ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

Novel Approaches to *Mortierella alpina* Identification and Arachidonic Acid Production Optimization

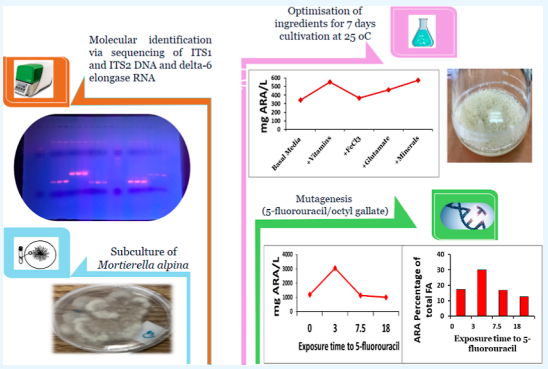
Violette S. Hanna, Mohamed N. Abd El-Ghany, Mohamed I. M. Ibrahim, Tahany M. Abdel-Rahman, and Hatem Tallima*

Cite This: *ACS Omega* 2024, 9, 34456−34463

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Arachidonic acid (ARA) is an integral constituent of cell structures and is instrumental for the nervous, muscular, and immune systems' functions. The sore need for this nutrient may be fulfilled via production based on the fungus *Mortierella alpina*. The identity of the *M. alpina* culture obtained from Assiut University, Egypt, was confirmed based on internal transcribed spacer DNA barcoding and elongation enzyme RNA sequencing. Liquid media glucose and peptone as carbon and nitrogen sources, respectively, and diverse micronutritional factors were adjusted for optimal biomass and ARA production. Shake flask cultivation at 25 °C for 7 days produced around 0.570 g of ARA per liter of culture. *M. alpina* treatment using mutagen 5-fluorouracil and octyl gallate-supplemented glucose−yeast−agar screening plates and shake-flask incubation at 25 °C, then at 20 °C, followed by aging at 10 °C, led to >3 g ARA/liter culture, a yield considered suitable for potential commercial production.



INTRODUCTION

Arachidonic acid (ARA), a 20 carbon (C), omega-6 polyunsaturated fatty acid (PUFA), is an essential nutrient.[1] The cis four double bonds confer on the FA a hairpin-like structure and, functionally, give the molecule the flexibility required to keep the fluidity of the cell membrane intact at physiological temperatures, explaining its abundance in phospholipids of cell membranes and its importance for signaling pathways.[2,3] ARA is the precursor of biological hormones known as eicosanoids, which are responsible for vital physiological processes such as inflammation, wound healing, mood, and appetite.[3,4] The free, unesterified form of ARA is also of great importance in fighting against microbes, schistosome invasion, and cancer.[5−9] ARA consumption was claimed to be mandatory for infants and toddlers for its contribution to brain development, visual acuity, and the immune system's proper function,[10,11] and for improving cognitive functions, notably in the elderly.[12,13]

Marine animal sources of PUFAs include many endangered species without efficient yields to satisfy market needs. Hence, a major orientation is aimed at producing some of these PUFAs (including ARA) from alternate microbial sources,[14] predominantly using the filamentous fungus *Mortierella alpina*, which has the ability to produce ARA-rich oil up to 70% of its biomass.[15] *M. alpina* is certified by the US Food and Drug Administration as the only source for dietary ARA.[16] Accordingly, a source for production of the sorely needed nutrient, ARA, is available in developed and developing countries.[17]

Taxonomic identification at the species level is required to ensure the reproducibility of industrial production.[18] Identification based solely on morphological analysis is misleading due to convergent evolution, cryptic speciation, and hybridization.[19−21] Accordingly, DNA sequence-based identification is used to detect fungal species. Commonly, the untranslated internal transcribed spacer (ITS) DNA regions are used for Zygomycetes identification.[22] The ITS regions can detect the difference between closely related species as they are highly variable and considered the fastest-evolving part of the rRNA (rRNA) cistron.[18,23,24] The mRNA sequence of protein-coding genes could be invaluable in the identification of fungus species. The present study is the first in confirming *M. alpina* isolate identity via checking the sequence of the *GLELO* gene, which encodes an endoplasmic reticulum-bound enzyme involved in FA biosynthetic pathways, elongase 2, or δ 6 elongase. It predominantly catalyzes the elongation of 18:$n-3$, γ-linolenic acid to 20:$n-3$, dihomo γ linolenic acid and is a rate-limiting step in ARA synthesis.[25−27]

Received: March 8, 2024
Revised: April 16, 2024
Accepted: June 19, 2024
Published: August 1, 2024

Several attempts were carried out to maximize *M. alpina* biomass and ARA production, whether through media optimization[15,17,28,29] or through strain mutation and genetic manipulation.[27] Culture optimization encompasses not only media ingredients but also the concentrations and ratios of added nutrients in a given medium. There are two main methods to study media optimization. First, empirical methods included changing one factor at a time, referring to basal media ingredients from previous literature and relying mainly on observational skills.[29−31] Second, statistical methods used mathematical approaches for the determination of the best concentration and combination of media ingredients.[32,33] However, neither empirical nor statistical methods provide us with a consensus on what basal media ingredients should be included to achieve the maximum production of ARA. Experts may agree on glucose as the C source, but nitrogen (N), bivalent metals, minerals, and other ingredients differ from one experiment to another, especially in scale-up production. In the present study, knowledge of the requirements for *M. alpina* ARA biosynthesis[16,34−42] dictated the selection of culture media and conditions. However, the ARA yield was not satisfactory for industrial production. Creating mutant isolates following established procedures[27,39,43,44] and cultivating the produced mutants using the user-friendly protocols recently defined[17] opened a road for ARA commercial production by start-up companies and groups.

MATERIALS AND METHODS

***M. alpina* Source and Maintenance.** *M. alpina* was obtained from Assiut University Mycological Centre (AUMC) Assiut, Egypt (https://www.aun.edu.eg/sp_units/en/aumcenter; https://www.aun.edu.eg/sp_units//sites/default/files/pdf/AUMC%20Catalog%202010.pdf), courtesy of Professor Ahmad Moharram, and grown on different media, potato dextrose (PDA) or sucrose (PSA) agar (infusion from 200 g unpeeled and washed potatoes/L. 20 g/L dextrose or sucrose and 20 g/L agar. pH 5.6), and Sabouraud dextrose agar (SDA; 40 g/L dextrose, 10 g/L peptone, 20 g/L agar. pH 5.6) supplemented with 100 mg of chloramphenicol (bacteriostatic agent) for 7 days at 28 °C. *M. alpina* was further maintained on PDA and SDA, stored at 4 °C and subcultured regularly.

