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New lipase-producing *Streptomyces* isolated from halo-alkaline habitat in Wadi El Natrun: polyphasic identification and statistical optimization of enzyme production

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Abstract

Background: Bioprospecting lipase producers in non-conventional habitats are the way to find special enzymes of diverse applications. Halo-alkaline marshes in Wadi El Natrun in Egypt are some of the most stable ecological systems in the world, and because of the double extremities of alkalinity and salinity, they harbor individual microbes capable of adapting stress conditions.

Results: Eight strains were recovered from the coastline soil of Al-Beida Lake in Wadi El Natrun and have been tested for lipase production. Among the eight isolates, the strain SBLWN_MH2 was the most active producer of lipase (7.5 U/ml). The crude SBLWN_MH2 lipase showed activity over a wide pH range (3.5 to 13) with an optimum pH at 10.5, and it was able to show more than 75% of its highest activity at pH elevated up to 13. The identification using phenotypic and genotypic methods strongly indicated that the strain SBLWN_MH2 belonged to the genus *Streptomyces* with a similarity of 99%. Thus, it has been given the suggested name *Streptomyces* sp. SBLWN_MH2 (MG593538). SBLWN_MH2 produced extracellular lipase in modified starch casein medium supplemented with different oils or Tween-80, and the potential production rate has been attained in the case of linseed oil after 3 days. Further experiments have been carried out to optimize medium composition through Box-Behnken design and response surface methodology, and it was possible to achieve more than 3.5-fold increase in lipase production.

Conclusions: The present study indicates that *Streptomyces* sp. SBLWN_MH2 is a potential lipase producer and could be fruitfully employed in the large-scale production of highly alkaline lipase.

Keywords: Isolation, *Streptomyces*, Polyphasic identification, Alkaline lipase, Medium optimization

1 Background

Lipases (EC 3.1.1.3) are hydrolytic enzymes that catalyze the cleavage of ester bonds in triglycerides of long-chain fatty acids. A tremendous number of applications relied upon lipases [1] and covered most industrial sectors due to their role as hydrolases or synthetases for many substrates [1, 2]. Their applications in food processing, acylation reactions, synthesis of esters, and separation of racemic mixtures are extended in many works of literature [2–4]. As such, lipases are the third-largest enzymes in total sales volume after proteases and amylases [5], and it has been predicted for the lipase market to reach 590.5 million dollars by 2020 [1].

Lengthy efforts have been accomplished to find persistent lipases that are used efficiently and economically in aqueous and non-aqueous solvents, and these efforts

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have been progressed through chemical, physical, and genetic modifications [6]. However, adequate efforts ought to be coordinated to explore lipase producers in environments of extreme conditions that represent the primary resource for natural lipases of promising characteristics.

The halo-alkaline environment in Wadi El Natrun in Egypt is one of the most stable, extremely alkaline, and least studied niches on earth [7]. With the double extremities of salinity and pH, it may harbor alkaliphiles and haloalkaliphiles [5] and poly-extremophiles [8] of particular genetic traits. Microbes in halo-alkaline habitats could survive through producing individual proteins being able to maintain activity and stability under the challenge of the high concentration of salt and pH, as are considered of potential importance in biotechnology [9].

A small number of investigations focused on enzymatic and biotechnological applications of such microbes [9]. Some of the valuable proteins produced by microbes in halo-alkaline habitats are alkaline enzymes such as proteases [10, 11]. Likewise, alkaline lipases have particular importance and could provide more applications in the synthesis of chiral molecules, biological pulping of wood, detergents, flavor synthesis, industries, and bioremediation as well as their use in food and feed formulations [5, 12, 13]. Salihu and Alam [13] reviewed many bacterial genera that were capable of producing alkaline lipases, and there was a scarce documentation for strains isolated from halo-alkaline habitats.

As such, the current investigation intended to bioprospect new lipase producers isolated from soda salt marshes in the Wadi El Natrun district in Egypt. It is one of the least studied double-extremities environments. Furthermore, to induce lipase production through different classical and statistical methods of optimization.

2 Methods

2.1 Isolation of *Streptomyces* producing lipase

The strain of the current study was isolated from the coastline soil of Al-Beida Lake in the Wadi El Natrun district in Egypt. The soil samples were collected few centimeters beneath the surface in the spring of 2016. Isolation has been done on agar plates of the starch casein medium [14] with 5% NaCl, and pH was adjusted at 8.5. The plates have been incubated for 5 days at 28 ± 2 °C. The colony of interest was picked up and streaked onto the surface of agar plates of the same isolation medium. After incubation for 5 days, the plates have been observed, and a touch of the colonial terminal growth of a separate colony was transferred to slants of the same medium and preserved by regular subculturing every 2 weeks. Long-term preservation of the isolate was attained by harvesting the spores of the 5-day-old culture in a sterile glycerol solution (10% V/V) and keeping the spore suspension in cryo-vials (1 ml/vial) at − 80 °C.

2.2 Lipase activity assay

Preliminary studies on lipolytic activity have been carried out on crude enzyme obtained from cultures of *Streptomycetes* in starch casein broth medium (50 ml medium in a 250-ml Erlenmeyer flask) modified by reducing the amount of starch to 3 g/l, the addition of NaCl (50 g/l), and adjusting pH at 8.2 before sterilization. Sterile olive oil (0.5 ml) has been added to the flask, and after incubation for 5 days (at 30 °C ± 2 and 150 rpm), a crude enzyme sample was prepared by transferring 1 ml of total fermentation medium into Eppendorf to be centrifuged for 10 min in cooling centrifuge at a temperature of 4 °C and 10,000 rpm.

The lipolytic activity was determined by the spectrophotometric method using p-nitrophenyl palmitate (p-NPP) as a substrate, as described by Chakraborty and Raj [15] with some modifications. In brief, 20 µl of p-NPP (10 mM) in isopropanol was added to a test tube containing 300 µl of buffer solution (50 mM of Tris buffer, pH 8). To this buffered substrate suspension, a sample of the crude enzyme (30 µl) has been added, and the test tube was then incubated in a water bath at 37 ± 2 °C for 30 min under continuous shaking (150 rpm).

Excess sodium carbonate solution (3 ml, 0.1 M) was added, to ensure sufficient alkaline medium, and then the contents of each tube were filtered using a nylon syringe filter (0.45 µ) before measuring the absorbance at 400 nm by Jasco UV-VIS spectrophotometer. For each sample, the blank was prepared typically like the sample without adding a p-NPP substrate. Readings of standard p-NP concentrations in Na_2CO_3 solution (0.1 M) were used to get product concentrations in samples.

