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ORIGINAL ARTICLE

Isolation, identification, and statistical optimization of a psychrotolerant *Mucor racemosus* for sustainable lipid production

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Abstract

Lipid accumulating fungi are promising tools as alternative lipid source with different applications. In this study, seven oleaginous fungal strains were isolated from dung samples from the Egyptian ecosystem which later investigated for lipid accumulation, and *Mucor racemosus* AH1 represented the highest one. Statistical optimization of *M. racemosus* AH1 for lipid production was carried out using Taguchi design. Accordingly, dry biomass, total lipids, and lipid content were 3.72 gL^{-1}, 1.21 gL^{-1}, and 32.4%, respectively. Fatty acid profile of the produced lipids at different temperatures from 5 to 35 °C was investigated using gas chromatography mass spectroscopy GC–MS. Results revealed that the best temperature range for unsaturated fatty acids production particularly polyunsaturated fatty acids (PUFAs) was between 10 and 20 °C in which unsaturated fatty acids (USFAs) were higher than saturated fatty acids (SFAs); 54.47% and 43.67%, respectively. In conclusion, a promising lipid accumulating and cold-adapted *M. racemosus* MG547571 are considered as hopeful source of USFAs particularly oleic and linoleic acids which can be recruited for pharmaceutical applications, additionally, the high lipid yield could be exploited for biodiesel production.

Keywords Psychrotolerant · Oleaginous fungi · *Mucor racemosus* · Taguchi design · Lipid production · GC–MS

1 Introduction

As a result, for population increase, a climate change, etc., there is a constant need for food, health, and energy sources, especially fuel. Lipids play an important role to offer a green alternative source of energy demands, as well as several industrial applications [1]. Economic development in industrial countries led to the rapid consumption of fossil fuels but in the same time it is a reason for air pollution by increasing greenhouse gasses in atmosphere and consequently causes climate change and global warming. The fossil fuel should be replaced by renewable energy sources such as solar energy, tidal energy, wind, and biofuel. Renewable energy sources are getting universal consideration and play an imperative role in meeting the future needs of the world [2]. Biofuels are derived from biomass and represent an essential contribution to our future energy supply [3]. Trans-esterification process is used to convert fats and oils in biological samples to biodiesel.

Biofuel production based on biomass has emerged as a major approach to enabling energy independence, reducing greenhouse gas emissions, revitalizing rural communities, and enhancing sustainable economic development. Oleaginous microorganisms can accumulate lipids of more than 20% (w/w) of their total dry biomass weight [4]. Oleaginous microorganisms are a group of species that belong to different varieties of microbial genera including bacteria, yeasts, algae, and fungi [5]. Recently, microbial lipids from filamentous fungi, yeasts, and microalgae are considered as a possible alternative source for biodiesel production, where contain high amounts of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFA) [6]. Fungi are the greatest group of microorganisms that have the ability to produce single cell oils (SCOs) due to their short life cycle and no need for light energy as well as their ability to

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utilize a wide range of carbon sources such as agro-industrial residues, lignocellulosic material, wastewater, and crude glycerol [7, 8]. There are many fungal species such as *Mortierella isabellina* and *Mortierella vinacea*, *Cunninghamella echinulata*, *Rhizopus oryzae*, *Aspergillus oryzae*, and *Mucor circinelloides* have been defined as SCO producers [9–12]. In some cases, the oleaginous microorganisms can do more than one benefit over their productivity of lipid which can be recruited in multipurpose fields, moreover, it could help in biorefinery, bioremediation, decolorization [13, 14], and medically important agents [15–17]. Therefore, the searching for a new oleaginous fungus is required due to several advantages of microbial lipids particularly fungi. This study is aimed at isolating and identifying a promising oleaginous fungal isolate with much higher ability to produce high quantities of lipids using of Taguchi design as a statistical tool for optimization of lipid production. Also, studying the effect of different temperatures on fatty acids types.

2 Material and methods

2.1 Isolation and screening of fungal isolates

The dung samples were collected carefully into complete sterile plastic bags, and the samples were diluted as 1: 100 (g/v) in a sterile saline solution. Malt extract agar medium (MEA) was purchased from Sigma Aldrich, Germany; used as growth medium while the production medium composed of glucose 100 gL^{-1} and yeast extract 10 gL^{-1}, with pH adjusted to 5.4. The 10% (v/v) mycelial suspension of isolated culture was inoculated in 100 mL flask containing 25 mL of malt extract broth and incubated at 30 °C for 7 days [18–20]. Fungal isolates were isolated from different dung sample from Giza, Egypt. Isolation was carried out according to method used by Hashem et al. [21]. Screening of fungal isolates was carried out by using Nile-red dye, and reparation and staining methods of dye were performed according to Lim et al. [22] by whom, a stock solution of 0.4 mg/mL (w/v), then, the working stain was prepared through 200-fold dilution to reach 2 µg/mL as a final working concentration. The stained lipid bodies were photographed using fluorescence microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (U-CMT, Olympus, Tokyo, Japan) [23].

2.2 Identification of the most potent fungal isolate

Morphological identification of the selected fungal isolate was carried out depending upon the culture characteristics (color, texture appearance, and diameter of the colonies), as well as the microscopic investigations using both the light and scanning electron microscope which was performed to confirm the morphological characteristics of fungal isolates [24–31]. Finally, the molecular approach had been used to confirm the fungal identification using the 18S-rRNA (partial sequence depending on a specific pair of primers). Additionally, ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) was used for sequencing of PCR products. The final sequence was aligned with similar sequences using the NCBI BLAST search program in the National Center for Biotechnology Information (NCBI). Evolutionary analyses were performed using MEGA-x [32–34].

2.3 Dry weight determination, lipid extraction, and lipid quantification

Biomass production was determined by harvesting the cells by filtration followed by drying at 55–60 °C overnight or until constant weight then determined. The extraction of lipid was performed by adding 40 mL of chloroform–methanol (2:1) to 1 g of grinded dry biomass, then, this mixture was agitated for 20 min at 20 °C and then filtered with Whatman paper no. 1. The solvent containing lipids was separated then evaporated then lipids were determined [12, 35]. The extracted lipid was quantified using sulfo-phospho vanillin method (SPV) [12, 36, 37]. Phosphovanillin reagent was prepared by mixing of vanillin 6% with phosphoric acid 85%. The test sample was prepared by diluting of 20 µL of samples with 180 µL of sulfuric acid and incubated at 100 °C for 10 min., then cooled at room temperature, after that, phosphovanillin reagent and kept for a while till color development, and finally the test sample was measured at 530 nm [38].

2.4 Methyl ester preparation and fatty acid analysis

Methylation was carried out to the extracted lipid to convert fatty acids to fatty acid methyl ester (FAMEs) [39]. Two layers were formed after cooling. The lower layer which contains fatty acids methyl esters (FAMEs) was separated with chloroform, and the final FAMEs product was obtained by evaporating chloroform from the solution. FAMEs were analyzed by GC–MS [37, 40].