Fungal Strain Identification. Morphological identification was thoroughly performed, whereby morphological characteristics of the fungus were observed on different nutritional agar media in 9 cm Petri dishes. Mycelial hyphae were examined using a light microscope. Molecular identification was based on an ITS. A transcriptional unit composed of three nuclear ribosomal genes (rRNA) (18S, 5.8S, and 28S) and an untranslated ITS region (ITS1 and ITS2) is commonly used in fungal identification (Figure S1). In order to confirm morphology-based identification of *M. alpina*, six universal primers (forward and reverse) were designed for the ITS1 and ITS2 regions (Figure S1) and synthesized at IDT (Integrated DNA Technologies, Coralville, IA, USA). Primers' sequences are shown in Table S1, and location, in Figure S2. Additional molecular identification was based on the GLELO gene product. The primer pair (forward and reverse, Table 1) generated for cloning *GLELO* mRNA was synthesized at IDT.

DNA/RNA Extraction. For DNA extraction, approximately 0.1 g of freshly grown mycelium (7−10 days old) was ground in 400 μL 50 mM Tris-HCl, pH 7.2-based lysis buffer, and DNA extracted using the chloroform−phenol−isoamyl alcohol (24:25:1) (Sigma, St. Louis, MO, USA) method, as described,[45] and detailed in Supporting Information 1. Approximately 0.1 g of mycelium was harvested from a 7 to 10 days-old culture, ground in 1 mL PureZOL reagent (Bio-Rad, Hercules, CA, USA), and RNA extracted, as described previously[46] and detailed in Supporting Information 1. The purity and concentration of extracted DNA and RNA were spectrophotometrically determined by using triplicate OD_{260}−OD_{280} absorbance values (GeneRay, Biometra, Göttingen, Germany).

Table 1. Primer Sequences Used for *GLELO* RNA Cloning and Reverse Transcription-Amplification

name	sequence
elongase forward	5′ ATG GAG TCG ATT GCG CAA TTC 3′
elongase reverse	5′ CAG TCC ATA GTT GGC CTG GT 3′

Polymerase Chain Reaction Amplification. The ITS1 and ITS2 regions were amplified in four different reactions using the GoTaq Flexi DNA Polymerase polymerase chain reaction (PCR) kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and details shown in Supporting Information 2. Reverse transcription PCR was used for elongase-2 mRNA reverse transcription and amplification using the GeneAmp EZ r*Tth* RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and details shown in Supporting Information 2.

Agarose Gel Electrophoresis. Amplicon products were separated on a 2% agarose gel. The AmpliSize molecular ruler was used as a ladder (10 ng/band/μL) (50 to 2000 bp) (Bio-Rad). DNA gel extraction and purification were performed using the Quantum Prep Freeze 'N Squeeze DNA Gel Extraction kit (Bio-Rad) according to the manufacturer's instructions and as described previously.[47]

Sanger Sequencing. Amplicon products were sequenced bidirectionally at MCLAB (South San Francisco, CA, USA).

Media for Optimum ARA Production. Media components and *M. alpina* cultivation conditions were adjusted, aiming for optimal ARA production. Chemicals were purchased from Sigma-Merck (Darmstadt, Germany). MEM Eagle Vitamin Mixture (100×) was obtained from Lonza Bioscience (Verviers, Belgium). Batches were carried out in duplicates or triplicates. The selection of culture media ingredients was based on the biochemistry of lipid accumulation in oleaginous micro-organisms.[16,34−42] Culture media ingredients were added in a cumulative manner, as shown in Table 2. To peptone, yeast extract (SERVA Electrophoresis GmbH, Heidelberg, Germany), glucose, chloramphenicol, ferric chloride, $NaHPO_4$, magnesium sulfate heptahydrate, L-glutamine (Sigma), and MEM Eagle Vitamin Mixture (100×) were added. Each 100 mL of prepared culture medium was inoculated with 1 agar fungal disk and incubated for 7 days at 25 °C and 150 rpm.

Fatty Acid Determination. Fatty acid percentages and content were determined by gas chromatography−mass spectroscopy, as described.[48,49]

Mutagenesis and ARA Output. Spores were collected from freshly subcultured *M. alpina*, suspended in 10 mL Dulbecco phosphate-buffered saline, pH 7.1, and incubated with 20 μg/mL 5-fluorouracil (Ebewe Pharma, Unterach am Attersee, Austria) at room temperature with slight shaking for 0, 3, 7.5, and 18 h before spreading on glucose (20 g/L), yeast (10 g/L) (agar 20 g/L), and chloramphenicol (100 mg/L)

https://doi.org/10.1021/acsomega.4c02294
ACS Omega 2024, 9, 34456−34463

Table 2. Culture Media Cumulative Components and Their Concentrations in 100 mL

0.5 g peptone	0.5 g peptone	0.5 g peptone	0.5 g peptone	0.5 g peptone
0.3 g yeast extract	0.3 g yeast extract	0.3 g yeast extract	0.3 g yeast extract	0.3 g yeast extract
3 g glucose	3 g glucose	3 g glucose	3 g glucose	3 g glucose
	2 mL vitamin mixture	vitamins	vitamins	vitamins
		1 μL $FeCl_3 \cdot 6H_2O$[a]	1 μL $FeCl_3 \cdot 6H_2O$	100 mg glutamic acid
			100 mg glutamic acid	0.3 g NaH_2PO_4
				0.03 g $MgSO_4$

[a]$FeCl_3 \cdot 6H_2O$ final concentration is 2 mg/L and is added on the third day (late log phase).

plates, supplemented with 15 mg/L octyl gallate. Octyl gallate is a strong inhibitor of *M. alpina* δ-6 front-end desaturase, which converts linoleic acid (18:*n*2) to γ linoleic acid (18:*n*3) and thus selects cultures overexpressing the enzyme.[44,50] Octyl gallate was prepared by acid-catalyzed esterification of octyl alcohol and gallic acid equimoles at 70 °C for 4 h at pH 5.4 maintained by the dropwise addition of 1 N HCl. The solid product was recrystallized in 96% ethanol and checked for purity by measuring the melting point (100 °C). The yield was about 82%. The cultures were maintained at 25 °C for 7 days. Disks were cultivated in duplicates. Shake flask cultivation conditions, including stationary aging at 10 °C for 7 days, and extraction of lipids using the Folch method were exactly as described.[17]

RESULTS

Fungal Strain Identification. Cultures showed cottony colonies with a milky appearance (Figure 1A). The examination of mycelial hyphae by light microscopy revealed that the mycelia are coenocytic (contains multiple nuclei) and do not contain septate cells (Figure 1B,C). Based on the phylogenetic analysis and morphological features, the fungal strain is preliminarily characterized and indicated as *M. alpina*.