The coefficient of extinction (ε) of p-NP under the conditions described has been determined as 21 mM^{-1} cm^{-1}. One unit of enzyme activity has been defined as the amount of enzyme needed to liberate 1 µM min^{-1} of p-NP under the standard assay conditions [16]. Activity at different pH values has been achieved by using different buffers (at a concentration of 50 mM) to cover a broad pH range from 3 to 13.

Citrate phosphate buffer has been used to cover pH range from 3 to 7. For pH range from 7.5 to 9, the tris-HCl buffer has been used. Carbonate/bicarbonate buffer has been used to cover pH range from 9.5 to 10.5, while disodium hydrogen phosphate/sodium hydroxide buffer has been used to cover pH range from 11 to 12. Potassium chloride/sodium hydroxide buffer has been used to cover pH range from 12.5 to 13.

2.3 Characterization of isolated *Streptomyces*

2.3.1 Phenotypic characterization

In terms of the International Streptomyces Project (ISP), morphological, biochemical, cultural, and physiological characteristics of the isolated *Streptomycetes* were performed [17]. The microscopic depiction was performed with coverslip culture. Formation of aerial, substrate mycelium and spore arrangements on mycelium were monitored under a phase-contrast microscope (Nikon Eclipse E600, USA) at 200 magnification and scanning electron microscopy (JEOL JSM 5300, JEOL Techniques Ltd., Japan).

The culture characteristics such as growth, the coloration of aerial and substrate mycelia, and the formation of soluble pigment have been investigated on seven different media as recommended by ISP. Biochemical characterization, namely melanin pigment production, nitrate reduction, gelatin liquefaction, and starch hydrolysis, has been also performed as suggested by ISP. Physiological characterizations such as the growth rate in NaCl (2–13%) and survival at 50 °C have been also evaluated. Further, the capability of isolates to utilize various carbon and nitrogen sources was estimated using the ISP 9 agar medium. Based on the above characteristics, the genus-level identification of the potential strain was made by Bergey's manual of systematic bacteriology [18].

The disk diffusion susceptibility method has been carried out according to Jorgensen and Turnidge [19]. The test has been performed by applying the SAH1-CWMSG-isolate inoculum to a starch nitrate agar plate. Eight standard antibiotic disks, e.g., tetracycline, neomycin, novobiocin, vancomycin, cefodizime, nalidixic acid, rifamycin, and streptomycin, have been placed on the inoculated agar surface. Plates were incubated for 24–48 h at 28 °C before the measurement of the results. The zones of growth inhibition around each of the antibiotic disks have been measured to the nearest millimeter.

2.3.2 Phylogenetic identification

2.3.2.1 DNA isolation and PCR amplification An overnight culture of the strain SBLWN_MH2 grown at 28 °C was used for the preparation of genomic DNA. DNA extraction has been done using the protocol of the Gene JET Genomic DNA purification Kit (Thermo K0721) following the manufacturer's instructions of the kit. The PCR amplification of the 16S rDNA region was carried out following the Maxima Hot Start PCR Master Mix (Thermo K1051).

The 16S rDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify a 1500-bp fragment of the 16S rDNA region. The domain bacteria-specific primer 27F (forward primer) was 5′ AGAGTTTGATCMTGGCTCAG3′, and the universal bacterial primer 1492R (reverse primer) was 5′TACGGYTACCTTGTTACGACTT3′ [20–22].

The PCR reaction was performed with 5 µl of genomic DNA as the template, 1 µl of 16S rRNA forward primer, 1 µl of 16S rRNA reverse primer, 18 µl water, nuclease-free, and 25 µl Maxima® Hot Start PCR Master Mix (2×) in a 50-µl reaction mixture as follows: activation of 2 Taq polymerase at 95 °C for 2 min, 35 cycles of 95 °C for 1 min, and 65 °C and 72 °C for 1 min each were performed, finishing with a 10-min step at 72 °C. After completion, the PCR products were electrophoresed on 1% agarose gels containing ethidium bromide (10 mg/ml) to ensure that a fragment of the correct size had been amplified.

2.3.2.2 DNA sequencing, phylogenic analysis, and tree construction The 16S rRNA sequence analysis and phylogenetic tree construction by the neighbor-joining method have been performed for species-level confirmation of *Streptomyces* sp.

The amplification products have been purified with the K0701 GeneJET™ PCR Purification Kit (Thermo). Afterward, the samples become ready for sequencing in an ABI Prism 3730XL DNA sequencer and analysis on GATC Company. The sequencing reaction has been performed with the primers 518F 5′ (CCA GCA GCC GCG GTA ATA CG) 3′ and 800R 5′ (TAC CAG GGT ATC TAA TCC) 3′ using a PRISM BigDye Terminator v3.1 Cycle Sequencing Kit. The DNA samples containing the extension products have been added to Hi-Diformamide (Applied Biosystems, Foster City, CA). The mixture has been incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by the ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequence alignment has been prepared with the DNAS TAR software programs (DNASTAR. INC., Madison, WI).

Phylogenetic data were obtained by aligning the nucleotides of different 16S RNA retrieved from the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), using the CLUSTAL W program version 1.8 with standard parameters. The sequences with 98–100% homology have been considered for molecular taxonomy analysis. Multiple sequence alignment has been performed for the 16S rRNA sequence generated in this study and sequence of the GenBank database with the CLUSTAL W program [23].

The phylogenetic tree has been constructed using the neighbor-joining and maximum-parsimony tree making methods in the Molecular Evolutionary Genetics Analysis (MEGA version 5.0) software [23] based on bootstrap values of 500 replications.