2.5 Optimization of lipid production using a statistical design

Taguchi design was used for optimization factors affecting lipid production such as carbon source, nitrogen source, temperature, incubation time, and pH. Optimization process was carried out with two stages. The first stage includes selecting the best condition for each factor using L16 array of Taguchi design. Factors are carbon source (glucose, sucrose, starch, and CMC), nitrogen source (peptone, yeast extract, sodium

nitrate, and ammonium nitrate), initial pH (4 and 7), and incubation period (4, 7, and 10 days). Second stage includes testing effect of five factors on lipid production to reach optimum conditions of *M. racemosus* AH1 for lipid production. Table 1 shows different factors and their levels for lipid production, where factors include temperature (15, 20, 25, 30, and 35 °C), initial pH (5, 6, 7, 8, and 9), incubation time (8, 9, 10, 11, and 12 days), different concentrations of soluble starch (10, 20, 30, 40, and 50 gL^{-1}), and different concentrations of yeast extract (1, 2, 3, 4, and 5 gL^{-1}). Table 2 illustrates L25 array of Taguchi design which contains different factors and their levels to produce high quantities of lipids by *M. racemosus AH1*.

2.6 Statistical analysis

All the experiments including both first and second optimization steps were performed in triplicates, and statistical analysis was carried out using Minitab software (version 18). The values are given as means ± SD (standard deviations). Levels of significance were considered at $p \leq 0.05$. Statistical analysis is investigated by ANOVA (one-way analysis of variance) Tukey method for the obtained results.

3 Result and discussion

3.1 Isolation and screening of fungi

Totally, seven fungal isolates were recovered from the dung sample using dilution agar plating. The seven fungal isolates were screened for lipid accumulation using Nile-red dye and photographed using fluorescence microscope. Result showed the fungal isolate AH1 is the most potent for lipid accumulation where contains many lipid bodies under light microscope (Fig. 1A), and under fluorescence microscope was appeared with red color bodies as shown in Fig. 1B. Furthermore, fungal isolate AH1 was incubated for different intervals 7, 14, and 21 days, and the lipid quantity was quantified using SPV methods as shown in Table 3. Results illustrated that the time at 7 days is the best for lipid production where dry biomass, total lipids, and lipid content were 2.22 gL^{-1}, 0.72 gL^{-1}, and 32.43%, respectively. At incubation time, 14 and 21 days lead to decrease total lipid to 0.59 and 0.42 gL^{-1}, respectively [10, 41]. In the long run, the decrease in lipid accumulation may be due to the nutrient shortage, byproducts accumulation, pH change, lipid peroxidation, and other environmental changes that make the fungus consume the storing lipids to recompense the nutrients' shortage to enable it to survive longer [42]. Thus, the fungal isolate AH1 is considered oleaginous fungus because accumulates lipid more than 20% of its dry weight.

Table 1 Different factors and their levels for lipid production

Factor	L1*	L2	L3	L4	L5
Temperature (°C)	15	20	25	30	35
Initial pH	5	6	7	8	9
Time (days)	8	9	10	11	12
Soluble starch (gL^{-1})	10	20	30	40	50
Yeast extract (gL^{-1})	1	2	3	4	5

*L means level

Table 2 L25 array by Taguchi design of the selected nutritional factors for maximum lipid production by the fungal isolate

Experiment no	Temp. (°C)	pH	Time (day)	Starch (gL^{-1})	Yeast extract (gL^{-1})
1	15	5	8	10	1
2	15	6	9	20	2
3	15	7	10	30	3
4	15	8	11	40	4
5	15	9	12	50	5
6	20	5	8	30	4
7	20	6	9	40	5
8	20	7	10	50	1
9	20	8	11	10	2
10	20	9	12	20	3
11	25	5	8	50	2
12	25	6	9	10	3
13	25	7	10	20	4
14	25	8	11	30	5
15	25	9	12	40	1
16	30	5	8	20	5
17	30	6	9	30	1
18	30	7	10	40	2
19	30	8	11	50	3
20	30	9	12	10	4
21	35	5	8	40	3
22	35	6	9	50	4
23	35	7	10	10	5
24	35	8	11	20	1
25	35	9	12	30	2

3.2 Identification of the most potent fungi

Classical and molecular identification were carried out for the two fungal isolates AH1. Routine identification is one of the most techniques which used for identification of fungi [24, 43]. Figure 2 shows macroscopic and microscopic characteristics of AH1 fungal isolate, where showed that 4-day age culture appeared moderate growth, light in color (Fig. 2A), sporangia globose, light brown, encrusted walls, up to 80 mm. Columellae ellipsoidal to pyriform, up to

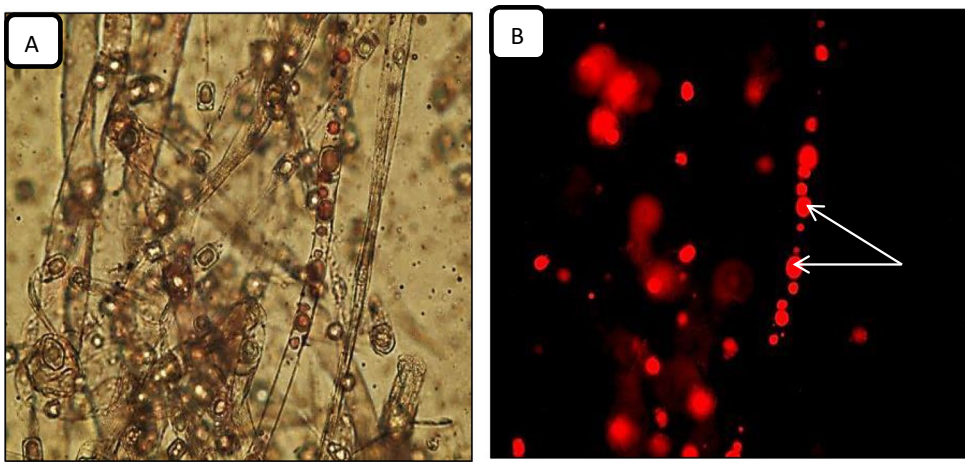

Fig. 1 Lipid bodies produced by fungal isolate AH1 under light microscopy (**A**) and fluorescence microscopy (**B**)

Table 3 Quantitative determination of lipid production at different times

Time/days	Dry biomass gL^{-1}	Total lipids gL^{-1}	Lipid content %
7	**2.22 ± 0.13[a]**	**0.72 ± 0.05[a]**	**32.43 ± 0.66[a]**
14	2.76 ± 0.11[b]	0.59 ± 0.02[b]	21.37 ± 0.27[b]
21	2.99 ± 0.09[c]	0.42 ± 0.03[c]	14.04 ± 0.49[c]

40-mm long (Fig. 2B&D). Sporangiospores are angular, subglobose to ellipsoidal and up to 10 µm in length (Fig. 2C). At the molecular level, the top hit was showed 98% identical similarity with *M. racemosus* strain CBS 636.67 18S ribosomal RNA gene, partial sequence with the highest alignment score 2028 and 79% query covered (Fig. 2E). Fungal isolate AH1 was identified as *M. racemosus* and recorded in gen bank with accession number MG547571. *M. racemosus* was used for lipid production [44, 45]. Another species of Mucor have the ability to accumulate lipid more than 20% as *M. circinelloides* [46–48], *M. hiemalis* [49], and *M. indicus* [50].

