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An efficient strategy for screening polyunsaturated fatty acid-producing oleaginous filamentous fungi from soil

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ABSTRACT

Screening oleaginous microorganisms capable of accumulating considerable lipids is essential for industrial lipid production. Here we demonstrated forty-seven filamentous fungal isolates were obtained from eight soil samples using a new screening strategy with both triphenyltetrazolium chloride (TTC), a redox indicator used for testing oil presence, and cerulenin, an inhibitor of fatty acid synthase (FAS), supplemented in screening medium. Among these fungal isolates, nineteen have high lipid content (> 20% dry biomass weight) and were affiliated with the genus *Mortierella* by morphology identification and phylogenetic analysis based on ITS gene sequences. Notably, one strain designated as SL-4 reached 32% of its biomass weight as lipid, displaying the highest potential. Two candidates with high lipid content as well as biomass production were selected for exploring the effect of different carbon and nitrogen sources on morphology, biomass and lipid accumulation.

1. Introduction

Polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA), gamma linolenic acid (GLA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have crucial structural and functional roles in higher eukaryotes and are recommended for prevention or treatment of chronic diseases, such as cardiovascular and inflammatory (Bellou et al., 2016; Hulbert et al., 2005). Conventional sources of PUFAs are plant seed and fish oils. However, high cost and relative scarcity limit their further application. In recent years, the use of single cell oils (SCOs) as an alternative source of essential fatty acids has received increasing interest due to their high productivity and low land requirement (Fang et al., 2016).

Microbial oil, also known as SCOs, is produced by oleaginous microorganisms, which contain > 20% lipids of their cell biomass. And those microorganisms can achieve excess accumulation by changing the microbial metabolic pathway specifically (Kosa and Ragauskas, 2011; Ledesma-Amaro, 2015; Ratledge, 1991). Compared with plant seed and fish oils, the composition of microbial oils offers more variety, enriched in ARA, GLA, EPA and DHA. In addition, the production of microbial oils presents many other advantages such as shorter life cycle, less dependence of location and climate, lower labor requirement, easier scaling-up, non-arable land usage, and is renewable and environment friendly (Kiran et al., 2014; Li et al., 2008). However, due to the high cost of fermentation, the study of microbial oils is mainly focused on the production of special nutritional and industrial oils with high economic value.

Currently, the study of oleaginous microorganisms primarily concentrates on yeast, mold and microalgae. Among different oleaginous microorganisms, filamentous fungi have drawn increasing attention in recent years due to multiple advantages, such as capable of accumulating long chain PUFAs as well as high biomass (Zheng et al., 2012). Notable oleaginous filamentous fungi include *Mortierella alpina*, *Mortierella isabellina*, and *Mucor circinelloides* (Fakas et al., 2009; Ratledge and Wynn, 2002; Wynn et al., 2001). As high-yield ARA-producing strains, *Mortierella alpina* has been one of the most promising filamentous fungi in industrial production. *Mucor circinelloides*, a GLA-rich oil-producing filamentous fungus, is chiefly used for commercial production of SCOs around the world. Since these two successful examples of SCOs have been commercialized, new potential strains of SCOs have

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been searched for through new screening strategies, especially high throughput screening method.

In previous studies, oleaginous microorganisms, especially filamentous fungi, were generally isolated through random selection based on low temperature stress or microscopic observation after staining with liposoluble dyes. However, the conventional approaches are time-consuming. In our study, we created a new screening strategy to identify new potential strains more rapidly and efficiently by using indicator triphenyltetrazolium chloride (TTC) and fatty acid synthase inhibitor cerulenin. TTC is an oxidant that can be reduced from colorless TTC to red triphenylformazan (TF). It has been generally used to assess desaturases activity by colorimetry, and proved to be an effective isolation procedure for high ARA-producing fungi through their degree of staining, which means the gradation of red color was positively correlated with lipid content (Yadav et al., 2015; Zhu et al., 2004). Cerulenin, derived from the filamentous fungus *Cephalosporium caerulens*, shows a broad spectrum of antimicrobial activity and inhibits fatty acid synthase (FAS) activity by covalent modification of the active site (Johansson et al., 2008), and has already been successfully used in screening for high ARA-yield mutant strains (Li et al., 2015).