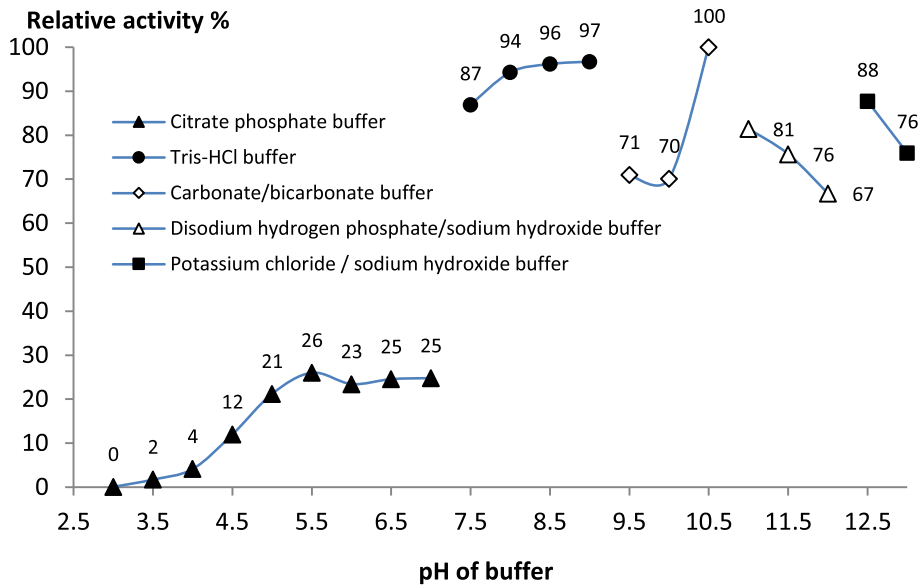

Fig. 1 Relative activity of *Streptomyces* sp. SBLWN_MH2 lipase at different buffered pH values

2.4 Induction of lipase production by different substrates

Fermentation was done in Erlenmeyer flasks (250 ml), each containing 50 ml of starch casein broth medium [14] modified by reducing the amount of starch to 3 g/l, the addition of NaCl (50 g/l), and adjusting pH at 8.2 before sterilization. Different substrates (oils and Tween-80) were separately autoclaved and added to the flasks in a concentration of 0.5 ml/flask (1%) for oils and 0.1 ml/flask (0.2%) for Tween-80. Each flask has been then inoculated with 1 ml spore suspension prepared by adding 3 ml of sterile Tween-80 solution (0.1%) to a 5-day-old slant of the *Streptomyces* sp. SBLWN_MH2 and releasing the spores using a sterile needle.

The flasks have been incubated at 28 ± 2 °C and 150 rpm for different time courses. Fermentation has been done in duplicate flasks for each treatment, and after a separate analysis of lipase concentration in each flask, the net results of duplicates have been expressed as arithmetic mean ± standard error.

2.5 Statistical optimization of lipase production

Response surface methodology using Box-Behnken design, analysis of variance (ANOVA) of data, regression analysis to get polynomial coefficients and equations, and three-dimensional response surface plots have been achieved using the software "Design Expert" statistical package (version 7.0.0, Stat-Ease Inc., Minneapolis, USA). Calculations of standard error, ANOVA single two factors at a confidence level of 95%, have been conducted through Microsoft Office Excel 2007.

Table 1 Cultural characteristics of the *Streptomyces* sp. SBLWN_MH2 at 14 and 21 days

	Medium no.	Growth	Color		
			Aerial mycelium	Substrate mycelium	Diffusible pigments
1	Tryptone yeast extract broth (ISP 1)	Moderate	White	Beige	None
2	Yeast-malt extract agar (ISP 2)	Moderate	Light white	Beige	None
3	Oatmeal agar (ISP 3)	Moderate	Light white	Beige	None
4	Inorganic-trace salt-starch agar (ISP 4)	Moderate	Light white	Beige	None
5	Glycerol asparagine agar (ISP 5)	Moderate	Light white	Beige	None
6	Peptone yeast extract iron agar (ISP 6)	Weak	Light white	Beige	None
7	Tyrosine agar (ISP 7)	Moderate	Light gray	Dark brown	Brown
8	Nutrient agar	Weak	Light white	Beige	None
9	Czapek's agar	Moderate	White	Light brown	None

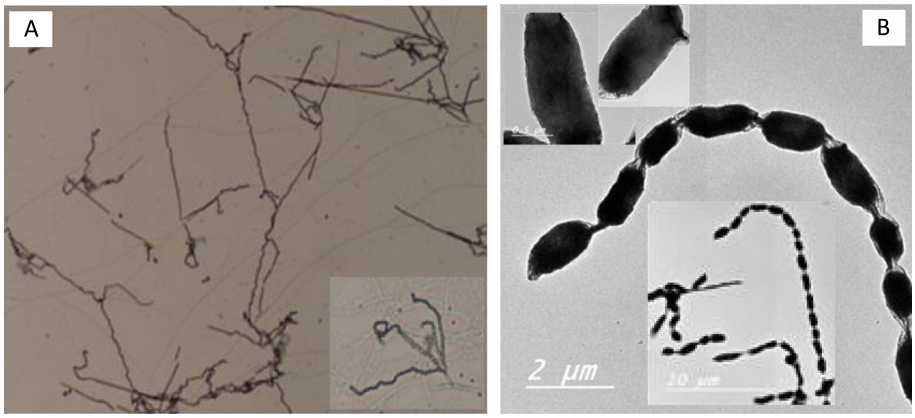

Fig. 2 a Light microscopy images of the aerial mycelium showing a spiral spore chain type (G x 400) for 14 days at 28 °C. **b** Scanning electron micrographs are showing smooth spore surface ornamentation (× 7, 500) of *Streptomyces* sp. SBLWN_MH2 grown on a starch nitrate medium for 21 days at 28 °C

3 Results

3.1 Isolation of *Streptomyces* producing lipase

Isolation plates from samples collected at latitude and longitude of 30° 26′ 20″ N and 30° 14′ 44.0″ E showed repeated occurrence (basing on apparent cultural characteristics) of eight different strains of *Streptomyces* (their colony count exceeded 10^3 c.f.u./gm), and so those strains were further streaked on the surface of agar plates before being transferred to slants of the same isolation medium.

Screening of lipolytic activities among the isolates showed the observable potency of the strain designated as SBLWN_MH2, which produced the highest yield (7.5 U/ml) of lipase. Preliminary investigation on the crude lipase from SBLWN_MH2 strain to get an approximate profile of enzyme activity at different buffered pH values showed detectable relative activity over broad pH values ranged between 3.5 and 13 as depicted graphically in Fig. 1.

The results have also shown an abrupt increase in relative activity by the change in pH buffer from 7 to 7.5, and the greater activity has been reached at pH 10.5. Surprisingly, with rising pH up to 13 with potassium chloride/sodium hydroxide buffer, the enzyme could exhibit more than 75% of its highest activity.