3.3 Statistical optimization of lipid production by M. racemosus MG547571 using Taguchi design

Oil production represented an important research field with special interest in food biotechnology in particular polyunsaturated fatty acids (PUFAs) [51], as known, plants were considered sources of variety of bioactive compounds [52, 53], and fungi and other microbes are not less important than plants to produce valuable compounds [15, 54]. In particular, oleaginous microorganisms, including fungi and microalgae, are able to grow fastly on several substrates and they were considered as the main producers of PUFAs that could be recruited in many different industrial applications [55]. Lipids are essential for all organisms as one of the three macronutrients. Oleaginous fungi mainly yeasts and zygomycetes can accumulate up to 80% of their biomass as lipids. Lipids in all forms including SFAs, MUFAs, and PUFAs can contribute to synthesize biodiesel through acylglycerides. As well, lipids can conjugate with other biomolecules such as glycolipids which have high potential as biosurfactant [56].

According to recent studies, incubation temperature, initial pH value, the incubation period, carbon, and nitrogen source were listed among the most important factors affecting lipid production [57, 58]. Therefore, these factors are studied in this study. L16 Taguchi design was used to select the best carbon interacted with nitrogen source. L16 design contains runs from 1 to 16. Table 4 illustrates that the run no. 10 was the best for lipid production, and this run includes soluble starch with yeast extract which produced lipid 0.93 gL^{-1}, dry biomass 2.8 gL^{-1}, and lipid content 33.07%. Aoki et al. [59] reported that 3% of soluble starch is the best for lipid production by *M. hiemalis* HA-30. In addition, results showed that yeast extract is the best nitrogen source for lipid production by *M. racemosus* MG547571. In the same accordance with our results, [18] reported that yeast extract is the best for lipid production by *Mucor sp.* Lipids and starch have a specific relation because they represent the major energy forms especially in plants, and according to Yu et al. [60] who proved the role of fatty acid β-oxidation and the regulatory network controlling fatty acid synthesis, and they reveal the mechanistic basis by which starch and lipid metabolic pathways interact and undergo cross talk to modulate carbon allocation, and energy homeostasis.

pH factor is considered one of the most factors affected lipid production. Total lipid and lipid content at pH 4 and 7 were (0.33 gL^{-1} and 19.06%) and (0.91gL^{-1} and 31.11%), respectively, as shown in Fig. 3A. [18] demonstrated that *Mucor spp.* produced the maximum yields of g-linoleic acid and total PUFA when the medium pH was 6.5. Moreover, [59] reported that pH at 6 is the optimum for EPA production by *M. hiemalis* HA-30. From

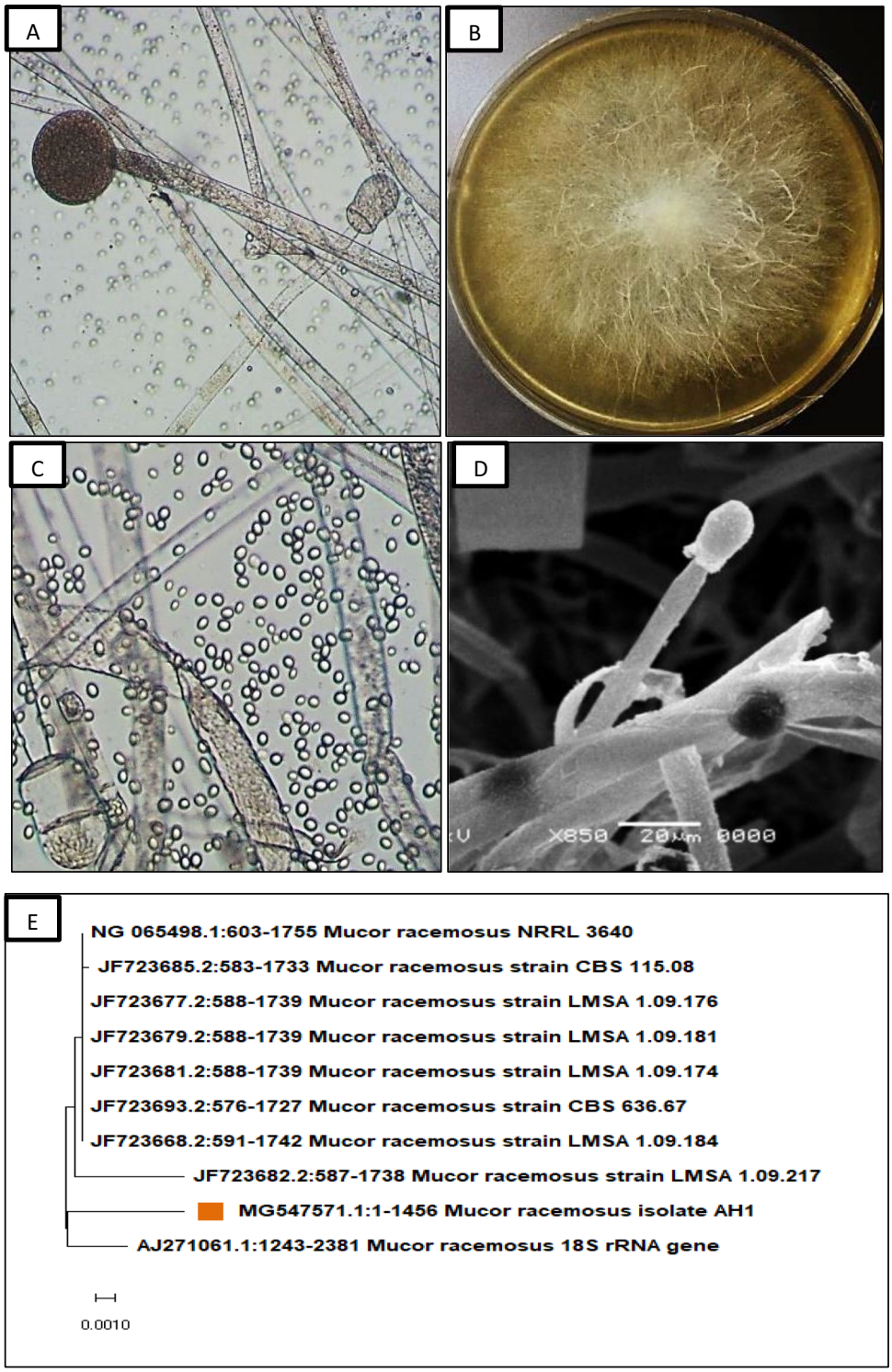

Fig. 2 Classical and molecular identification of 4-day age culture of *M. racemosus* AH1 (A–C): (**A**) colony on MEA. (**B**) Sporangium and sporangiophore (×400). (**C**) Sporangiospore. (**D**) Scanning electron microscopy (×850). (**E**) Phylogenetic tree

the previous results, it is obvious to find that pH at 7 was the best center point pH for lipid production to use it in the next step of optimization. Four, seven, and ten days were performed to detect center point of incubation period which will be used in Taguchi design**.** Figure 3B shows ten days are the most favorable center point of incubation period for lipid production by *M. racemosus* MG547571.

Taguchi design is very important for optimization processes. This design was used to achieve the highest lipid production by optimizing the medium conditions [57, 58].

