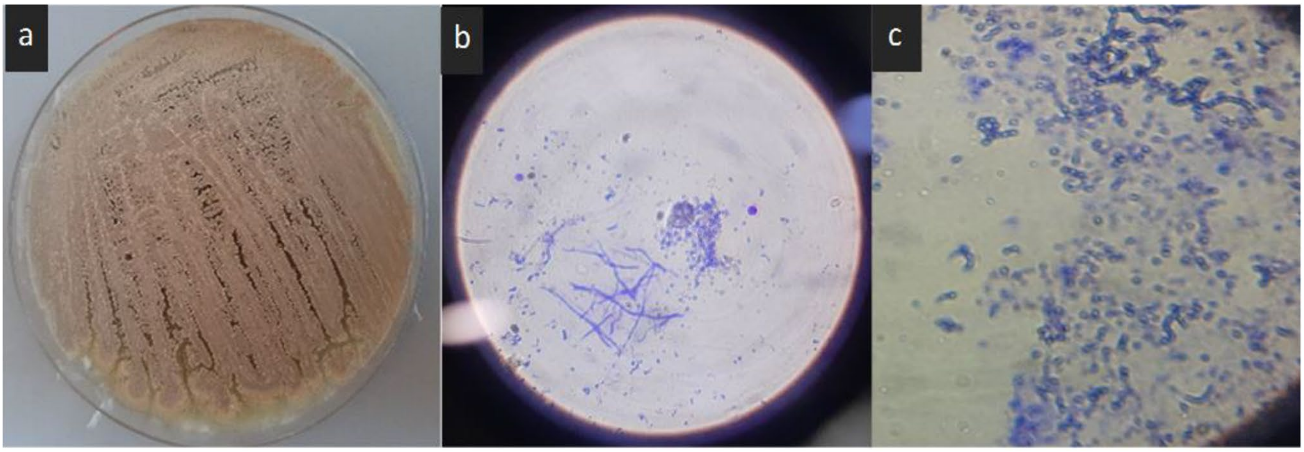
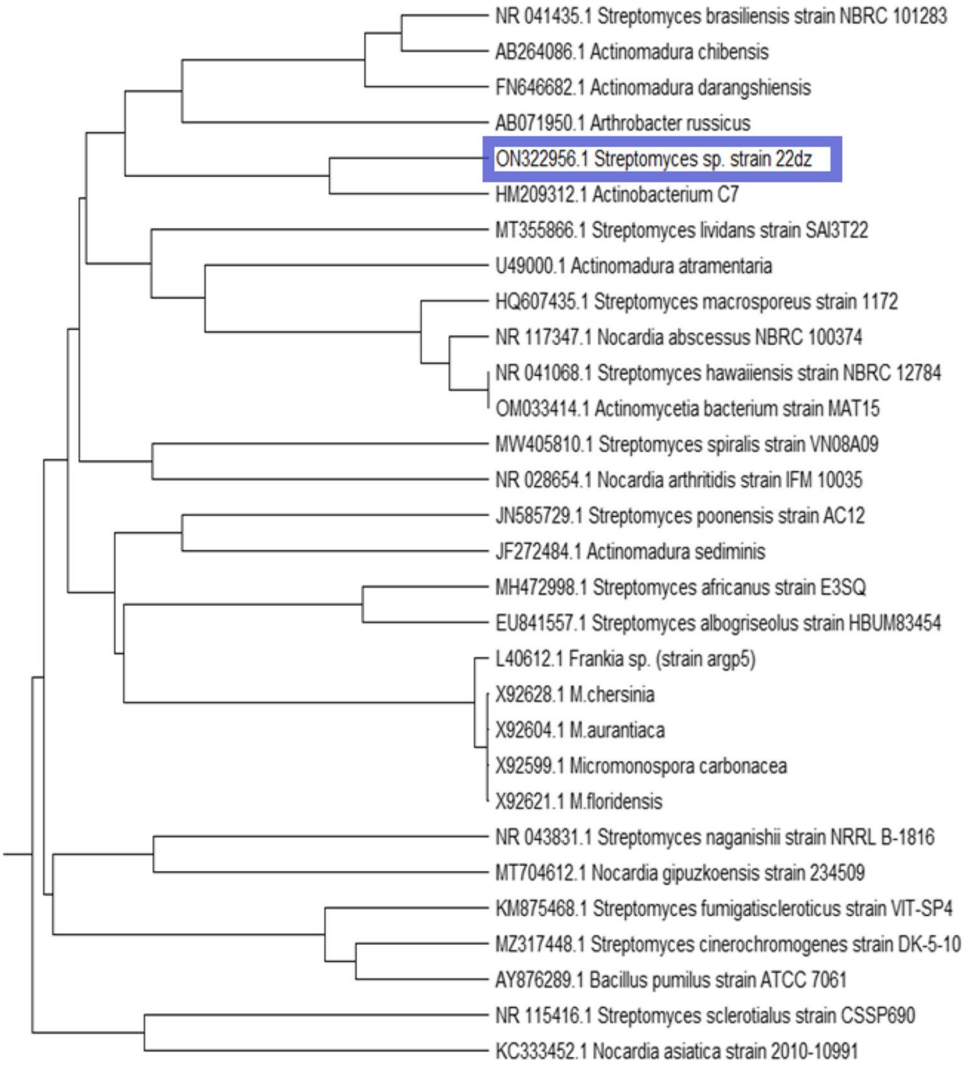