3.2 Identification of SBLWN_MH2 isolate

3.2.1 Conventional taxonomy

3.2.1.1 Morphological properties The traditional methods confirmed that the strain displayed features like other memberships of the genus *Streptomyces*. The results presented in Table 1 showed that the growth of the *Streptomyces* sp. SBLWN_MH2 isolate differs from frail to reasonable based on medium construction. The growth was reasonable on medium ISP 6 and nutrient agar (NA) and frail on the further media. The color of aerial mycelium varied from bright white to white. Therefore, the aerial mycelium has been assigned to the white group. The substrate mycelium varied from one medium to another medium, based on the medium contents.

The color of substrate mycelium was dark brown with ISP 7 and Czepcks media, while the color is beige on the other media. The soluble pigment was brown on ISP 7, while no pigment has been observed in the other media. Spore masses were matched against the seven color wheels of Tresner and Backus [24], as used in the ISP [17].

Figure 2a showed that the spore chains of the isolate have a spiral type when observed by a light microscope.

3.2.1.2 Physiological and biochemical characteristics As indicated in Table 2, SBLWN_MH2 isolate produced melanin pigment, degraded starch, and gave positive results for nitrate reduction on the medium used. On the other hand, the results were negative for milk coagulation and gelatin liquefaction.

All *Streptomyces* cell wall contains the LL-diaminopimelic acid (LL-DAP) type as an ordinary constituent [25], and the LL-DAP type has been noticed in the promised isolate cell wall. Moreover, the data in Table 2 delineated that all the tested sugar have been utilized by the selected isolate using medium no. 9.

SBLWN_MH2 isolate was growing well in a medium containing glycine while most of the tested amino acids gave moderate growing, as shown in Table 2. However, no growth has been recorded on a medium containing

Table 2 Morphological, physiological, and biochemical properties and amino acid utilization of *Streptomyces* sp. SBLWN_MH2

Characteristics	Results
Morphological characteristics	
Spore chains	Spiral
Spore surface	Smooth
Color of aerial mycelium	White series
Physiological characteristics	
Melanin production	Positive
Action of milk	No coagulation in 14 days
Nitrate reduction	Positive
Gelatin liquefaction	Negative
Starch hydrolysis	Positive
Cell wall	LL-diaminopimelic acid
Carbon utilization	
No carbon	–
D-glucose	+
D-xylose	±
L-arabinose	–
D-fructose	–
Raffinose	–
D-mannitol	+
Meso-inositol	±
Salicin	ND
Sucrose	±
Temperature tolerance (°C)	
28–30 °C	Abundant
40 °C	Weak
50 °C	No growth
Amino acids	
DL-methionine	–
DL-iso-leucine	+
L-arginine	+
L-lysine	–
L-glutamic	–
L-histidine	+
Ph-alanine	+
L-aspargine	+
L-valine	+
L-cysteine	+
Glycine	++
Prolene	+
Ornithine	+
Tyrosine	+
DL-serine	+
NaCl % resistance	
0	Gray, abundant

Table 2 Morphological, physiological, and biochemical properties and amino acid utilization of *Streptomyces* sp. SBLWN_MH2 *(Continued)*

Characteristics	Results
3–5	White, good
7–10	Light gray, weak
13	No growth
Antibiotic susceptibility (mm)	
Rifamycin (RD, 5 μg)	14[b]
Vancomycin (VA, 30 μg)	40[c]
Streptomycin (S, 10 μg)	00[a]
Neomycin (N, 30 μg)	30[c]
Tetracycline (TE, 5 μg)	20[c]
Nalidixic acid (NA, 30 μg)	15[b]
Novobiocin (NV, 30 μg)	40[c]
Cefodizime (CDZ 30 μg)	00[a]

ND not detected
"+++" = good growth, "++" = moderate growth, "+" = weak growth, "–" = negative
[a]Susceptible
[b]Intermediate
[c]Resistant

DL-methionine, L-lysine, and L-glutamic acid as a nitrogen basis.

Moreover, the data in Table 2 presented that the abundant growth of SBLWN_MH2 isolate was observed from 28 to 30 °C and weak growth at 40 °C, while there was no growth at 50 °C. On the other hand, the strain grew very well in the presence of 0–5% NaCl, but the growth was weak with 7–10% NaCl, and there is no growth using 13% NaCl. The characteristics of SBLWN_MH2 *Streptomyces* isolate have been contrasted with distributed portrayals of different *Streptomyces* types of conventional properties [17]. Antibiotic susceptibility of the SBLWN_MH2 isolate has been presented in Table 2.

In conclusion, SBLWN_MH2 isolate has a place with spiral hyphae, a smooth spore surface, and the white series with actual melanin color. In particular, SBLWN_