Table 4 Effect of carbon and nitrogen source on lipid production by *M. racemosus* MG547571 using L16 array Taguchi design

Exp. no	Carbon source	Nitrogen source	Dry biomass gL^{-1}	Lipid gL^{-1}	Lipid %
1	**Glucose**	**Peptone**	1.60 ± 0.08^{abc}	0.41 ± 0.016^{c}	25.93 ± 0.32^{b}
2	**Glucose**	**Yeast extract**	0.31 ± 0.02^{c}	0.07 ± 0.004^{gh}	22.50 ± 0.49^{c}
3	**Glucose**	**Sod. nitrate**	0.39 ± 0.06^{c}	0.05 ± 0.007^{h}	14.04 ± 0.31^{fg}
4	**Glucose**	**Amm. nitrate**	0.68 ± 0.08^{c}	0.09 ± 0.013^{gh}	13.04 ± 0.48^{fg}
5	**Sucrose**	**Peptone**	2.52 ± 0.11^{ab}	0.67 ± 0.027^{b}	26.73 ± 0.68^{b}
6	**Sucrose**	**Yeast extract**	1.03 ± 0.17^{bc}	0.23 ± 0.038^{e}	22.50 ± 0.58^{c}
7	**Sucrose**	**Sod. nitrate**	0.64 ± 0.04^{c}	0.12 ± 0.003^{fg}	18.66 ± 0.60
8	**Sucrose**	**Amm. nitrate**	1.24 ± 0.08^{abc}	0.15 ± 0.006^{f}	12.37 ± 0.40^{gh}
9	**Starch**	**Peptone**	1.53 ± 0.02^{abc}	0.35 ± 0.009^{d}	23.14 ± 0.48^{c}
10	**Starch**	**Yeast extract**	$\mathbf{2.80 \pm 0.05^{a}}$	$\mathbf{0.80 \pm 0.015^{a}}$	$\mathbf{28.57 \pm 1.43^{a}}$
11	**Starch**	**Sod. nitrate**	0.97 ± 0.08^{bc}	0.15 ± 0.009^{f}	15.24 ± 0.32^{ef}
12	**Starch**	**Amm. nitrate**	0.75 ± 0.05^{c}	0.08 ± 0.001^{gh}	10.26 ± 0.50^{h}
13	**CMC**	**Peptone**	1.16 ± 0.04^{abc}	0.08 ± 0.004^{gh}	7.20 ± 0.37^{i}
14	**CMC**	**Yeast extract**	0.65 ± 0.05^{c}	0.11 ± 0.006^{fg}	16.54 ± 0.37^{de}
15	**CMC**	**Sod. nitrate**	1.81 ± 2.24^{abc}	0.05 ± 0.040^{h}	4.47 ± 1.92^{j}
16	**CMC**	**Amm. nitrate**	0.60 ± 0.04^{c}	0.04 ± 0.004^{h}	6.29 ± 0.39^{ij}

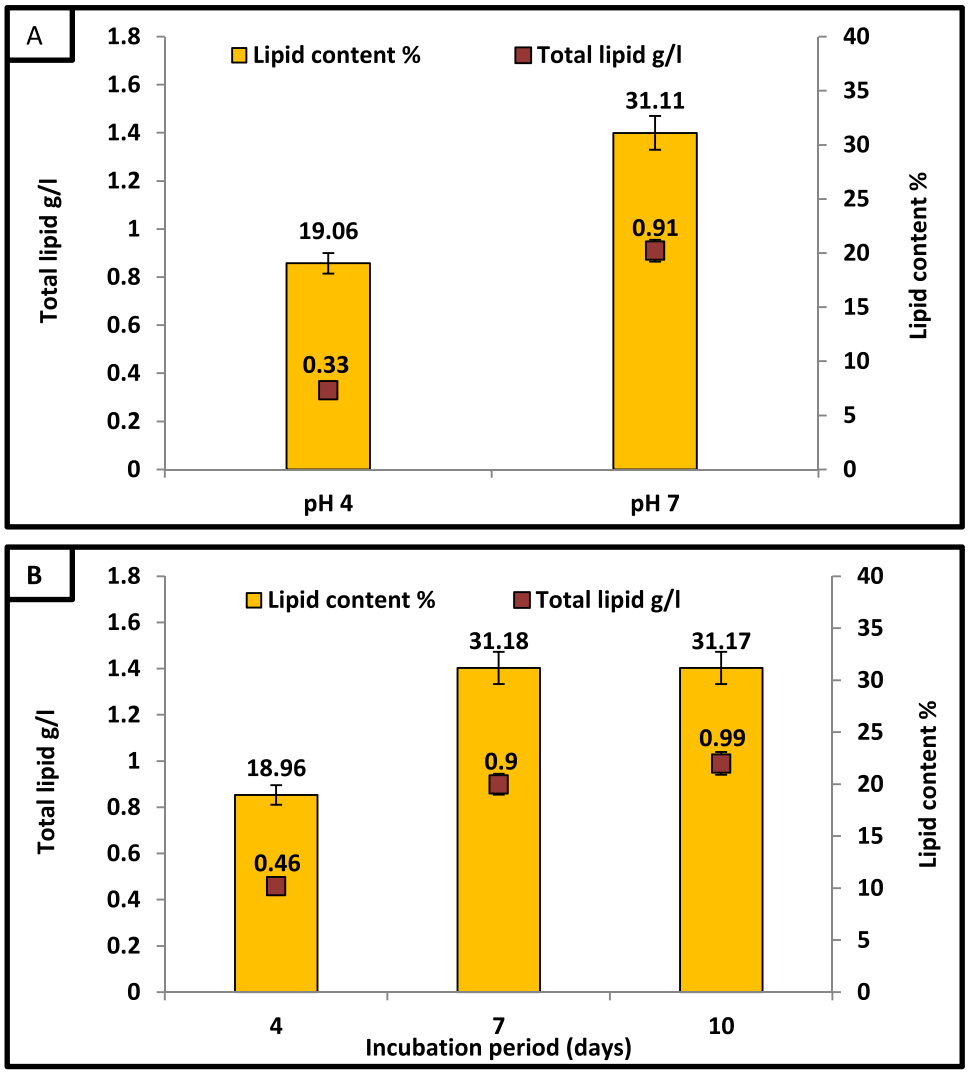

Fig. 3 Effect of different pH levels (**A**) and incubation periods (B) on lipid production by *M. racemosus* MG547571

Accumulation of lipids mainly depends on the carbon and nitrogen source, pH, and temperature. Therefore, these factors were selected for enhancement of lipid production by *M. racemosus* MG547571. Generally, oleaginous fungi can accumulate lipids at more than 20% of their cell dry weight. Figure 4 illustrates different experiments designed by Minitab 18 software for lipid production by *M. racemosus* MG547571. Results showed experiment 7 is the best for lipid production which includes conditions, temperature at 20ºC, initial pH at 6, incubation period for 10 days, soluble starch with concentration 40 g, and yeast extract with concentration 5 g. Dry biomass, total lipid, and lipid content at these optimal conditions were 3.72 gL^{-1}, 1.21 gL^{-1}, and 32.4%, respectively. This demonstrates *M. racemosus* MG547571 is an oleaginous microorganism and the possibility to be used for industrial production of microbial oils. Moreover, we noticed there in no growth of *M. racemosus* in five runs from 21 to 25, these runs belong to temperature at 35 °C where this fungus could not grow at this degree while can grow from 4 to 34 °C.

To evaluate the effect of each factor individually upon lipid production process, Taguchi design has ability to calculate the impact percent in production process. In this experiment, impact percent was calculated for each factor against the value of lipid produced. Table 5 displays effectiveness percent of all 5 tested factors which exhibited that incubation temperature was the most effective for lipid production with percent 37.9%. Whereas different concentrations of starch, different concentrations of yeast, and time had low effect on lipid production with percent 19.68, 18.26, and 15.45% respectively. Initial pH was the lowest effectiveness (8.71%) on lipid production.