Three primers, ITSF1, ITS1, and ITS5, were used as forward primers, with ITS2 as the common reverse primer, to amplify the ITS1 region situated between the small-subunit rRNA genes and the 5.8S region, producing three PCR products of between 257 to 295 bp. Searches using the Basic Local Alignment Search Tool for nucleotide (BLASTn) database sequences revealed the alignment of the amplicon (query) with the ITS1 region of *M. alpina* isolates, with similarities up to 97% (Figure S3). The primer pair (ITS3 + ITS4) enabled the amplification of the ITS2 region situated between the 5.8S region and the large-subunit rRNA genes, generating a 418 bp long PCR product. Alignment of the product with the published ITS2 region of *M. alpina* isolates revealed >95% similarity, confirming the successful identification of the fungus of interest as *M. alpina* (Figure S3).

In the present study, a novel technique for fungal species detection was developed using the mRNA of the *GLELO* gene. Following RNA extraction from 0.1 g of fresh mycelium, the mRNA of the *GLELO* gene was successfully amplified using a primer pair (Table 1) designed based on the published NCBI sequence for *GLELO* mRNA (EU639657). The generated PCR product was 408 bp long, as predicted. BLAST searches showed alignment of the amplicon with *GLELO* mRNA of *M. alpina* isolates with 91 and 94% identity at the DNA (Figure S4) and RNA (Figure 2) levels, respectively.

Effect of Cultivation Conditions on *M. alpina* Biomass and Fatty Acid Production. The evaluation of cumulative media, as dictated by the fungus metabolism requirements, was examined by two factors: the fungus biomass and percent yield of saturated (palmitic and stearic) and unsaturated (oleic, linoleic, dihomo-γ linoleic, and arachidonic) FA. The consensus basal medium of glucose, peptone, and yeast extract was supplemented with vitamins and then with ferric chloride, glutamate, and minerals in a sequential manner. Data based on duplicate or triplicate cultures for each addition showed that *M. alpina* biomass production increased by 1.7 fold following the cumulative addition of vitamins, $FeCl_3$, glutamate, and minerals into the basal growth medium (Figure 3).

Group B vitamin supplementation to the culture medium stimulated a slight increase in the biomass (15 g/L) and engendered the balanced production of arachidonic (20:4), palmitic (16:0), and oleic (18:1) acids (551, 495, and 498 mg/L respectively). The yield of biomass continued to increase with the addition of Fe^{3+} reaching 17 g/L. However, Fe^{3+} ions showed exclusive increased oleic acid yield (18:1). The positive effects on biomass and oleic acid (18:1) were not associated with an increase in ARA (20:4) levels (365 mg/L). The addition of glutamate to the culture did not further modify the biomass yield but favored the production of palmitic, stearic (18:0), and oleic acids (Figure 4, Tables 2 and 3). The biomass (18.7 g/L) and oleic acid (810 mg/L culture) levels reached a maximum value following the addition of minerals to vitamins and glutamate, supporting the validity of adhering to the fungus metabolism requirements upon selection of culture ingredients (Figures 3 and 4; Table 3). The final culture medium enhanced the production of dihomo-γ linoleic acid compared to the basal medium added with vitamins, Fe^{3+}, and glutamates, and elicited an increase in ARA percentage to around 15% (Figure S5).

Mutagenesis Impact on *M. alpina* Fatty Acids Percentage and Yield. Analysis of duplicate cultures with

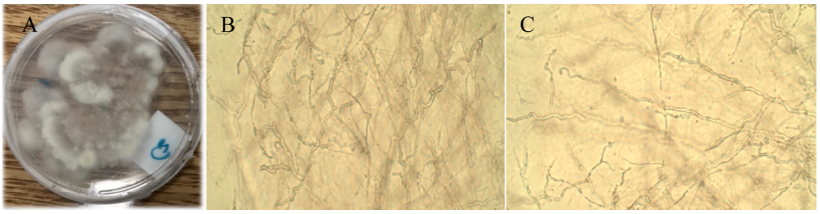


Figure 1. Showing (A) cottony colonies with milky appearance and (B,C) magnified snaps of coenocytic and not septate mycelial hyphae of two culture generations, ×400.

https://doi.org/10.1021/acsomega.4c02294
ACS Omega 2024, 9, 34456−34463

```
Amplicon   1   ESIAQFLPSKMPQDLFMDLASAIGVRAAPYVDPLEAALVAQAEKYFPTIVHHTRGFLVAV  60
               ESIAQFLPSKMPQDLFMDLA+AIGVRAAPYVDPLEAALVAQAEKY PT+VHHTRGFLVAV
AJD76153.1  2   ESIAQFLPSKMPQDLFMDLATAIGVRAAPYVDPLEAALVAQAEKYIPTVVHHTRGFLVAV  61

Amplicon   61  ESPLARELPLMNPFHVLLIALAYLVTVFMGMQIMKNFERFEVKTFSLFHNFCLVSXSAYM  120
               ESPLARELPLMNPFHVLLI LAYLVTVF+GMQIMKNFERFEVKTFSL HNFCL  SAYM
AJD76153.1 62   ESPLARELPLMNPFHVLLIVLAYLVTVFVGMQIMKNFERFEVKTFSLLHNFCLVSISAYM  121
```

Figure 2. Protein BLAST confirms the similarity of the isolate amplicon with the PUFA elongation enzyme, partially from *M. alpina*. Sequence ID: AJD76153.1, with 94% identities and 96% positives.

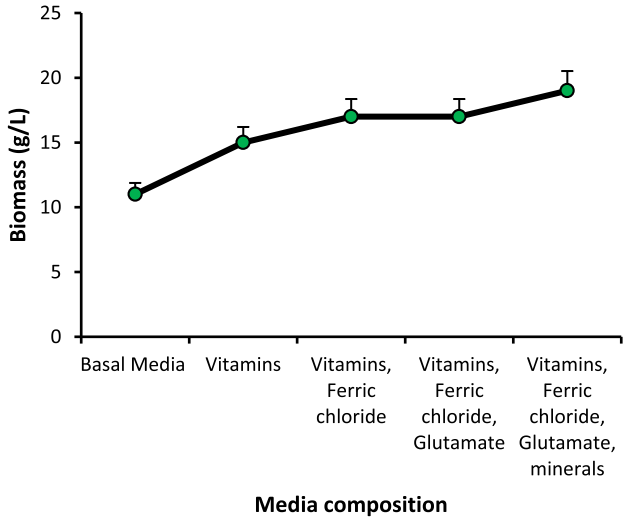


Figure 3. Graphical representation of the influence of the cumulative addition of nutrients to basal media on fungal biomass. Vertical bars denote the SE around the mean biomass g/L of replicate cultures.

similar results indicated the remarkable increase in ARA percent (Table 4) and content (up to 3.1 g/L, Figure 5) in the *M. alpina* culture derived from spores exposed to 5-fluorouracil for 3 h. Spores longer exposure to 5-fluorouracil led to cultures showing increases in palmitic and stearic acid percentages at ARA expense (Table 4).