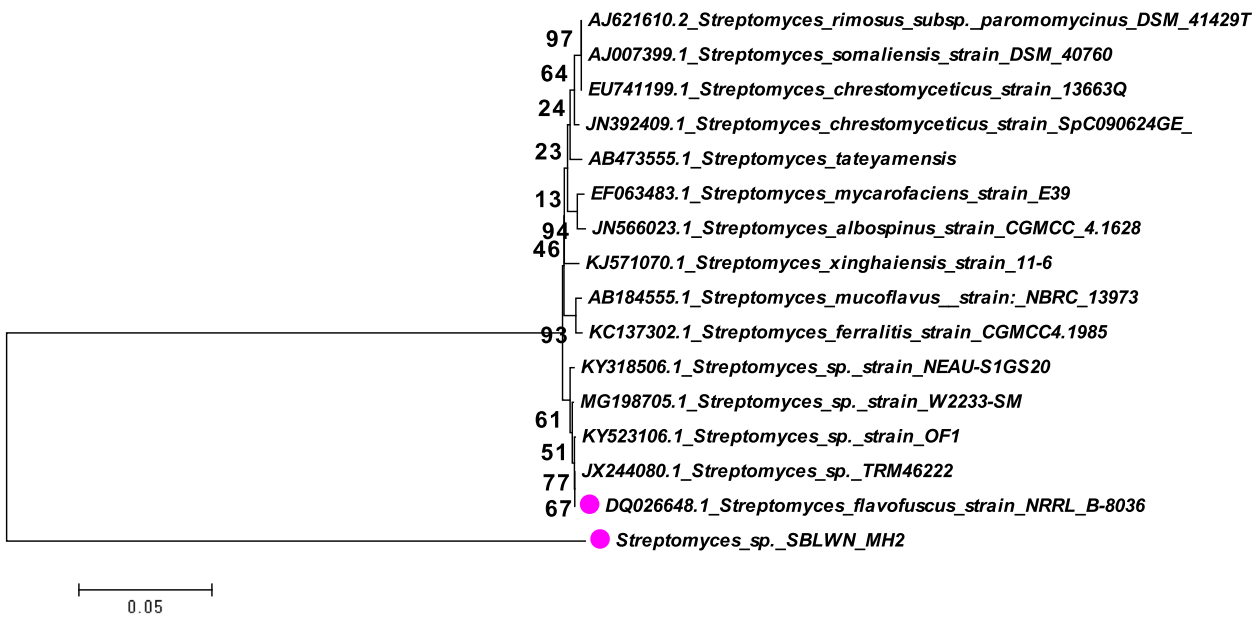

Fig. 3 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship between *Streptomyces* sp. SBLWN_MH2 and 19 isolates and closely related type strains of the genus *Streptomyces*. Bootstrap values (calculated as percentages from 500 replications) are indicated. The tree has been drawn to scale with branch lengths measured in the number of substitutions per site

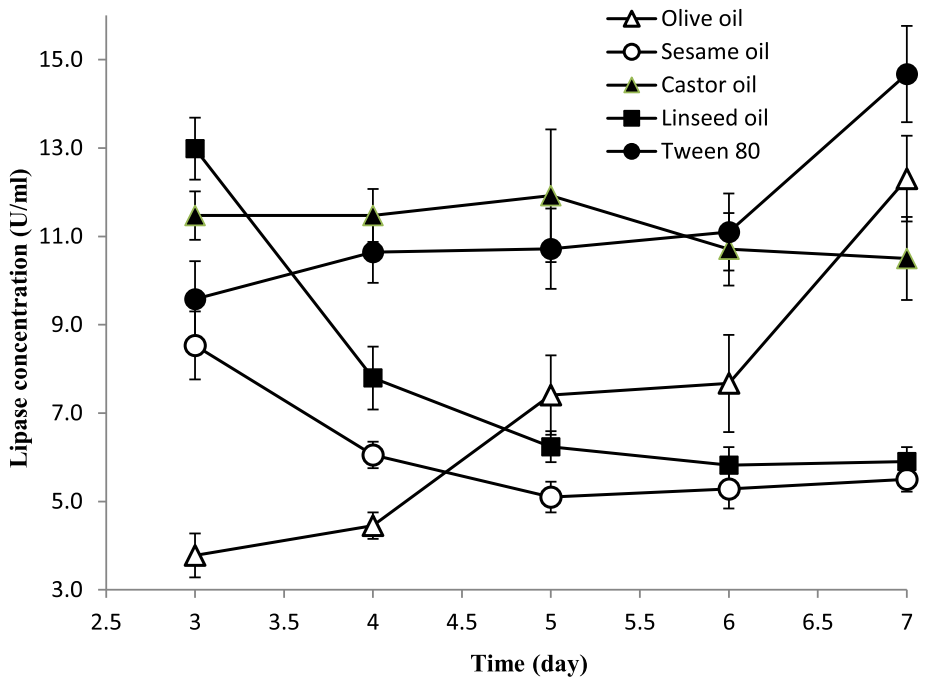

Fig. 4 Induction of lipase production by different substrates. Bars in the figure represent the standard error between replicates

MH2 isolate does not take over any appreciated *Streptomyces* strains.

3.2.2 Genotypic identification

The 16S rRNA is a useful asset for phylogenetic investigation and species differential of the *Streptomyces* species. Hence, in this study, examinations dependent on the 16S rRNA data for this strain were carried out corresponding to phenotypic methods.

3.2.2.1 PCR amplification of the 16S rRNA gene The 16S rRNA genetic material of SBLWN_MH2 isolate has been intensified utilizing the *Streptomyces*-explicit PCR primers formerly referenced in the "Methods" section. The PCR product of the strain indicated a band at 1.400 kb (information is not shown).

Some factors, e.g., the reaction conditions, annealing temperature, primers, and gene districts flanking the real site properties, have been influenced by the PCR specificity. In this study, the F27/R1492 primer pair was used to amplify the DNA isolate indicated by Edwards et al. [20] and Awad et al. [21]

3.2.2.2 Phylogenetic analysis The 16S rRNA nucleotide sequence of the SBLWN_MH2 isolate comprised of 1268 bp and matched in the GenBank database through the NCBI BLAST (www.ncbi.nlm.nih.gov). Assessment among the 16S rRNA sequence of this isolate with those individuals in the genomic database bank has been accomplished. This assessment demonstrated an elevated level of sequence similarity (99%) with numerous *Streptomyces* species.

Figure 3 represented and demonstrated the phylogenetic tree that resulting from distance matrices of fifteen nucleotide sequences by the neighbor-joining technique. The missing data and gaps in all positions have been wiped out, and the phylogenetic analysis has been accompanied by MEGA6 [23].

In conclusion, the phenotypic joined with genotypic methods of SBLWN_MH2 isolate designated that the neighboring strain is *Streptomyces flavosucs* NRRL-

Table 3 Two-factor ANOVA analysis for lipase induction by different substrates at different time courses

Source of variation	SS	df	MS	*F*	*p* value	*F* crit
Different times	11.91815	4	2.979537	0.501844	0.734906	3.006917
Different oils	117.512	4	29.37799	4.94814	0.008648	3.006917
Error	94.99486	16	5.937179			

SS sum of squares, *MS* mean square

Table 4 Box-Behnken design for modulating the effects of starch, linseed, and Tween-80 concentrations on lipase production by *Streptomyces* sp. SBLWN_MH2

Run	A: Starch conc. (g/l)	B: Linseed oil conc. (%)	C: Tween-80 conc. (g/l)	Lipase concentration (U/ml)
1	1	1	2	27.7
2	1	3	2	6.8
3	1	2	4	11.0
4	3	2	2	14.4
5	1	2	0	17.6
6	3	2	2	15.5
7	3	3	0	20.4
8	5	2	0	16.7
9	3	3	4	16.8
10	3	1	4	25.5
11	5	3	2	23.2
12	5	1	2	13.2
13	3	2	2	13.3
14	3	1	0	14.7
15	5	2	4	22.2

B8036, but not closely related to the phenotypic method. Therefore, *Streptomyces* sp. SBLWN_MH2 has been proposed as its name as a new species.