Table 5 Response table for of factors affecting lipid production by *M. racemosus* MG547571

Level	Temperature	pH	Period	Starch	Yeast extract
1	0.279	0.204	0.142	0.066	0.172
2	0.575	0.298	0.173	0.129	0.157
3	0.223	0.247	0.265	0.364	0.248
4	0.076	0.239	0.265	0.364	0.248
5	0.0	0.165	0.196	0.345	0.426
Delta	0.575	0.132	0.234	0.298	0.275
Rank	1	5	4	2	3
Impact %	37.9	8.71	15.45	19.68	18.26

Interactions between different levels of selected factors are very important to select optimum level in each factor. Figure 5 shows different levels of temperature were interacted with different levels of initial pH, incubation period, different concentrations of soluble starch, and different concentrations of yeast extract; temperature at 20 ºC was the optimum for lipid production. Also, pH levels were interacted with different levels of temperature, incubation period,

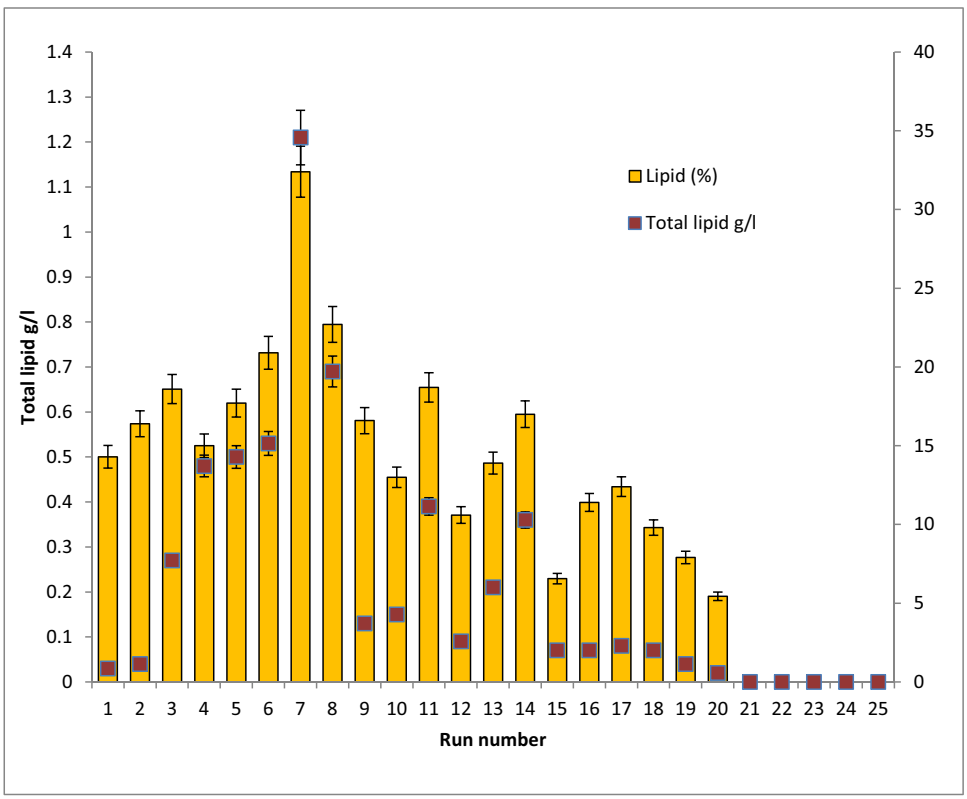

Fig. 4 Effect of main parameters on lipid production by *M. racemosus* MG547571 using Taguchi design

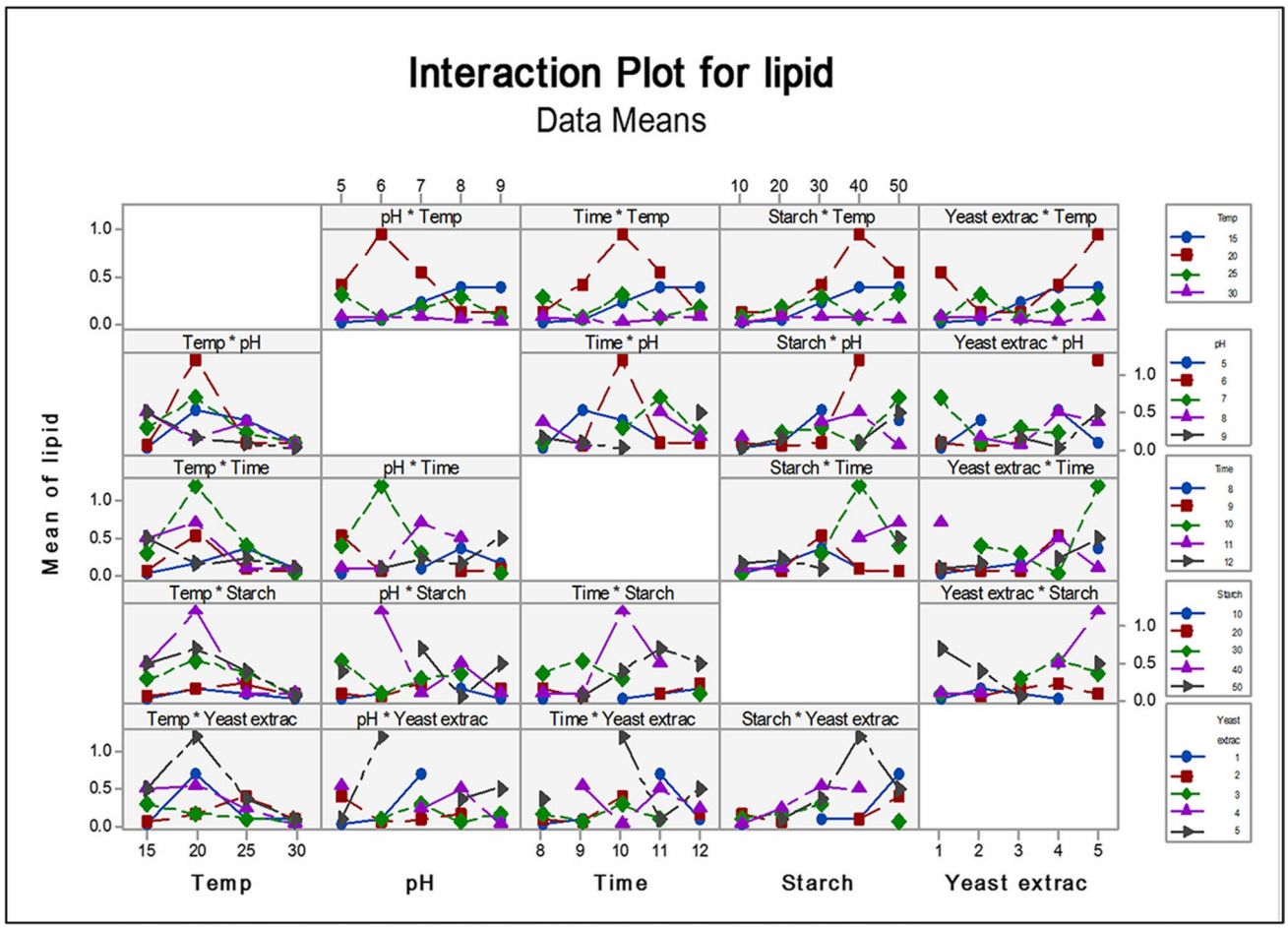

Fig. 5 Interactions between different levels of the all factors for lipid production by *M. racemosus* MG547571

different concentrations of soluble starch, and different concentrations of yeast extract; pH 6 was the optimum for lipid production. In addition to, incubation period levels were interacted with different levels of temperature, initial pH, different concentrations of soluble starch, and different concentrations of yeast extract, 10 days were the optimum for lipid production. Different levels of soluble starch and yeast extract were interacted with different levels of temperature, initial pH, and incubation period; optimum concentration of soluble starch and yeast extract was 40 and 5gL^{-1}, respectively. Finally, optimization of *M. racemosus* MG547571 for lipid production is significantly affected by using Taguchi design. Figure 6 shows lipid content, and total lipids before optimization were 20.76 and 0.51 gL^{-1}, but lipid content and total lipids were increased sharply to 32.40 and 1.21 gL^{-1}, respectively, using Taguchi design.