Fig. 5 Morphology of SC1 isolate obtained from roots; **a** top view of SC1isolat colony in in ISP-3 medium; **b** and **c** microscopic morphology (100×) of spore chain arrangement and Gram staining respectively

Fig. 6 Phylogenetic tree for *Streptomyces* sp. strain 22dz 16S ribosomal RNA gene



optimization of extracellular lipase production from *Botryococcus sudeticus* UTEX 2629 [43]. Optimization of the production conditions of the lipase produced by *Bacillus cereus* from rice flour through Plackett-Burman design (PBD) and response surface methodology (RSM) [44], but little work has been done in relation to optimizing lipase production by actinobacterial strains. However, the principal difference in this work was that enzyme production was optimized using a low-cost substrate such as OMW (Table 2). Moreover, the bio degradation of these wastes in the medium, as a result of the lipolytic activity, was also followed.

3.4.1 Performance and model fitting with RSM

The significance of the quadratic model created from the experimental data may be calculated according to analysis of variance (ANOVA) (Table 3). The statistical significance of the model is indicated by the *F*-value of 10.50, which equals a *p*-value of 0.001. The model includes X_1 (temperature), X_3 (incubation time), X_4 (inoculum size), X_1X_2 (temperature vs. pH), X_1X_3 (temperature vs. incubation time), X_1X_4 (temperature vs. inoculum size), X_1X_5 (temperature vs. olive wastewater), X_2X_4 (pH vs. inoculum size), X_3X_4 (incubation time vs. inoculum size), X_3X_5 (incubation time vs olive wastewater), $X_1{}^2$ (temperature^2), and $X_4{}^2$ (inoculum size^2) are significant terms (Table 3) with a *p*-values less than 0.05. On the other hand, the *p*-values were above 5% for the factors X_2 (pH), X_5 (olive wastewater), X_2X_3 (pH vs. incubation time), X_2X_5 (pH vs. olive wastewater), X_4X_5 (inoculum size vs. olive wastewater), $X_2{}^2$ (pH^2), $X_3{}^2$ (incubation time^2), and $X_5{}^2$ (olive wastewater^2), proving that these variables and their interactions are not significant. The model correctly explains the experimental data, as indicated by the lack-of-fit with an *F*-value of 0.1888, which suggests that this is not significant in comparison to the pure error. According to Table 3's determination coefficient value (R^2) of 0.9051, the model is acceptable for predicting values from the experimental data. Since the R^2 value is greater than 0.75, the model is sufficient in explaining the majority of

Table 3 Estimated regression coefficients for the quadratic polynomial model and the analysis of variance