3.2.2.3 The accession number of the nucleotide sequence The 16S rRNA gene of *Streptomyces* sp. SBLWN_MH2 has been protected in GenBank under the ID: MG593538.

3.3 Kinetics of lipase production using different substrates

Kinetics of lipase induction by different lipid substrates was studied. The results depicted in Fig. 4 showed that lipase production varied considerably with different inducing substrates, namely olive oil, sesame oil, castor oil, linseed oil, and Tween-80. Tween-80 induced the highest yield of the enzyme (14.7 ± 1.1 U/ml) after a considerable long period of

Table 5 ANOVA for response surface in 2-factor interaction (2FI) model

Source	Sum of squares	df	Mean square	*F* value	*p* value Prob > *F*	
Model	374.55	6	62.43	7.02	0.0074	**Significant**
A: Starch conc.	018.60	1	18.60	2.09	0.1860	
B: Linseed oil conc.	024.15	1	24.15	2.72	0.1379	
C: Tween-80 conc.	004.65	1	04.65	00.52	0.4901	
AB	238.70	1	238.70	26.85	0.0008	
AC	036.60	1	036.60	04.12	0.0770	
BC	051.84	1	051.84	05.83	0.0422	
Residual	071.12	8	008.89			
Lack of fit	068.70	6	011.45	09.46	0.0986	**Not significant**
Pure error	002.42	2	001.21			
Design specifications						
Std. Dev.	02.980		R^2 (correlation coefficient)			0.8404
Mean	17.270		Adj R^2			0.7207
C.V. %	17.270		Adeq precision			9.2910
PRESS	265.65					

Adj adjusted, *Adeq* adequate signal

7 days, which revealed a slow rate of production (14.7 U/ml per 7 days) which was about 2 U/ml/day. Although linseed oil induced the second-highest yield (13 ± 0.7 U/ml) at 3 days, it was the most potent inducer considering the production rate (13 U/ml per 3 days), around 4.3 U/ml/day.

Other substrates induced various amounts of lipases at different times, and all were of observable lower inducing potentiality compared with linseed oil or Tween-80. To confirm the significance of the effects of lipid substrate, and time course, analysis of variance (ANOVA) for the obtained data was conducted and presented in Table 3. ANOVA analysis showed a highly significant effect of the type of lipid substrate on lipase production (p value = 0.0086).

3.4 Interactions of carbon nutrition, oil, and surfactant in lipase production

To modulate linseed concentration for the optimal induction of lipase produced by *Streptomyces* sp. SBLWN_MH2, it was of interest to consider the nutritional role of linseed oil as a carbon source which may interfere with the role of the primary carbon source in the medium, starch. Also, to test the ability of Tween-80 to act as a surfactant, it was not possible to neglect its role as an inducer of lipase, which may interfere with that of linseed oil.

Therefore, the levels of linseed oil, starch, and Tween-80 and possible interactions between their roles have been observed modulated through response surface methodology built on the Box-Behnken design. The Box-Behnken design is a three-level fractional factorial design [26]. The three factors of starch, linseed, and Tween-80 concentrations (coded by A, B, and C, respectively) have been applied at three levels in the design consisting of 15 runs and three center points. Table 4 showed the design with corresponding lipase concentrations for each run.

Analysis of variance (ANOVA) of the design was conducted and presented in Table 5.

ANOVA results in Table 5 showed a very significant model of two-factor interaction. The model has a p value of 0.0074 and an F value of 7.02, which indicated that the model is significant. There is only a 0.74% chance that a "model F value" this large could occur due to noise. Significant interactions between factors A and B and also between B and C were observed (p value = 0.0008 and 0.0422, respectively).

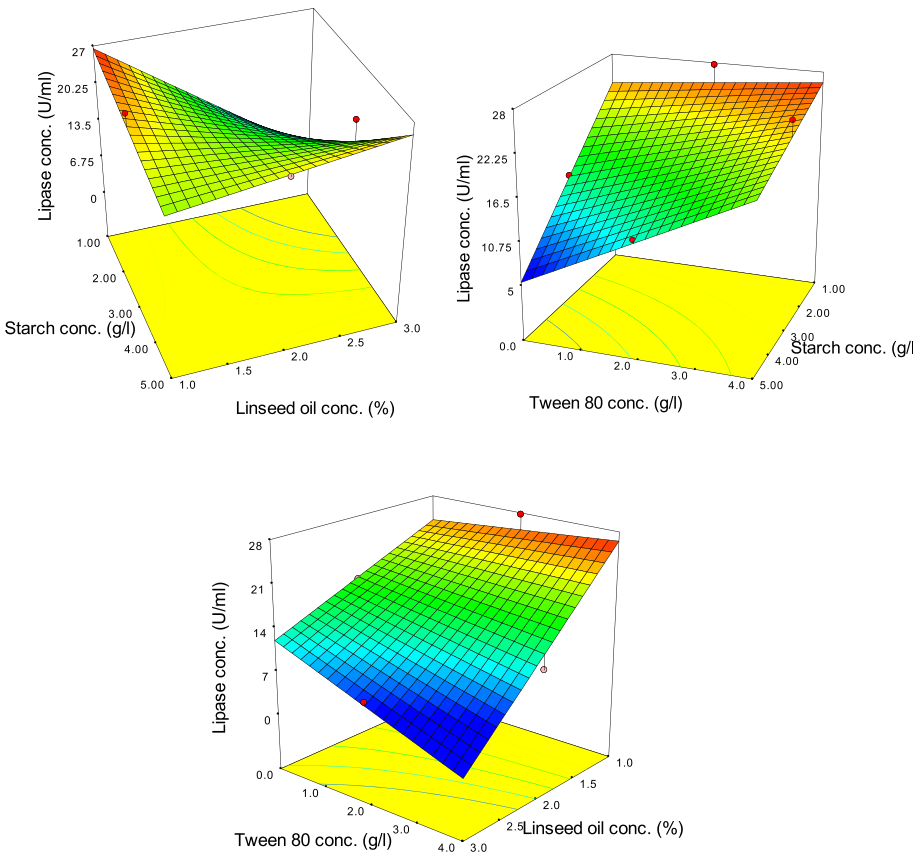

Fig. 5 The three-dimensional surface plot for the lipase production relative to a combination of two factors