DISCUSSION

ARA is a fundamental cog in the life machine, which is a conditional essential FA for the human body. It is integrated into numerous and diverse physiological functions, including membrane fluidity, ion channels, apoptosis induction, receptors, and enzyme activity. It regulates cell membrane fluidity, thus maintaining cell integrity and vascular permeability. Additionally, ARA influences many integral proteins involved in fundamental cellular signaling, ensuring the maintenance of cell vitality. The importance of ARA has further extended to encompass psychological functions, including supporting neural plasticity, enhancing memory, and learning hippocampal capabilities.[4,7,13] Recently, ARA has been advocated as a potential tumoricide and schistosomicide.[5,6,8,9] It is thus of importance to provide the market with its growing requirements and needs in the form of edible and affordable ARA supplementation based on the fungus *M. alpina*.

Taxonomic identification was a prerequisite step before the cultivation processes. The fungus taxonomy was confirmed based on morphological observation of the milky−cottony appearance of the fungal growth and the observation of microscopic snaps of coenocytic (not septate) mycelial hyphae, which are the hallmarks of Zygomceytes.[51] From the molecular aspect, the fungus species was confirmed through DNA barcoding based on the ITS regions, considered one of the most suitable methods for molecular fungal identification.[22,23] Indeed, the ITS region was selected rather than the 18S

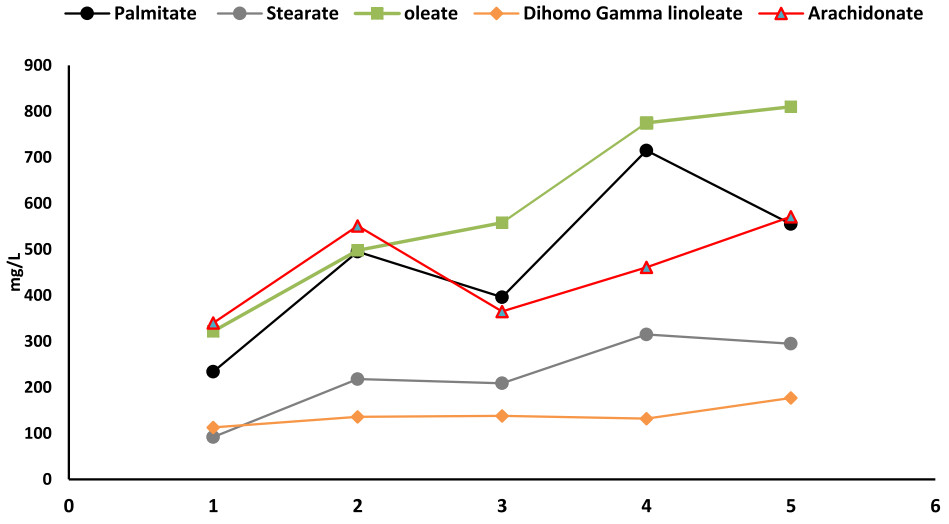


Figure 4. Each point represents the mean mg/L predominant saturated and unsaturated fatty acids content of three cultures in response to basal culture medium (1), and to the addition on the basal medium of vitamins (2), $FeCl_3$ (3), glutamate (4), and minerals (5), with negligible SE values around the mean, shown in Table 3.

https://doi.org/10.1021/acsomega.4c02294
ACS Omega 2024, 9, 34456−34463

Table 3. Cultures Predominant Fatty Acids Content

	basal media	+vitamins	+vitamins + $FeCl_3$	+vitamins + $FeCl_3$ + glutamate	+vitamins + $FeCl_3$ + glutamate + minerals
	mean mg/L culture ± SE				
palmitic acid, 16:0	234 ± 0.07	495 ± 0.21	395 ± 0.10	715 ± 0.07	555 ± 0.04
stearic acid, 18:0	92 ± 0.05	218 ± 0.08	209 ± 0.11	315 ± 0.03	295 ± 0.04
oleic acid, 18:1	322 ± 0.05	498 ± 0.21	558 ± 0.10	775 ± 0.07	810 ± 0.12
dihomo-γ LA, 20:3	113 ± 0.05	136 ± 0.03	138 ± 0.04	132 ± 0.09	177 ± 0.07
arachidonic acid, 20:4	340 ± 0.06	551 ± 0.12	365 ± 0.11	461 ± 0.06	571 ± 0.06

Table 4. Percentage Fatty Acids in *M. alpina* Cultures Derived from Spores Exposed to 5-Fluorouracil[a]

	area sum percentage following h exposure			
fatty acid/exposure hour	0	3	7.5	18
myristic, 14:0	2.25	0.13	1.19	2.39
pentadecanoic, 15:0	0.61	0.52	0.43	0.92
palmitic, 16:0	18.55	18.66	**28.63**	**27.5**
margaric, 17:0	0.51	0.45	0.47	0.7
stearic acid, 18:0	7.07	7.49	**12.15**	**11.87**
oleic acid, 18:*n* − 1	**25.28**	15.1	15.05	**20.01**
linoleic, 18:*n* − 2	7.36	6.56	6.22	6.58
γ-linolenic, 18:*n* − 3	6.02	2.98	2.68	5.08
linolenic, 18:*n* − 3	0.32	0.36	0.5	0.54
arachidic, 20:0	0.34	0.37	0.39	0.21
cis-11-eicosenoic, 20:*n* − 1	5.72	6.11	9.74	5.77
cis-11,14-eicosadienoic	0.18	0.66	0.11	0.18
homo-γ-linolenic, 20:*n* − 3	4.07	3.09	1.61	2.54
arachidonic, 20:*n* − 4	17.28	**30.02**	16.89	12.45
behenic, 22:0	0.47	0.76	0.38	0.18
erucic, 22:*n* − 1	1.14	1.32	1.71	0.88
eicosapentaenoic, 22:*n* − 5	ND	ND	0.21	0.21

[a]The data are typical of two independent experiments. ND = not detectable.

because the level of variability in the *M. alpina* ITS region is higher than that of 18S rDNA.[22,24] ITS sequencing of the ITS region is additionally more user-friendly and cost-effective than other molecular tools, such as DNA−DNA hybridization. Of interest is the alignment of the amplicons with *M. alpina* isolate from Korea (KJ921606[52]) and Libya (MZ298831.1[45]) revealed 96 and 98% identity, respectively. Nevertheless, it was herein proposed that further proof for accurate identification should involve the enzyme RNA sequence. The elongation enzyme RNA was cloned, reverse transcribed into DNA, and sequenced. BLAST studies revealed 94% identity at the amino acid level with the elongation enzyme of *M. alpina* species, thus providing definitive evidence regarding the identity of the isolate used in the present study.