3.4 Cellular lipid composition

M. racemosus MG547571 was grown at different low temperatures 5, 10, 15, 20, 25, and 30 °C due to this strain is psychrotolerent and to compare between fatty acids produced at different low temperatures. Table 6 shows ratios of USFAs were more than SFAs, where USFAs at 5, 10,

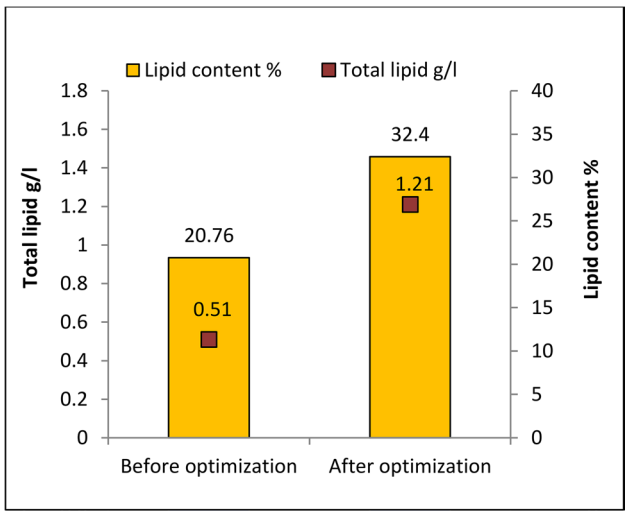

Fig. 6 Lipid production before and after statistical optimization

Table 6 Fatty acid profile of *M. racemosus* MG547571 predicted by GC–MS at different temperatures

Fatty acid name	FA type	Fatty acid % at different temperatures					
		5 °C	10 °C	15 °C	20 °C	25 °C	30 °C
Myristic acid	SFAs	12.78	4.0	3.55	5.14	2.72	—
Pentadecanoic acid	SFAs	3.09	1.97	1.59	2.24	1.01	1.15
Palmitoleic acid	MUFAs	6.29	4.66	4.45	4.61	—-	1.95
Palmitic acid	SFAs	23.74	30.61	29.84	34.88	33.1	30.97
Methyl 8-heptadecenoate	MUFAs	—-	1.24	1.12	0.94	—	—
Margaric acid	SFAs	0.89	2.56	2.12	1.75	—	—
6,9,12-Octadecatrienoic acid	PUFAs	—	—	—	—	—	1.95
Gama lenolenic acid	PUFAs	3.83	7.52	6.28	6.47	—	—
Linoleic acid	PUFAs		12.58	11.74	10.34	—	—
Oleic acid	MUFAs	25.41	25.71	30.61	22.99	46.92	47.46
11-Octadecanoic acid	MUFAs	—	—	—	—	3.2	—
Stearic acid	SFAs	0.93	5.89	5.79	3.68	5.34	4.16
7,10,13-Eicosatrienoic acid,	PUFAs	—	0.12	0.14	0.12	—	—
11-Eicosenoic acid	MUFAs	—-	0.09	0.13	0.11	—	—
Arachidic acid	SFAs	—-	0.10	0.18	0.10	—	—
10-Octadecenoic acid	MUFAs	—-	0.35	--	—	—	—
SFAs %		**52.92**	45.8	43.67	49.86	43.42	36.28
USFAs %		35.53	52.27	**54.47**	45.58	50.12	51.36
MUFAs %		31.7	32.05	36.31	28.65	50.12	**51.36**
PUFAs %		3.83	**20.22**	18.16	16.93	—-	—-

15, 20, 25, and 30 °C were 35.53, 52.27, 54.47, 45.58, 50.12, and 51.36, respectively. On the other hand, SFAs at the same grades of temperatures were 52.92, 45.8, 43.67, 49.86, 43.42, and 36.28%, respectively. One of the primary responses to low temperature culture is to increase number of fatty acids, with the majority of classes experiencing a substantial rise in abundance at 5, 10, 15, and 20 °C but at 25 and 30 °C showed a decrease in number of fatty acids. The second response to low-temperature culture was the high abundance of PUFAs, with levels peaking at 10–20 °C culture and then disappearing with the increase in temperature at 25 and 30 °C. USFAs particularly PUFAs at low temperatures 5, 10, 15, and 20 °C were higher than at 25 and 30 °C. This result was in accordance to [61] who informed that *M. racemosus* at 15 °C produced the most USFAs. The reason for incorporating the most highly USFAs is predominantly to maintain the fluidity of the membrane during cold conditions. The rise in the entire complement of fatty acids within *M. racemosus* during cold temperature growth indicates that FAs are being sent for storage. The most efficient growth of *M. racemosus* in terms of fatty acid due to the minimal loss of biomass coupled with the substantial increase in total PUFAs production particularly GLA and LA.

Oleic and palmitic acid are the major fatty acid produced by *M. racemosus* AH1; where oleic acid at 5, 10, 15, 20, 25, and 30 °C was 25.41, 25.71, 30.61, 22.99, 46.92, and 47.46%, respectively. Also, palmitic acid at 5, 10, 15, 20, 25, and 30 °C was 23.74, 30.61, 29.84, 34.88, 33.1, and 30.97%, respectively. GLA at 5, 10, 15, and 20 °C was 3.83, 7.52, 6.28, and 6.47%, respectively; and disappeared at 25 and 30 °C. LA at 10, 15 and 20 °C was 12.58, 11.74, and 10.34%, respectively; and disappeared at 5, 25, and 30 °C. Total PUFAs which produced from *M. racemosus* AH1 at 5, 10, 15, and 20 °C were 3.83, 20.22, 18.16, and 16.93%, respectively; and disappeared at 25 and 30 °C. Consequently, the best temperature range for unsaturated fatty acid production particularly polyunsaturated fatty acids was between 10 and 20 °C.

4 Conclusion

In the current study, a promising psychrotolerant oleaginous *M. racemosus* MG547571 isolated from cow dung. Taguchi method was carried out to make optimization process which can help in obtaining more confident and precise result, also save the time and cost. Moreover, Taguchi method in this study was effective for lipid production by fungi. *M. racemosus* could produce lipid 1.21 gL^{-1} with lipid content 32.4%. Moreover, fatty acid profile of lipid produced at different low temperatures illustrated that USFAs were higher than SFAs. Eventually, *M. racemosus* is a promising for USFAs which can be used for pharmaceutical applications. Furthermore, the high content of lipid produced is remarkably noticed that could be considered for the large-scale production as future perspectives and subsequently submitted to biodiesel

production as an alternative source for the renewable energy. As future perspectives, we planned to use large-scale production of lipid by co-cultures of different fungal isolates using undesirable agricultural wastes to hit two birds with one stone. Consequently, our perspectives may contribute to finding new alternatives for food and energy demands in addition to waste recycling which retains the environment clean, thus, it will contribute to resolving the problems of climate change.