"Adeq precision" estimates the signal to noise ratio. A ratio larger than 4 has been expected. The design ratio of 9.291 designates an acceptable signal, and so the model can be used to navigate the design interplanetary. Response surface methodology (RSM) was then constructed based on the data of Box-Behnken design to develop a mathematical equation that describes the model and predicts the response from studying factors [27] in a three-dimensional (surface) plot of the response to the combination of two of the studied factors. The final equation describing the effect of the three factors on lipase production is the following 2-factor interaction equation:

Lipase concentration (U/ml) = + 38.20417 − 8.475A − 9.725B + 1.7125C + 3.8625AB + 0.75625 AC − 1.8 BC

The three-dimensional surface plot for the response (lipase concentration) relative to the combination of two of the studied factors has been depicted (Fig. 5). An apparent interaction between starch and linseed oil in lipase production was observed by following up the effect of starch concentration on lipase production at two different levels of linseed oil as graphically represented in Fig. 6.

Change in linseed level from 1 to 3% has altered the shape of the relation between starch concentration and lipase production. At the lowest level of linseed oil (1%), the highest amount of lipase has been obtained at a low starch concentration (1 g/l), and more increase in starch resulted in a deterioration in lipase production (negative effect of starch on lipase production). Nevertheless, at 3% linseed oil, the increase in starch concentration was accompanied by an enhancement in lipase production (positive effect), and so the highest amount of lipase was attained at the highest starch quantity (5 g/l). Accordingly, the highest lipase activity was obtained at high levels of 5 g/l starch and 3% linseed oil. The most noteworthy lipase yield could be achieved by reducing the levels of starch and linseed oil simultaneously (1 g/l and 1%, respectively) which may offer an economic advantage in production systems.

In the same manner, the interaction between linseed oil and Tween-80 has been realized in the plot representing the effect of linseed oil concentration on lipase production at two different levels of Tween-80, as depicted in Fig. 7. The plot in Fig. 7 showed an alteration in the shape of the relation between linseed oil concentration and lipase production under a change in Tween-80 level from 0.0 to 4 g/l. An increase in linseed oil was of a weak activating role in lipase production in the absence of Tween-80 (0.0 g/l), whereas it was about

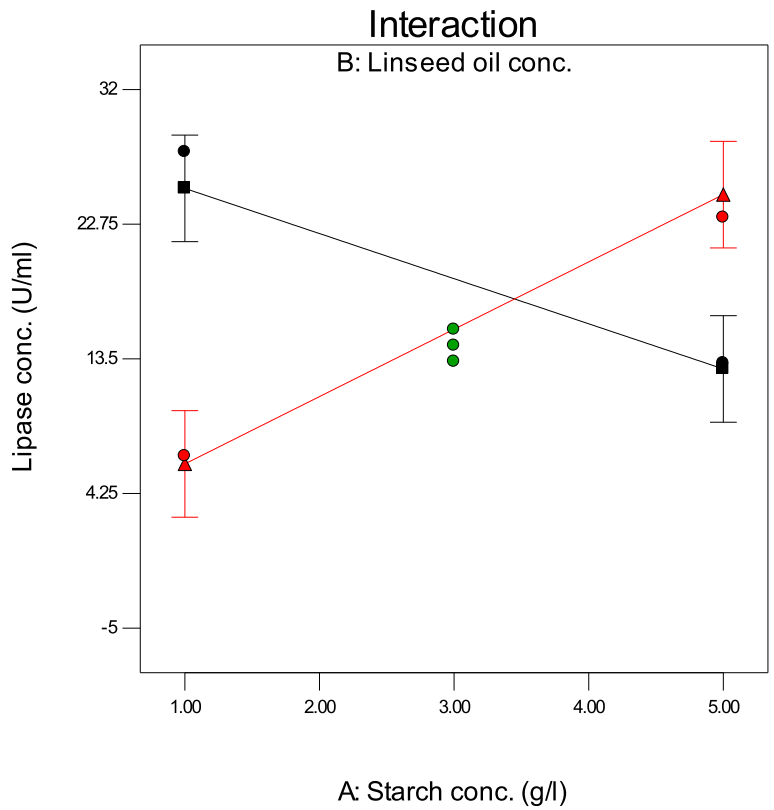

Fig. 6 The effect of starch concentration on lipase production at linseed oil levels of 1% (black-colored line) and 3% (red-colored line). Bars on the plot represent the least significant difference

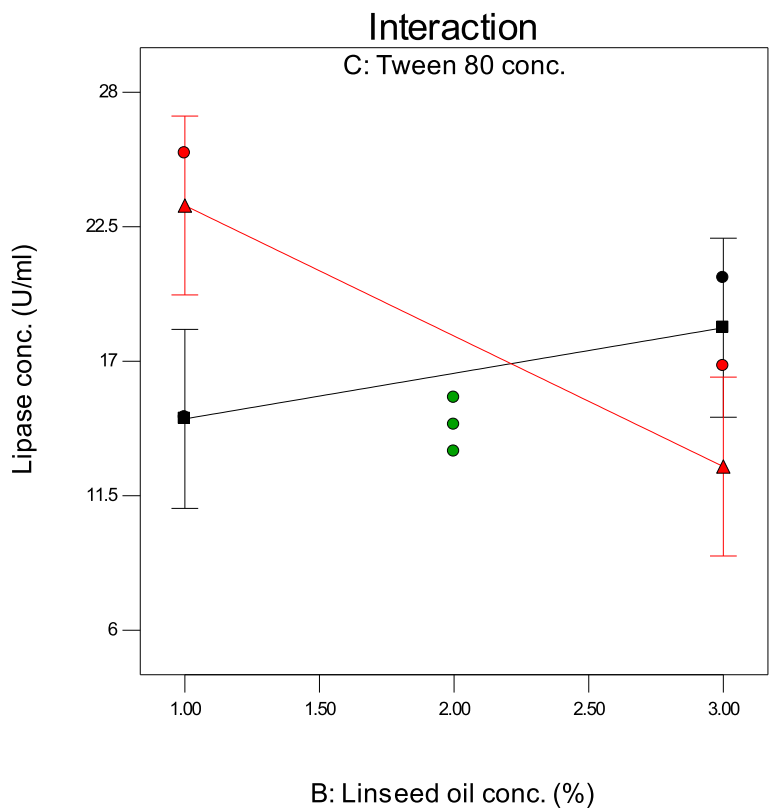

Fig. 7 The effect of linseed oil concentration on lipase production at Tween-80 levels of 0.0 g/l (black-colored line) and 4.0 g/l (red-colored line). Bars on the plot represent the least significant difference

the remarkable inactivation effect of the high levels of Tween-80 (4 g/l).