Processes of ARA production from *M. alpina* are well-known, as there is a consensus on some basic needs for the fungus to proliferate and promote FA production. However, the know-how of inducing the optimum amount of biomass and ARA accumulation is challenging due to the number of variables that control the fungal lipid fermentation in oleaginous microorganisms. The complexity begins with the variability in *M. alpina* strains, place of origin, regulation of environmental conditions (including pH, temperature, and incubation period), and ends with the nutrients and supplements added to the growth media, including carbon and nitrogen source and ratio, and other factors such as minerals, vitamins, glutamate, and iron, which play a crucial role in microbial activities. The choice of culture medium ingredients was based on studying the lipid biosynthesis pathway in oleaginous microorganisms, in addition to literature consensus and basic experimental trials observing the fungal strain preferences.[15,16,34−42] The basal medium provided a C/N ratio of 6, deemed optimal for fungal growth and PUFA production.[17,53,54] Besides peptone, the addition of yeast extract, which is rich in proteins,[55] was necessary because N is

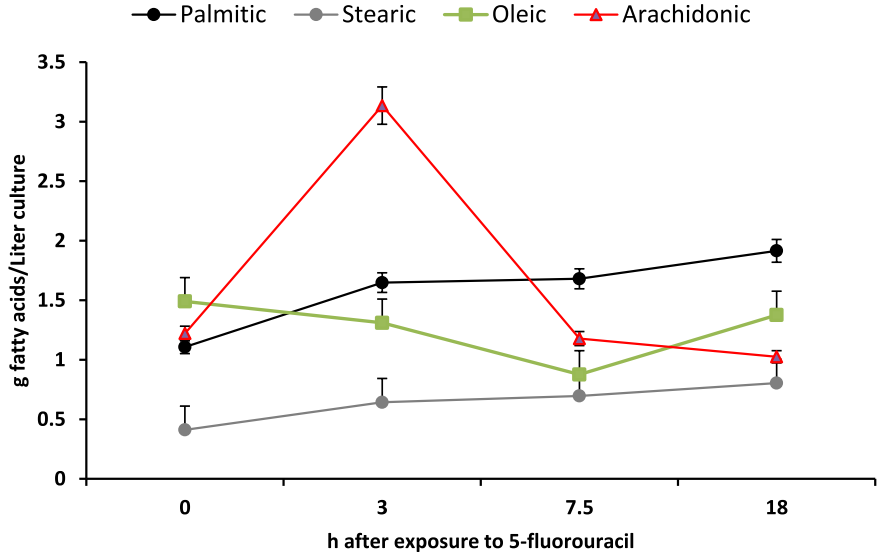


Figure 5. Fatty acid content (g/L culture) following 5-fluorouracil/octyl gallate mutagenesis. Each point represents the mean FA content obtained in two independent experiments, and vertical bars denote SE around the mean.

https://doi.org/10.1021/acsomega.4c02294
ACS Omega 2024, 9, 34456−34463

a limiting factor for fungal growth.[53,56,57] The basal medium and 7-day shake cultivation at 25 °C produced fungal biomass and ARA levels suitable for a cost-effective start-up. However, the addition of expensive and difficult-to-obtain vitamin mixtures led to a remarkably larger biomass and ARA yield, in accordance with Zeng et al.,[58] who suggested that the group B-vitamins are cofactors for ARA synthesis key enzymes. It was recently shown that vitamin addition was dispensable, provided that 0.6 g (versus 0.3 g) of yeast extract/100 mL were incorporated in the cultivation medium,[17] as yeast extract is a rich source of group B vitamins.[55,57]

Focusing on the ARA level, it kept increasing throughout the addition of nutrients, except in the case of iron supplementation. The addition of iron is a crucial requirement for the fungus' desaturases. Desaturases are membrane-bound, iron-containing enzymes which are involved in the lipid accumulation process.[59] However, FA analysis in the current study showed a high proportion of oleate at the expense of ARA levels following iron supplementation. Surprisingly, the coexistence of iron with vitamins reduced levels of palmitic acid and ARA, counteracting the positive effect of vitamins. It is worth mentioning that a previous study observed a correlation between the increase in Fe^{3+} with the increase in oleic and linoleic acid in *Mortierella ramanniana*, while >40 mg/L of Fe^{3+} showed an unfavorable effect on *Mortierella* sp., leading to ARA inhibition.[60] The glutamate restored the capacity for accumulating ARA by 1.2-fold and favored palmitic acid levels by 1.8 fold. The glutamate works on activating acetyl-CoA carboxylase, the backbone of FA elongation processes,[61−63] and thus is considered a crucial ingredient for *M. alpina* cultivation media.[17]

The addition of $NaHPO_4$ and magnesium sulfate led to a considerable increase in biomass and ARA yield as compared with cultures in basal medium. The addition of bivalent cations and minerals acts as cofactors for many enzymes (e.g., acetyl-CoA carboxylase) involved with the lipid accumulation process. The most used inorganic minerals are calcium, phosphorus, magnesium, sodium, sulfur, iron, manganese, zinc, and copper in minute amounts in the media.[31,38,59,60,64] The complete medium ARA yield was in the range of 500 mg/L, which is considered to be unsuitable for commercial production. Accordingly, the culture medium ingredients and cultivation conditions were modified. Cultures in shake flasks were incubated for 7 days at 25 °C, then for 7 days at 20 °C, followed by stationary aging at 10 °C, leading to a threefold higher ARA yield.[17]

To further increase the ARA content for potential commercial uses, mutagenesis of the isolate was attempted as recommended.[27,39,43,44,50] The procedures described by Zhang et al.[44] were followed with modifications leading to a further threefold increase in ARA percentage and content in cultures of spores exposed to 5-fluorouracil for 3 h, likely due to excessive activation of desaturase enzymes as octyl gallate screening selects mutants with exceptionally high FA desaturase activity.[44,66] Longer exposure of spores to 5-fluorouracil apparently stimulated the activity of FA synthase more than elongase 1 (MALCE1) or desaturases, leading to the accumulation of palmitic acid and an increase in stearic acid percentage and content at ARA expense (Table 4, Figure 5). Longer exposure to the mutagen impaired ARA yield, indicating that fungus mutant frequency decreases with increasing mutant exposure time, not only dosage,[65] likely as a result of increased 5-fluorouracil-mediated inhibition of DNA and some RNA synthesis. The achieved high ARA yield support Chang et al.[16] recommendation toward combining mutation breeding with culture optimization to enhance the ARA level in *M. alpina*, whereby the achieved ARA yield was comparable to that obtained in fermenters for industrial production.[15,44,49] Additionally, the *M. alpina* oil obtained compares well in FA composition with commercial oils, such as ARASCO, SUNTGA 40S, and RAO,[15] which are not available in developing countries because the production hardly supplies the demand in Europe, Australia, and North America. The present study may help promote ARA production on a commercial level in developing countries to meet at least the requirements of infant formulas.