The paper established an efficient screening strategy for polyunsaturated fatty acids-producing filamentous fungi using TTC and cerulenin efficient screening. Moreover, the effect of different organic carbon and nitrogen sources on biomass and lipids profile was also investigated, providing valuable information for further fermentation optimization.

2. Materials and methods

2.1. Samples collection

Seven soil samples were collected from different geographic areas in China, including Qilian Mountain (Qinghai Province), Lhasa (Tibet Autonomous Region), Tianshui (Gansu Province), Hami (Xinjiang Uygur Autonomous Region), Liang Mountain (Sichuan Province), Lingyuan (Liaoning Province) and Huhhot (Inner Mongolia Autonomous Region). No permissions were required prior to conducting the research. All of these sampling positions are located in high altitude areas, where the temperature is relatively low. Besides, we also collected three soil samples from plain areas, including Wuxi (Jiangsu Province), Suzhou (Jiangsu Province) and Chengdu (Sichuan Province), where the average temperature is higher than the previous areas. The soil samples were captured from a depth of 5–10 cm underground after the surface litter was scraped away. Soil samples were placed into 50 mL sterile centrifuge tube and stored at 4 °C immediately.

2.2. Tests of proper concentration of cerulenin and TTC for strain isolating

Mortierella alpina (#32222, American Type Culture Collection, Manassas, Virginia), with accumulation of lipid content accounting for 27% of dry cell weight in broth fermentation medium, was inoculated on potato dextrose agar (PDA) plates (BD Difco TM Potato Dextrose Agar cat# 213400) at 28 °C for 7 days. 6 mL sterile water was added to two plates. Spores were gently scraped off the surface with a sterile loop, and then filtrated through a 40-micron cell strainer (Miracloth, Calbiochem). Spores were concentrated by centrifuging at 12,000 $\times g$ for 10 min and suspended in a small volume of sterile water. Then spore suspension was prepared as described in previous studies (Wang et al., 2011; Chen et al., 2015).

100 μL spore-suspension (10^6/mL) of *M. alpina* ATCC 32222 were inoculated at PDA plates supplemented with various cerulenin concentrations (0 mg/L, 0.022 mg/L, 0.22 mg/L, 2.2 mg/L), then the fungi were incubated at 28 °C for 3 days. And the growth status of colonies was monitered to achieve the proper concentration of cerulenin for preliminary screening.

To test optimal TTC concentration, the spores suspension of *M. alpina* ATCC 32222 were inoculated at PDA plates supplemented with different TTC concentrations (0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L, 2 g/L), followed by incubation at 28 °C for 3 days. Similarly, the proper concentration of TTC should also be identified through observation of growth status of the colonies.

2.3. Preliminary screening of oleaginous filamentous fungi

Ten grams of each sample was suspended in 90 mL sterile water. Serial 10-fold dilutions of the original suspensions were made, and then 100 μL soil suspension from 10^{-1} to 10^{-4} dilutions was spread evenly on the surface of PDA supplemented with TTC, cerulenin and chloramphenicol (20 μg/mL), respectively. Each dilution was plated in triplicate. The plates were then incubated at 28 °C with Avoiding light treated.

After 3–20 days of incubation, the fungal colonies were transferred to fresh PDA plates according to its color (red colonies were selected) and diameter , then incubated at 28 °C for further purification. The pure isolates were maintained on PDA slants at 4 °C and subcultured once every 3 months.