Source	Sum of squares	Df*	Mean square	*F*-value	*p*-value	
Model	100.45	20	5.02	10.50	< 0.0001	Significant
X_1-temperature	2.56	1	2.56	5.36	0.0304	
X_2-pH	0.0498	1	0.0498	0.1040	0.7502	
X_3-incubation time	16.66	1	16.66	34.81	< 0.0001	
X_4-inoculum size	4.42	1	4.42	9.24	0.0060	
X_5-olive wastewater	0.8352	1	0.8352	1.75	0.2000	
X_1X_2	3.15	1	3.15	6.59	0.0176	
X_1X_3	2.83	1	2.83	5.92	0.0235	
X_1X_4	39.34	1	39.34	82.22	< 0.0001	
X_1X_5	9.77	1	9.77	20.42	0.0002	
X_2X_3	0.4011	1	0.4011	0.8383	0.3698	
X_2X_4	6.93	1	6.93	14.49	0.0010	
X_2X_5	0.0494	1	0.0494	0.1032	0.7510	
X_3X_4	2.42	1	2.42	5.06	0.0349	
X_3X_5	3.87	1	3.87	8.10	0.0094	
X_4X_5	1.78	1	1.78	3.72	0.0669	
$X_1{}^2$	2.74	1	2.74	5.72	0.0257	
$X_2{}^2$	1.32	1	1.32	2.75	0.1113	
$X_3{}^2$	0.6602	1	0.6602	1.38	0.2527	
$X_4{}^2$	3.28	1	3.28	6.86	0.0157	
$X_5{}^2$	0.0470	1	0.0470	0.0983	0.7569	
Residual	10.53	22	0.4785			
Lack-of-fit	688	20	0.3441	0.1888	0.9857	Not significant
Pure error	3.65	2	1.82			
Cor total	110.97	42				
R^2	0.9051					
Adjusted R^2	0.8189					
Adeq. precision	15.1500					

[*] Degree of freedom

the variability in the trial data [45]. The proposed model is verified by the adjusted R^2 value of 0.8189. The precision of the model can be determined by the signal-to-noise ratio (adeq. precision), and it should be greater than 4 [46]. The value of adeq. accuracy is 15.15 (Table 3), indicating a high level of accuracy and adequate response ratio. However, this value and the coefficient of variation (CV %; 19.86 %) suggest that the model has reliability and reproducibility [47, 48].

Figure 7 shows the results of the predicted values of the production of lipase versus the actual values of the RSM. The figure indicates that the actual and predicted values of lipase production correlate well and the linear distribution in the model shows a good fit. The figure clearly shows that the model is quite realistic, even though the deviation is small from actual to predicted values. The model's R^2 value is 90.51 %, which indicates the good accuracy between the model and the data. Therefore, the model can be used to navigate the design data space. Based on its statistical properties, it can be summarized that this model is appropriate for establishing the main impacts of the factors [49–51].

The second-order polynomial quadratic regression equation was calculated for the lipase production based on the coded factors [Eq. (3)] is as follows:

$$\begin{aligned}\text{Lipase activity} = {} & +2.65 - 0.4X_1 + 0.05X_2 - 1.02X_3 + 0.52X_4 - 0.22X_5 \\ & + 0.88X_1X_2 - 0.84X_1X_3 - 3.14X_1X_4 + 1.56X_1X_5 - 0.31X_2X_3 \\ & + 1.32X_2X_4 + 0.11X_2X_5 - 0.77X_3X_4 - 0.984X_3X_5 + 0.66X_4X_5 \\ & + 0.65X_1^2 + 0.45X_2^2 + 0.32X_3^2 + 0.71X_4^2 + 0.08X_5^2\end{aligned} \tag{3}$$

where, X_1: temperature (°C), X_2: pH, X_3: incubation time (days), X_4: inoculum size (spores/mL), and X_5: olive wastewater concentration (%, v/v). Minus signs preceding the terms denote an antagonistic effect, while plus signs denote a synergistic effect. The coded factor development model is useful to identify the most significant factors that will affect the response [52].