CONCLUSIONS

The present study provides an additional method for confirming the identity of *M. alpina* cultures via GLELO gene product sequencing, which can be used independently or along with the ITS regions. The adjustment of growth media constituents led to an increase in biomass and ARA production. Improvement of culture parameters and a successful mutagenesis trial paved the road for commercial production of a cost-effective supplement, aiming for an improved life quality for children, the elderly, vegans, vegetarians, and residents of developing countries.

ASSOCIATED CONTENT

Data Availability Statement

All data have been shown in the manuscript and Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02294.

DNA/RNA extraction, PCR amplification, fatty acid determination, ITS regions (ITS1 and ITS2) located within the three rRNA genes, forward ITS5, ITS1F, and ITS1, and reverse ITS2, ITS3, and ITS4 hybridization locations on the DNA locus, primer sequences used for ITS region amplification, BLASTn, and fatty acid percentage in culture as assessed by gas chromatography−mass spectrometry (PDF)

AUTHOR INFORMATION

Corresponding Author

Hatem Tallima − *Department of Chemistry, School of Sciences and Engineering, The American University in Cairo, New Cairo 11835 Cairo, Egypt*; orcid.org/0000-0003-3714-641X; Email: htallima@aucegypt.edu

Authors

Violette S. Hanna − *Biotechnology Department, Faculty of Science, Cairo University, Giza 12613, Egypt*

Mohamed N. Abd El-Ghany − *Botany and Microbiology Department, Faculty of Science, Cairo University, Giza 12613, Egypt*

Mohamed I. M. Ibrahim − *Food Toxicology and Contaminant Department, National Research Centre, Giza 12622, Egypt*; orcid.org/0000-0003-0623-5225

Tahany M. Abdel-Rahman − *Botany and Microbiology Department, Faculty of Science, Cairo University, Giza 12613, Egypt*

Complete contact information is available at:

https://doi.org/10.1021/acsomega.4c02294
ACS Omega 2024, 9, 34456−34463

https://pubs.acs.org/10.1021/acsomega.4c02294

Author Contributions

Violette S. Hanna: conceptualization, methodology, investigation, visualization, validation, formal analysis, data curation, writing—original draft, writing—review and editing. **Mohamed N. Abd El-Ghany:** conceptualization, supervision, project administration, methodology, investigation, data curation, and writing—review and editing. **Mohamed I. M. Ibrahim:** visualization, validation, methodology, investigation, formal analysis, data curation, writing—review and editing. **Tahany M. Abdel-Rahman:** conceptualization, supervision, investigation, formal analysis, data curation. writing—review and editing. **Hatem Tallima:** conceptualization, visualization, validation, resources, project administration, methodology, investigation, supervision, funding acquisition, formal analysis, data curation, writing−original draft, writing—review and editing.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The research was funded by Cairo University and the National Program for Research & Innovation in Health and Biomedical Sciences-Academy of Scientific Research and Technology (PRISM-ASRT). Fatty acid percentage and content were analyzed at the National Research Centre, Central Laboratories Network, Chromatographic Laboratories, Dokki, Giza, Egypt. The exceptional administrative support of Eng, Asmaa Abdallah, PRISM Program Manager, The Academy of Scientific Research and Technology, and the excellent technical assistance of Abdel Badih Foda are herein acknowledged.

■ ABBREVIATIONS

ARA, arachidonic acid; C/N, carbon−nitrogen ratio; FA, fatty acid(s); ITS, untranslated internal transcribed spacer; PCR, polymerase chain reaction; PUFA(s), polyunsaturated fatty acid(s); rRNA, ribosomal RNA