The optimum parameters and lipase productivity defined by the Box-Behnken design and RSM have been illustrated in Table 6.

4 Discussions

Wadi El Natrun district is an elongated depression that is 23 m below sea level and located about 90 km northwest of Cairo, Egypt [28]. The halo-alkaline habitat in Wadi El Natrun district in Egypt is one of the most stable highly alkaline environments on earth [7], and all lakes had pH values of 8.5–9.5 and salinity from 283 to 540 g/l with inferior content of Ca and Mg and so appear to be unique among saline lakes [28]. Al-Beida Lake, one of the salt marshes in Wadi El Natrun district, was undertaken in isolation course to investigate its residents of *Streptomycetes* in the spring of 2016.

SBLWN_MH2 strain exhibited noticeable relative activity over widespread pH values stretched between 3.5 and 13, as shown in Fig. 1. Among alkalophilic lipases, enzymes that have an optimum pH over ten were seldom reported [13, 29]; thus, the lipase from the Wadi El Natrun strain SBLWN_MH2 was viewed as one of the profoundly highly alkaline remarkable enzymes. Also,

Table 6 The optimum parameters and lipase productivity defined by the Box-Behnken design and RSM

Fermentation parameter	Amount
Parameters for maximum lipase production	
Soluble starch conc. (g/l)	1.00
Casein (g/l)	0.30
K_2HPO_4 (g/l)	2.00
KNO_3 (g/l)	2.00
NaCl (g/l)	50.00
$MgSO_4.7H_2O$ conc. (g/l)	0.05
$FeSO_4.7H_2O$ (g/l)	0.01
Anhydrous $CaCO_3$ (g/l)	0.02
Linseed oil (%)	1.00
Tween-80 conc. (g/l)	4.00
Medium pH	8.20
Time course (day)	3.00
Lipase productivity (U/ml)	
Yield	26.54
SE mean	03.25
95% CI low	19.04
95% CI high	34.05
SE Pred	04.41

the previous investigations about lipases produced by microbes of halo-alkaline habitat are very scarce [13, 30], and there was a complete absence of *Streptomycetes* species in the previous composition of bacterial communities in Wadi El Natrun [31]. Therefore, in-depth investigations to describe and identify the strain SBLWN_MH2 on phenotypic and genotypic bases have been intended.

Locci [18] mentioned the strain photos under an ordinary microscope have been assembled as rectus-flexible (RF), retinaculum apertum (RA), and spiral (S). Figure 2b shows that the individual spores are tube-shaped with a smooth surface, which was resolved by the classifications of Tresner et al. [32], who found that the spore surface is one of the distinct portrayals for each sort of culture. Other categories like the previous physiological properties were verified and measured to establish the species taxonomy of the novel isolate prescribed by Locci, [33].

In this case, the taxonomy of *Streptomyces* dependent on traditional methods is insufficient most of the time. Consequently, genotypic methods, dominatingly rRNA genetic material structures, have been presented [22]. It very well may be utilized as a phylogenetic strategy corresponding to the traditional methods [34].

Kinetics of lipase induction results by different lipid substrates were illustrated in Fig. 4 and indicated that lipase activity diverse significantly with different substrates. Many investigators have been engaged with increasing lipase productivity [3, 35, 36] through studying various parameters and activators, yet they neglected the comparison between the inducing potential of different substrates. Sande et al. [37] studied the hydrolytic activity of lipase from *Colletotrichum gloeosporioides* using different substrates, and the maximum activity was shown in the case of olive oil, while the lowest activity was in the case of linseed (flaxseed) and castor oils. Differently, Lakshmi et al. [38] found that sesame oil was the best substrate among the different oils tested.

Supakdamrongkul et al. [39] found that castor oil potentiated the highest yield of lipase by the fungus *Nomuraea rileyi*, while the linseed oil induced the lowest production yield. Along these lines, the hydrolytic activity profile of various lipases against various substrates ought to be utilized as a basic illustrative character in enzyme production. Linseed oil contains high content (over 50%) of omega-three fatty acids in the form of α-linolenic acid [40]. As linseed oil was the most appropriate substrate for the current lipase, utilization of the current lipase in the extraction of omega-three fatty acids from linseed oil is emphatically anticipated [37].

The advantage of the Box-Behnken design in profiling interaction between variables was associated with the further advantage of defining the parameters for attaining 26.5416 U/ml of lipase (more than 3.5-fold increase in initial yield of 7.5 U/ml) which is more suitable for bioreactors studies.

5 Conclusion

Lipases are the third major enzymes in total sales bulk after proteases and amylases. A new potent lipase producer was isolated from soda salt marsh in Wadi El Natrun in Egypt. Phenotypic and genotypic identification of the new producer showed that it was a member of the genus *Streptomyces* with no perfect similarity with any known species of the genus and designated as *Streptomyces* sp. SBLWN_MH2 (MG593538). The enzyme was highly alkaline, and its optimum production medium has been addressed after being developed on statistical bases. The suitability of the strain for future large-scale production and new applications of lipase has clearly prospected. The study highlighted the soda salt ecologies as a valuable resource in industrial bio-catalysis.

Abbreviations

BLAST-N: Basic local alignment search tool; CDA: Czapek-Dox agar; CSPY: Casein starch peptone yeast extract agar; DNA: Deoxyribonucleic acid; MEA: Malt extract agar; PCR: Polymerase chain reaction; GC: Guanine and cytosine

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Authors' contributions

MM contributed to the design and implementation of the research, verified the analytical methods of enzyme characterization, wrote the part of enzyme optimization, fabricated the XYZ sample for statistical optimization designs, and contributed to the final version of the manuscript. HA contributed to the design and implementation of the research, carried out the experiment of isolation and identification of the strain with support from MM, took the lead in writing the isolation and identification part, and contributed to the final version of the manuscript. All authors have read and approved the final article.

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Availability of data and materials

The datasets generated during the current study are available in the GenBank repository, NCBI. These include *Streptomyces* sp. SBLWN_MH2 (MG593538).

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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