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Declarations

Ethics approval Research involving Human Participants and/or Animals is not applicable.

Informed consent Informed consent is not applicable.

Conflicts of interest The authors declare no competing interests.

References

1. Ghazani SM, Marangoni AG (2021) Microbial lipids for foods. Trends Food Sci Technol 119:593–607. https://doi.org/10.1016/j.tifs.2021.10.014
2. Murillo G, Sun J, Ali SS, Yan Y, Bartocci P, He Y (2018) Evaluation of the kinematic viscosity in biodiesel production with waste vegetable oil, ultrasonic irradiation and enzymatic catalysis: a comparative study in two-reactors. Fuel 227:448–456
3. Murillo G, Ali SS, Sun J, Yan Y, Bartocci P, El-Zawawy N, Azab M, He Y, Fantozzi F (2019) Ultrasonic emulsification assisted immobilized Burkholderia cepacia lipase catalyzed transesterification of soybean oil for biodiesel production in a novel reactor design. Renewable Energy 135:1025–1034
4. Ali SS, Al-Tohamy R, Koutra E, Kornaros M, Khalil M, Elsamahy T, El-Shetehy M, Sun J (2021) Coupling azo dye degradation and biodiesel production by manganese-dependent peroxidase producing oleaginous yeasts isolated from wood-feeding termite gut symbionts. Biotechnol Biofuels 14(1):1–25
5. Ali SS, Al-Tohamy R, Xie R, El-Sheekh MM, Sun J (2020) Construction of a new lipase-and xylanase-producing oleaginous yeast consortium capable of reactive azo dye degradation and detoxification. Bioresour Technol 313:123631
6. Akoh CC (2005) Handbook of functional lipids. CRC Press
7. Murillo G, He Y, Yan Y, Sun J, Bartocci P, Ali SS, Fantozzi F (2019) Scaled-up biodiesel synthesis from Chinese Tallow Kernel oil catalyzed by Burkholderia cepacia lipase through ultrasonic assisted technology: a non-edible and alternative source of bio energy. Ultrason Sonochem 58:104658
8. Ali SS, Sun J, Koutra E, El-Zawawy N, Elsamahy T, El-Shetehy M (2021) Construction of a novel cold-adapted oleaginous yeast consortium valued for textile azo dye wastewater processing and biorefinery. Fuel 285:119050
9. Wynn JP, Hamid AA, Li Y, Ratledge C (2001) Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi Mucor circinelloides and Mortierella alpina. Microbiology 147(10):2857–2864
10. Zhang Y, Adams IP, Ratledge C (2007) Malic enzyme: the controlling activity for lipid production? Overexpression of malic enzyme in Mucor circinelloides leads to a 2.5-fold increase in lipid accumulation. Microbiology 153(7):2013–2025
11. Pinzi S, Leiva D, López-García I, Redel-Macías MD, Dorado MP (2014) Latest trends in feedstocks for biodiesel production. Biofuels, Bioprod Biorefin 8(1):126–143
12. Suleiman W, El-Sheikh H, Abu-Elreesh G, Hashem A (2018) Recruitment of Cunninghamella echinulata as an Egyptian isolate to produce unsaturated fatty acids. Res J Pharm Biol Chem Sci 9(1):764–774
13. Ali SS, Al-Tohamy R, Koutra E, El-Naggar AH, Kornaros M, Sun J (2021) Valorizing lignin-like dyes and textile dyeing wastewater by a newly constructed lipid-producing and lignin modifying oleaginous yeast consortium valued for biodiesel and bioremediation. J Hazard Mater 403:123575
14. Abdel-Razek A, El-Sheikh H, Suleiman W, Taha TH, Mohamed M (2020) Bioelimination of phenanthrene using degrading bacteria isolated from petroleum soil: safe approach. Desalin Water Treat 181:131–140
15. Soliman MO, Suleiman WB, Roushdy MM, Elbatrawy EN, Gad AM (2021) Characterization of some bacterial strains isolated from the Egyptian Eastern and Northern coastlines with antimicrobial activity of Bacillus zhangzhouensis OMER4. Acta Oceanol Sin 41:1–8
16 Gad AM, Suleiman WB, Beltagy EA, El-Sheikh H, Ibrahim HA (2021) Antimicrobial and antifouling activities of the cellulase produced by marine fungal strain; Geotrichum candidum MN638741.1. Egypt J Aquat Biol Fish 25(6):49–60. https://doi.org/10.21608/ejabf.2021.210301
17. Suleiman WB, El Bous M, Ibrahim M, El Baz H (2019) In vitro evaluation of Syzygium aromaticum L. ethanol extract as biocontrol agent against postharvest tomato and potato diseases. Egypt J Bot 59(1):81–94
18. Ahmed SU, Singh SK, Pandey A, Kanjilal S, Prasad RB (2006) Effects of various process parameters on the production of γ-linolenic acid in submerged fermentation. Food Technol Biotechnol 44(2):283–287

19 Fouda A, Khalil A, El-Sheikh H, Abdel-Rhaman E, Hashem A (2015) Biodegradation and detoxification of bisphenol-A by filamentous fungi screened from nature. J Adv Biol Biotechnol 2(2):123–132

20. Ahmed NE, Salem SS, Hashem AH (2021) Statistical optimization, partial purification, and characterization of phytase produced from Talaromyces purpureogenus NSA20 using potato peel waste and its application in dyes de-colorization. Biointerface Research in Applied Chemistry 12(4):4417–4431

21. Hashem AH, Suleiman WB, Abu-Elrish GM, El-Sheikh HH (2020) Consolidated bioprocessing of sugarcane bagasse to microbial oil by newly isolated oleaginous fungus: Mortierella wolfii. Arab J Sci Eng 46(1):199–211

22. Lim SH, Ming H, Park EY, Choi JS (2003) Improvement of riboflavin production using mineral support in the culture of Ashbya gossypii. Food Technol Biotechnol 41(2):137–144

23. Hashem AH, Suleiman WB, Abu-elreesh G, Shehabeldine AM, Khalil AMA (2020) Sustainable lipid production from oleaginous fungus Syncephalastrum racemosum using synthetic and watermelon peel waste media. Bioresour Technol Rep 12:100569

24. Khalil AMA, Hashem AH (2018) Morphological changes of conidiogenesis in two Aspergillus species. J Pure Appl Microbiol 12(4):2041–2049

25. Khalil AMA, Hashem AH, Abdelaziz AM (2019) Occurrence of toxigenic Penicillium polonicum in retail green table olives from the Saudi Arabia market. Biocatal Agric Biotechnol 21:101314

26. Hasanin MS, Hashem AH (2020) Eco-friendly, economic fungal universal medium from watermelon peel waste. J Microbiol Methods 168:105802

27. Hasanin MS, Hashem AH, Abd El-Sayed ES, El-Saied H (2020) Green ecofriendly bio-deinking of mixed office waste paper using various enzymes from Rhizopus microsporus AH3: efficiency and characteristics. Cellulose 27(8):4443–4453