■ REFERENCES

(1) Abedi, E.; Sahari, M. A. Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. *Food Sci. Nutr.* **2014**, *2*, 443−463.
(2) Brash, A. R. Arachidonic acid as a bioactive molecule. *J. Clin. Invest.* **2001**, *107*, 1339−1345.
(3) Tallima, H. Clarification of Arachidonic Acid Metabolic Pathway Intricacies. *ACS Omega* **2021**, *6*, 15559−15563.
(4) Hanna, V. S.; Hafez, E. A. A. Synopsis of arachidonic acid metabolism: A review. *J. Adv. Res.* **2018**, *11*, 23−32.
(5) Das, U. N. Gamma-linolenic acid, arachidonic acid, and eicosapentaenoic acid as potential anticancer drugs. *Nutrition* **1990**, *6*, 429−434.
(6) Das, U. N. Arachidonic acid and other unsaturated fatty acids and some of their metabolites function as endogenous antimicrobial molecules: A review. *J. Adv. Res.* **2018**, *11*, 57−66.
(7) Tallima, H.; El Ridi, R. Arachidonic acid: Physiological roles and potential health benefits - A review. *J. Adv. Res.* **2018**, *11*, 33−41.
(8) Tallima, H.; Hanna, V. S.; El Ridi, R. Arachidonic acid is a safe and efficacious schistosomicide, and an endoschistosomicide in natural and experimental infections, and cysteine peptidase vaccinated hosts. *Front. Immunol.* **2020**, *11*, 609994.
(9) Tallima, H.; El Ridi, R. Mechanisms of arachidonic acid in vitro tumoricidal impact. *Molecules* **2023**, *28*, 1727.
(10) Hadley, K. B.; Ryan, A. S.; Forsyth, S.; Gautier, S.; Salem, N. The essentiality of arachidonic acid in infant development. *Nutrients* **2016**, *8*, 216.
(11) Forsyth, S.; Gautier, S.; Salem, N. The importance of dietary DHA and ARA in early life: a public health perspective. *Proc. Nutr. Soc.* **2017**, *76*, 568−573.
(12) Tokuda, H.; Kontani, M.; Kawashima, H.; Akimoto, K.; Kusumoto, A.; Kiso, Y.; Koga, Y.; Shibata, H. Arachidonic acid-enriched triacylglycerol improves cognitive function in elderly with low serum levels of arachidonic acid. *J. Oleo Sci.* **2014**, *63*, 219−227.
(13) Stacey, D.; Benyamin, B.; Lee, S. H.; Hyppönen, E. A metabolome-wide Mendelian randomization study identifies dysregulated arachidonic acid synthesis as a potential causal risk factor for bipolar disorder. *Biol. Psychiatry* **2024**, No. S0006-3223(24)01106-5, DOI: 10.1016/j.biopsych.2024.02.1005.
(14) Ratledge, C. Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. *Biochimie* **2004**, *86*, 807−815.
(15) Mamani, L. D. G.; Magalhaes, A.; Ruan, Z.; Carvalho, J. C. d.; Soccol, C. R. Industrial production, patent landscape, and market trends of arachidonic acid-rich oil of *Mortierella alpina*. *Biotechnol. Res. Innov.* **2019**, *3*, 103−119.
(16) Chang, L.; Lu, H.; Chen, H.; Tang, X.; Zhao, J.; Zhang, H.; Chen, Y. Q.; Chen, W. Lipid metabolism research in oleaginous fungus *Mortierella alpina*: Current progress and future prospects. *Biotechnol. Adv.* **2022**, *54*, 107794.
(17) Anter, A.; Abdel-Ghany, M. N.; Ibrahim, M. I. M.; Charmy, R. A.; Yosri, M.; Tallima, H.; Tallima, H. Development of user-friendly protocols for single-cell arachidonic acid production in developing countries. *Bioresour. Technol. Rep.* **2024**, *25*, 101737.
(18) Raja, H. A.; Miller, A. N.; Pearce, C. J.; Oberlies, N. H. Fungal identification using molecular tools: a primer for the natural products research community. *J. Nat. Prod.* **2017**, *80*, 756−770.
(19) Brun, S.; Silar, P. Convergent evolution of morphogenetic processes in *Fungi*. In *Evolutionary Biology: Concepts, Molecular and Morphological Evolution*; Pontarotti, P., Ed.; Springer: Berlin, Germany, 2010; pp 317−328.
(20) Hughes, K. W.; Petersen, R. H.; Lodge, D. J.; Bergemann, S. E.; Baumgartner, K.; Tulloss, R. E.; Lickey, E.; Cifuentes, J. Evolutionary consequences of putative intra-and interspecific hybridization in agaric fungi. *Mycologia* **2013**, *105*, 1577−1594.
(21) Naranjo-Ortiz, M. A.; Gabaldón, T. Fungal evolution: cellular, genomic and metabolic complexity. *Biol. Rev. Cambridge Philos. Soc.* **2020**, *95*, 1198−1232.
(22) Schwarz, P.; Bretagne, S.; Gantier, J. C.; Garcia-Hermoso, D.; Lortholary, O.; Dromer, F.; Dannaoui, E. Molecular identification of zygomycetes from culture and experimentally infected tissues. *J. Clin. Microbiol.* **2006**, *44*, 340−349.
(23) Ferrer, C.; Colom, F.; Frasés, S.; Mulet, E.; Abad, J. L.; Alió, J. L. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J. Clin. Microbiol.* **2001**, *39*, 2873−2879.
(24) Ho, S. Y.; Chen, F. Genetic characterization of *Mortierella alpina* by sequencing the 18S-28S ribosomal gene internal transcribed spacer region. *Lett. Appl. Microbiol.* **2008**, *47*, 250−255.
(25) Parker-Barnes, J.; Das, T.; Bobik, E.; Leonard, A. E.; Thurmond, J. M.; Chaung, L.; Huang, Y. S.; Mukerji, P. Identification and characterization of an enzyme involved in the elongation of n-6 and n-3 polyunsaturated fatty acids. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8284−8289.
(26) Takeno, S.; Sakuradani, E.; Murata, S.; Inohara-Ochiai, M.; Kawashima, H.; Ashikari, T.; Shimizu, S. Molecular evidence that the rate-limiting step for the biosynthesis of arachidonic acid in *Mortierella alpina* is at the level of an elongase. *Lipids* **2005**, *40*, 25−30.
(27) Kikukawa, H.; Sakuradani, E.; Ando, A.; Shimizu, S.; Ogawa, J. Arachidonic acid production by the oleaginous fungus *Mortierella alpina* 1S-4: A review. *J. Adv. Res.* **2018**, *11*, 15−22.