3.4.2 Interaction analysis between influencing factors

Three-dimensional response surface plots were used to investigate the interaction of significant factors and their influence on response (Fig. 8a–g) based on the BBD regression analysis. While maintaining other variables at their optimum level, in the response surface plot some two variables were constructed. The variation in lipase production in response and the significant p-value; 0.0176, 0.0235, <0.0001, 0.0002 respectively for the interaction

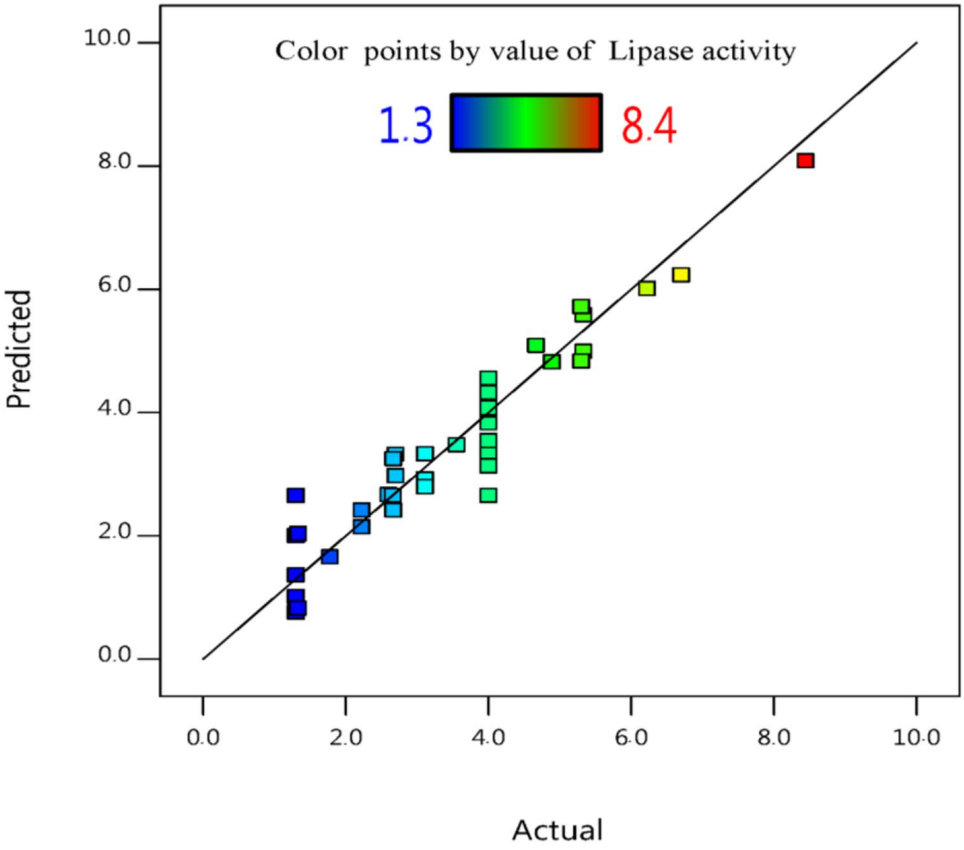


Fig. 7 Actual value vs. predicted value split from RSM design

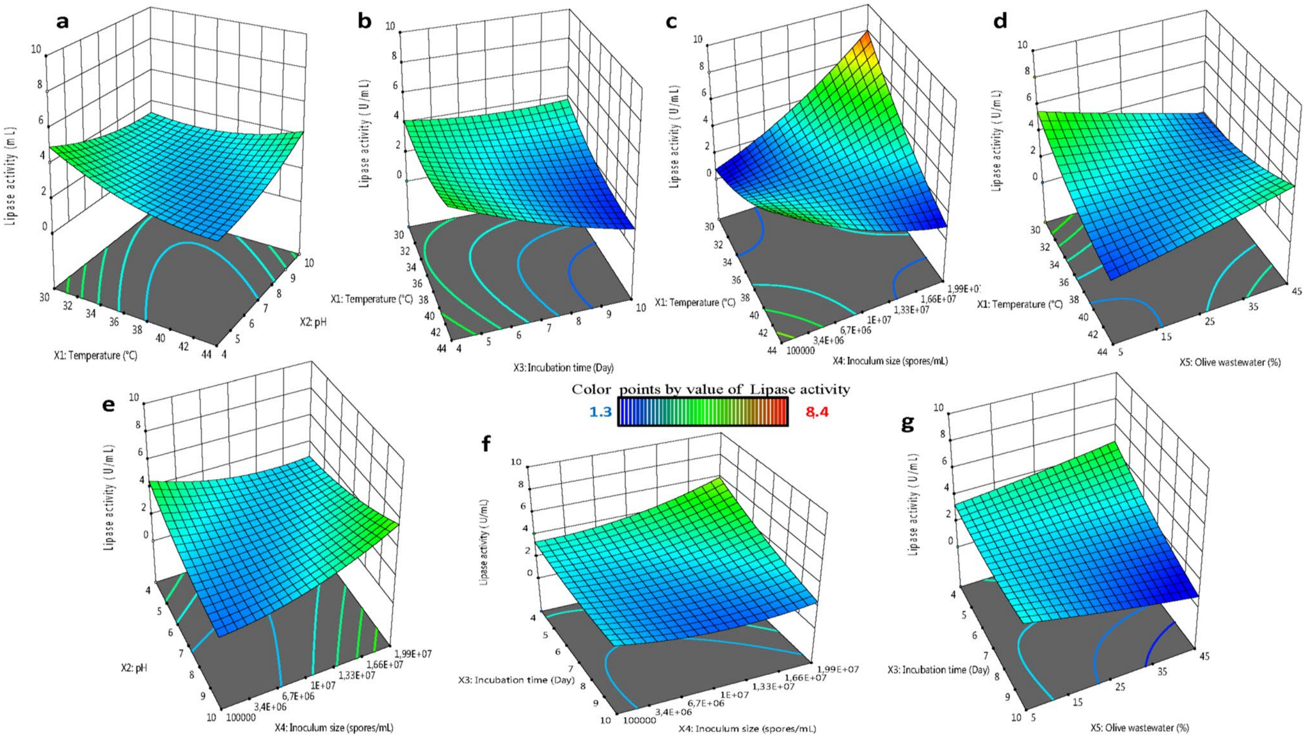


Fig. 8 Response surface analysis for lipase production by agitated submerged fermentation of *Streptomyces* sp strain DZ22 on olive wastewater and 3D plot showing the significant interaction between incubation temperature and initial pH (**a**), incubation temperature and incubation time (**b**), incubation temperature and inoculum size (**c**), incubation temperature and olive wastewater (**d**), and incubation time and inoculum size (**e**), initial pH and inoculum size (**f**), and incubation time and olive wastewater (**g**)

between X_1X_2 (temperature vs pH), X_1X_3 (temperature vs incubation time), X_1X_4 (temperature vs. inoculum size), and X_1X_5 (temperature vs olive wastewater concentration) demonstrated of positive interactions between these variables (Fig. 8a–d). Temperature was a major determinant of lipase production since it had strong quadratic and linear impacts ($p < 0.05$), confirming the results of the single-factor experiment (Table 3).

According to Sharma et al. and Behera et al. [8, 53], the positive interaction between incubation temperature and pH (X_1X_2) was found to be evident. Temperature is an important factor in the production process. Low or high temperatures can inhibit microbial development, which will then result in less enzyme synthesis, but Behnam et al. [54] signaled that temperature and incubation time interaction (X_1X_3) was insignificant for optimizing enzyme production by *Mucor indicus* via solid-state fermentation (SSF).According to previous studies [55, 56], the maximal enzyme production by *Rhizopus delemar* F2, *Aspergillus niger*, and *Aureobasidium pullulans* Y-2311-1 was influenced by the interaction of temperature and incubation period.