28. Hashem AH, Hasanin MS, Khalil AMA, Suleiman WB (2019) Eco-green conversion of watermelon peels to single cell oils using a unique oleaginous fungus: Lichtheimia corymbifera AH13. Waste Biomass Valor 11(11):5721–5732

29. Khalil A, Abdelaziz A, Khaleil M, Hashem A (2021) Fungal endophytes from leaves of Avicennia marina growing in semi-arid environment as a promising source for bioactive compounds. Lett Appl Microbiol 72(3):263–274

30 Hashem AH, Al Abboud MA, Alawlaqi MM, Abdelghany TM, Hasanin M (2021) Synthesis of nanocapsules based on biosynthesized nickel nanoparticles and potato starch: antimicrobial, antioxidant and anticancer activity. Starch-Stärke 74(1–2):2100165

31. Sharaf MH, Abdelaziz AM, Kalaba MH, Radwan AA, Hashem AH (2021) Antimicrobial, antioxidant, cytotoxic activities and phytochemical analysis of fungal endophytes isolated from ocimum basilicum. Appl Biochem Biotechnol. https://doi.org/10.1007/s12010-021-03702-w

32. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35(6):1547

33. Gad AM, Suleiman WB, Beltagy EA, El-Sheikh H, Ibrahim HA (2021) Characterization and screening of marine-derived fungi along the coastline of Alexandria, Mediterranean Sea, Egypt. Egypt J Aquat Biol Fish 25(5):215–239

34. Hasanin M, Al Abboud MA, Alawlaqi MM, Abdelghany TM, Hashem AH (2021) Ecofriendly synthesis of biosynthesized copper nanoparticles with starch-based nanocomposite: antimicrobial, antioxidant, and anticancer activities. Biol Trace Elem Res 1–14

35. Folch J, Lees M, Stanley GS (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226(1):497–509

36. Mishra SK, Suh WI, Farooq W, Moon M, Shrivastav A, Park MS, Yang J-W (2014) Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. Biores Technol 155:330–333

37. Suleiman W, El-Skeikh H, Abu-Elreesh G, Hashem A (2018) Isolation and screening of promising oleaginous Rhizopus sp and designing of Taguchi method for increasing lipid production. J Innov Pharm Biol Sci 5(1):8–15

38. Hashem AH, Khattab AM, Abdelraof M (2022) A facile one-pot bioconversion of frying oil waste to single cell oils and related products using fungi via response surface methodology. Biomass Convers Biorefin. https://doi.org/10.1007/s13399-021-02165-7

39. Miao X, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. Biores Technol 97(6):841–846. https://doi.org/10.1016/j.biortech.2005.04.008

40. Suleiman WB (2020) In vitro estimation of superfluid critical extracts of some plants for their antimicrobial potential, phytochemistry, and GC–MS analyses. Ann Clin Microbiol Antimicrob 19(1):1–12

41. Patel A, Arora N, Mehtani J, Pruthi V, Pruthi PA (2017) Assessment of fuel properties on the basis of fatty acid profiles of oleaginous yeast for potential biodiesel production. Renew Sustain Energy Rev 77:604–616

42. Ramana KV, Srivastava S, Singhal SS (2014) Lipid peroxidation products in human health and disease 2014. vol 2014. Hindawi

43. Benny GL (2008) Methods used by Dr. RK Benjamin, and other mycologists, to isolate Zygomycetes. Aliso: J Syst Evol Bot 26(1):37–61

44. Lu H, Zhang B-B, Wu Z-H (2010) Studies on mucor racemosus fermentation to manufacture Gamma-linolenic acid functional food douchi. Food Sci Technol Res 16(6):543–548

45. Kosa G, Zimmermann B, Kohler A, Ekeberg D, Afseth NK, Mounier J, Shapaval V (2018) High-throughput screening of Mucoromycota fungi for production of low-and high-value lipids. Biotechnol Biofuels 11(1):66

46. Tang X, Zan X, Zhao L, Chen H, Chen YQ, Chen W, Song Y, Ratledge C (2016) Proteomics analysis of high lipid-producing strain Mucor circinelloides WJ11: an explanation for the mechanism of lipid accumulation at the proteomic level. Microb Cell Fact 15(1):35. https://doi.org/10.1186/s12934-016-0428-4

47. Xia C, Zhang J, Zhang W, Hu B (2011) A new cultivation method for microbial oil production: cell pelletization and lipid accumulation by Mucor circinelloides. Biotechnol Biofuels 4(1):15. https://doi.org/10.1186/1754-6834-4-15

48. Zhang Y, Liu Q, Li P, Wang Y, Li S, Gao M, Song Y (2021) Enhanced lipid production by addition of malic acid in fermentation of recombinant Mucor circinelloides Mc-MT-2. Sci Rep 11(1):12674. https://doi.org/10.1038/s41598-021-92324-7

49. Mohamed H, El-Shanawany A-R, Shah AM, Nazir Y, Naz T, Ullah S, Mustafa K, Song Y (2020) Comparative analysis of different isolated oleaginous Mucoromycota fungi for their γ-linolenic acid and carotenoid production. BioMed Res Int 2020:1–13

50. Alavijeh RS, Karimi K, van den Berg C (2020) An integrated and optimized process for cleaner production of ethanol and biodiesel from corn stover by Mucor indicus. J Clean Prod 249:119321

51. Bellou S, Triantaphyllidou I-E, Aggeli D, Elazzazy AM, Baeshen MN, Aggelis G (2016) Microbial oils as food additives: recent approaches for improving microbial oil production and its polyunsaturated fatty acid content. Curr Opin Biotechnol 37:24–35

52. Suleiman WB, Helal EE (2022) Chemical constituents and potential pleiotropic activities of Foeniculum vulgare (Fennel) ethanolic extract; in vitro approach. Egypt J Chem 65:Article In Press
53. Kamel A, Suleiman WB, Elfeky A, El-Sherbiny GM, Elhaw M (2022) Characterization of bee venom and its synergistic effect combating antibiotic resistance of Pseudomonas aeruginosa. Egypt J Chem. https://doi.org/10.21608/EJCHEM.2021.99417.4626
54. Shawky M, Suleiman WB, Farrag AA (2021) Antibacterial resistance pattern in clinical and non-clinical bacteria by phenotypic and genotypic assessment. J Pure Appl Microbiol 15(4):2270–2279
55. Abedi E, Sahari MA (2014) Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. Food Sci Nutr 2(5):443–463
56. Passoth V (2017) Lipids of yeasts and filamentous fungi and their importance for biotechnology. In: Biotechnology of yeasts and filamentous fungi. Springer, pp 149–204
57. Enshaeieh M, Nahvi I, Madani M (2014) Improving microbial oil production with standard and native oleaginous yeasts by using Taguchi design. Int J Environ Sci Technol 11(3):597–604
58. Chiranjeevi P, Venkata Mohan S (2016) Optimizing the critical factors for lipid productivity during stress phased heterotrophic microalgae cultivation. Front Energy Res 4:26
59. Aoki H, Nishioka K, Mankura M, ENDo Y, FUJIMoTo K (1999) Production of eicosapentaenoic acid-enriched triacylglycerol by Mucor hiemalis HA-30. Biosci Biotechnol Biochem 63(2):356–360
60. Yu L, Fan J, Yan C, Xu C (2018) Starch deficiency enhances lipid biosynthesis and turnover in leaves. Plant Physiol 178(1):118–129
61. Broughton R (2011) Omega 3 fatty acids: identification of novel fungal and chromistal sources. University of London