(28) Shinmen, Y.; Shimizu, S.; Akimoto, K.; Kawashima, H.; Yamada, H. Production of arachidonic acid by *Mortierella* fungi. *Appl. Microbiol. Biotechnol.* **1989**, *31*, 11−16.
(29) Asadi, S. Z.; Khosravi-Darani, K.; Nikoopour, H.; Bakhoda, H. Evaluation of the effect of process variables on the fatty acid profile of single cell oil produced by *Mortierella* using solid-state fermentation. *Crit. Rev. Biotechnol.* **2015**, *35*, 94−102.
(30) Singh, A.; Ward, O. P. Production of high yields of arachidonic acid in a fed-batch system by *Mortierella alpina* ATCC 32222. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 1−5.
(31) Totani, N.; Hyodo, K.; Ueda, T. Minerals essential for growth of the filamentous fungus, *Mortierella alpina*. *J. Jpn. Oil Chem. Soc.* **2000**, *49*, 487−493.
(32) Nisha, A.; Rastogi, N. K.; Venkateswaran, G. Optimization of media components for enhanced arachidonic acid production by *Mortierella alpina* under submerged cultivation. *Biotechnol. Bioproc. Eng.* **2011**, *16*, 229−237.
(33) Liu, X.; Ji, X.; Zhang, H.; Fu, N.; Yan, L.; Deng, Z.; Huang, H. Development of a defined medium for arachidonic acid production by *Mortierella alpina* using a visualization method. *Appl. Biochem. Biotechnol.* **2012**, *168*, 1516−1527.
(34) Wynn, J. P.; Hamid, A. B. A.; Ratledge, C. The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. *Microbiology* **1999**, *145*, 1911−1917.
(35) Wynn, J. P.; Ratledge, C.; Hamid, A. A.; Li, Y. Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi *Mucor circinelloides* and *Mortierella alpina*. *Microbiology* **2001**, *147*, 2857−2864.
(36) Ratledge, C.; Wynn, J. P. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol.* **2002**, *51*, 1−52.
(37) Jenni, S.; Leibundgut, M.; Boehringer, D.; Frick, C.; Mikolásek, B.; Ban, N. Structure of fungal fatty acid synthase and implications for iterative substrate shuttling. *Science* **2007**, *316*, 254−261.
(38) Zhang, Y.; Ratledge, C. Multiple isoforms of malic enzyme in the oleaginous fungus, *Mortierella alpina*. *Mycol. Res.* **2008**, *112*, 725−730.
(39) Sakuradani, E. Advances in the production of various polyunsaturated fatty acids through oleaginous fungus *Mortierella alpina* breeding. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 908−917.
(40) Laoteng, K.; Čertík, M.; Cheevadhanark, S. Mechanisms controlling lipid accumulation and polyunsaturated fatty acid synthesis in oleaginous fungi. *Chem. Pap.* **2011**, *65*, 97−103.
(41) Li, X.; Yu, C.; Yao, J.; Wang, Z.; Lu, S. An online respiratory quotient-feedback strategy of feeding yeast extract for efficient arachidonic acid production by *Mortierella alpina*. *Front. Bioeng. Biotechnol.* **2018**, *5*, 83.
(42) Chang, L.; Tang, X.; Lu, H.; Zhang, H.; Chen, Y. Q.; Chen, H.; Chen, W. Role of adenosine monophosphate deaminase during fatty acid accumulation in oleaginous fungus *Mortierella alpina*. *J. Agric. Food Chem.* **2019**, *67*, 9551−9559.
(43) Sakuradani, E.; Ando, A.; Ogawa, J.; Shimizu, S. Improved production of various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 1−10.
(44) Zhang, H.; Lu, D.; Li, X.; Feng, Y.; Cui, Q.; Song, X. Heavy ion mutagenesis combined with triclosan screening provides a new strategy for improving the arachidonic acid yield in *Mortierella alpina*. *BMC Biotechnol.* **2018**, *18*, 23.
(45) Muftah Eltariki, F. E.; Tiwari, K.; Alhoot, M. A. Molecular characterization and genetic diversity of four undescribed novel oleaginous *Mortierella alpina* strains from Libya. *F1000Res.* **2021**, *10*, 895.
(46) Ridi, R. E.; Tallima, H. *Schistosoma mansoni* ex vivo lung-stage larvae excretory-secretory antigens as vaccine candidates against schistosomiasis. *Vaccine* **2009**, *27*, 666−673.
(47) Abdel Aziz, N.; Tallima, H.; Abou El Dahab, M.; El Ridi, R. Immunogenicity and protective capacity of *Schistosoma haematobium* recombinant cathepsin L against infection of hamsters with *S. haematobium*. *Vaccine Res.* **2019**, *6*, 1−8.
(48) Van der Walt, A. M.; Ibrahim, M. I. M.; Bezuidenhout, C. C.; Loots, D. T. Linolenic acid and folate in wild-growing African dark leafy vegetables (morogo). *Public Health Nutr.* **2009**, *12*, 525−530.
(49) Yuan, C.; Wang, J.; Shang, Y.; Gong, G.; Yao, J.; Yu, Z. Production of arachidonic acid by *Mortierella alpina* I_{49}-N_{18}. *Food Technol. Biotechnol.* **2002**, *40*, 311−315.
(50) Zhang, H.; Cui, Q.; Song, X. Research advances on arachidonic acid production by fermentation and genetic modification of *Mortierella alpina*. *World J. Microbiol. Biotechnol.* **2021**, *37*, 4.
(51) Ellis, D. Fungal hyphae and septation relevant to medical mycology. *Mycology Online*. www.mycology.adelaide.edu.au.
(52) Yadav, D. R.; Kim, S. W.; Babu, A. G.; Adhikari, M.; Kim, C.; Lee, H. B.; Lee, Y. S. First Report of *Mortierella alpina* (Mortierellaceae, Zygomycota) isolated from crop field soil in Korea. *Mycobiology* **2014**, *42*, 401−404.
(53) Higashiyama, K.; Fujikawa, S.; Park, E. Y.; Shimizu, S. Production of arachidonic acid by *Mortierella* fungi. *Biotechnol. Bioprocess Eng.* **2002**, *7*, 252−262.
(54) Ji, X. J.; Ren, L. J.; Nie, Z. K.; Huang, H.; Ouyang, P. K. Fungal arachidonic acid-rich oil: research, development and industrialization. *Crit. Rev. Biotechnol.* **2014**, *34*, 197−214.
(55) Grant, C. L.; Pramer, D. Minor element composition of yeast extract. *J. Bacteriol.* **1962**, *84*, 869−870.
(56) Lu, J.; Peng, C.; Ji, X. J.; You, J.; Cong, L.; Ouyang, P.; Huang, H. Fermentation characteristics of *Mortierella alpina* in response to different nitrogen sources. *Appl. Biochem. Biotechnol.* **2011**, *164*, 979−990.
(57) Li, L.; Jin, N. Effect of nitrogen concentration on the growth and fatty acid content of *Mortierella alpina*. *Int. J. Agric. Biol.* **2020**, *24*, 838−848.
(58) Zeng, Y.; Ji, X. J.; Chang, S. M.; Nie, Z. K.; Huang, H. Improving arachidonic acid accumulation in *Mortierella alpina* through B-group vitamin addition. *Bioprocess Biosyst. Eng.* **2012**, *35*, 683−688.
(59) Wang, M.; Chen, H.; Ailati, A.; Chen, W.; Chilton, F. H.; Todd Lowther, W.; Chen, Y. Q. Substrate specificity and membrane topologies of the iron-containing ω3 and ω6 desaturases from *Mortierella alpina*. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 211−223.
(60) Sajbidor, J.; Koželouhov'a, D.; Ĉert'ik, M. Influence of some metal ions on the lipid content and arachidonic acid production by *Mortierella* sp. *Folia Microbiol.* **1992**, *37*, 404−406.
(61) Bajpai, P. K.; Bajpai, P.; Ward, O. P. Arachidonic acid production by fungi. *Appl. Environ. Microbiol.* **1991**, *57*, 1255−1258.
(62) Lan, W. Z.; Qin, W. M.; Yu, L. J. Effect of glutamate on arachidonic acid production from *Mortierella alpina*. *Lett. Appl. Microbiol.* **2002**, *35*, 357−360.
(63) Yu, L. J.; Qin, W. M.; Lan, W. Z.; Zhou, P. P.; Zhu, M. Improved arachidonic acids production from the fungus *Mortierella alpina* by glutamate supplementation. *Bioresour. Technol.* **2003**, *88*, 265−268.
(64) Higashiyama, K.; Yaguchi, T.; Akimoto, K.; Fujikawaa, S.; Shimizu, S. Effects of mineral addition on the growth morphology of and arachidonic acid production by *Mortierella alpina* 1S-4. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1815−1819.
(65) Bos, C. J. Induction and isolation of mutants in fungi at low mutagen doses. *Curr. Genet.* **1987**, *12*, 471−474.
(66) Kubo, I.; Xiao, P.; Fujita, K. Antifungal activity of octyl gallate: structural criteria and mode of action. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 347−350.

https://doi.org/10.1021/acsomega.4c02294
ACS Omega 2024, 9, 34456−34463