In this study, the production of lipase was affected by the incubation temperature and inoculum size (X_1X_4), it was maximized by a combination of both of factors (it reached 8 U/mL), it was reported that inoculum size and temperature had a strong interactive effect on production of lipase when the temperature is decreasing, and the inoculum size is increasing. With regards to the interaction between temperature and olive wastewater concentration, increasing both factors improved the lipase production, but it has also been observed that after a certain limit, the lipase activity fell sharply. This may be attributed to substrate inhibition (feedback inhibition).

Figure 8f illustrates the effect of pH and inoculum size (X_2X_4) on the lipase activity when the temperature, time of incubation, and olive wastewater were fixed at the center level. The lipase activity increases significantly (p-value < 0.0094) with an increase in the pH and inoculum size. Both parameters could have affected the metabolism involved in lipase production. The pH in most culture media play an important role in determining the optimal bacterial cell physiological function and the transportation of various nutrients through the cell membrane for maximum enzymatic yield [57]. Initial pH of the culture broth is in fact one of the most crucial environmental parameters influencing the growth and the lipase production at the same time [58].

Figure 8e illustrates the combination of the effect of the interaction between incubation time and inoculum size (X_3X_4) on lipase activity. According to Abu et al. [28], the interaction of incubation time vs. inoculum size has a very

Table 4 Validation of quadratic model for lipase production with *Streptomyces* sp. strain 22 dz (SC1) optimized by response surface methodology (RSM)

Run	Temperature °C	pH	Incubation time Day	Inoculum size spores/mL	Olive wastewater %	Lipase predicted U/mL	Lipase actual U/mL	SD
1	30	4	9	$1.7*10^7$	6	8.82	8.4	0.42
2	30	7.5	9	$2.0*10^7$	7	8.45	8.1	0.35

important influence on lipase activity, where the lipase activity increase when the inoculum size increase and the incubation time decrease.

Using data from the surface response graph (Fig. 8g), a trend can be explained in the lipase activity according from the interaction of incubation time and olive wastewater (X_3X_5). These parameters significantly influenced lipase production with a p-value of 0.0349. Lipase activity was primarily dependent on incubation time, inoculum size, and olive wastewater, as both quadratic and linear effects on lipase activity were highly significant ($p < 0.05$), affirming and confirming the single-factor experiment results (Table 3).

3.4.3 Validation of a model

In this investigation, a new position of the experiment was created with the selected optimal settings to predict and verify the accuracy of the mathematical model. This was done after creating the optimal conditions and forecasting the reaction under these conditions. To establish the maximum lipase production at various levels of variables, a novel range and variable levels were examined. On the basis of the maximal lipase production observed in the complete model, the ranges of variable levels were established. The ranges of values used are shown in Table 4, and the variables' ideal values were chosen based on the database created by the software Design Expert 10, turning a second trial run (Table 4), combining the experimental conditions of the two runs with the actual lipase value measured under these conditions. This suggested that a validity rate of around 98.9 % had been obtained. We can make the conclusion that the model was effective at predicting optimal lipase production conditions since there was no significant difference between the predicted and actual lipase production.

Optimal values for the five variables included the following: 30°C (temperature, X_1), 4 (pH, X_2), 9 days (incubation time, X_3), $1.7*10^7$ spores/mL (inoculum size, X_4), and 6 %, v/v (olive wastewater, X_5) with a predicted maximum lipase activity of 8.82 U/mL, which is close to the actual activity of 8.4 U/mL.

4 Conclusion

The ability of olive oil mill effluent as a carbon source in a fermentation medium for improved lipase production from bacterial isolates by different experimental conditions allows for the beneficiation of a resource often considered to be a potential environmental pollutant. In this study, RSM has shown a powerful for the optimization of cultivation conditions, using diluted olive oil wastewater as substrate (6 %, v/v) with optimal production occurring at 30 °C as incubation temperature, pH 4, an inoculum size of $1.7*10^7$ spores/mL, and 9 days of fermentation. Under these optimized conditions, lipase activity was 8.82 U/mL, with lipase activity increasing by 60.43 % compared to the standard non-optimized conditions used. The response surface methodology was found to be more satisfactory and efficient compared to other methods based on its effectiveness to study multiple variables simultaneously with few numbers of experiments, thus economizing time and costs.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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