2.4. Lipids fermentation based on culture conditions of Mortierella

After the preliminary plate screening, the hyphae of pure fungal isolates were inoculated into 100 mL broth medium (Wang et al., 2011; Hao et al., 2015) (20 g/L of Glucose, 5 g/L of Yeast extract, 1 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4{\cdot}7H_2O$ and 10 g/L of KNO_3) in 250 mL flask , and cultured at 28 °C for 36 h with shaking at 200 rpm. After two rounds, the hyphae were broken into small fragments and inoculated into broth fermentation medium (50 g/L of Glucose, 5 g/L of Yeast extract, 1 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4{\cdot}7H_2O$ and 10 g/L of KNO_3). After 7 days' culture at 28 °C, 200 rpm, the fungal cells from the fermentation broth were harvested by filtering (200 mesh) and washed with distilled water twice. Samples were dried in vacuum freeze drier (Labconco, America) and weighted dry cell weight (DCW).

2.5. Preparation of fatty acid methyl esters (FAMEs) and GC–MS analyses

Cells were harvested and lyophilized for fatty acids analysis. Lipids were extracted from approximately 50 mg dry fungal sample with chloroform/methanol (2:1, *v*/v), and pentadecanoic acid (C15: 0, Sigma) was added as an internal standard for quantitative analysis. Fatty acid methyl esters (FAMEs) were obtained through the treatment of 10% HCl/methanol (*w*/w) (Bligh and Dyer, 1959).

FAMEs were analyzed through gas chromatography–mass spectrometry (GC–MS) (SPL-2010 Plus and GCMS-QP2010 Ultra; Shimadzu Co., Kyoto, Japan) with a 30-m by 0.25-mm Rt-wax column (film thickness, 0.25 μm). The temperature program was as follows: 40 °C for 3 min, ramp to 120 °C at 40 °C per min, ramp to 190 °C at 35 °C per min, hold for 5 min, ramp to 220 °C at 5 °C per min, hold for 16 min. Helium was the carrier gas. Fatty acid quantification was carried out using peak height area integrals using software named ‘GC solution’ (Metcalfe et al., 1966).

2.6. Phylogenetic analyses

Genomic DNA was extracted using the Biospin Fungus Genomic DNA Extraction Kit according to the manufacturer's instructions. The universal primer ITS1: TCCGTAGGTGAACCTGCGG ITS4: TCCTCCGCT TATTGATATGC was used to amplify DNA genes in the ITS1, 5.8 s ribosomal RNA, and ITS2 region. Polymerase chain reactions (PCR) was performed with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min, with final extension at 72 °C for 10 min (Yang et al., 2014). Amplified PCR products were purified using the Thermo GeneJET PCR Purification kit (Thermo Fisher Scientific, Vilnius, Lithuania) for sequencing (The

Table 1
Effect of various cerulenin concentrations on mycelial growth.

Concentration (mg/L)	Growth description
0	Normal growth with intact colony.
0.022	Growth was slightly inhibited with intact colony.
0.22	Growth was severely inhibited with little mycelium can be seen.
2.2	No growth was observed.

Beijing Genomics Institute , China). The sequencing results were aligned and compared to nucleotide sequences of similar strains stored in NCBI nucleotide collection database with the BLASTn program (http://www.ncbi.nlm.nih.gov/blast/).

Phylogenetic trees were constructed by the neighbor-joining (NJ) method using software MEGA (Molecular Evolutionary Genetics Analysis; version 5.05). The nucleotide sequences of similar strains used in tree construction were obtained from the NCBI nucleotide collection database. Nearly complete ITS1, 5.8 s r RNA, and ITS2 region sequences of both similar strains and isolated fungal strains with high-lipid content were covered for the tree construction. The bootstrap values were obtained from 1000 replications of NJ analyses.

3. Results

3.1. Proper concentration of cerulenin and TTC for preliminary screening

Gradient concentrations were set to determine the optimal concentration of cerulenin for preliminary screening (Table 1). It was found that spore germinated slowly with the cerulenin concentration increasing. At the concentration was at 0.22 mg/L or 2.2 mg/L, *M. alpina* ATCC 32222 presented slow growing, suggesting adverse effect to the growth of some oleaginous filamentous fungi. Thus, in order to address our requirement of screening more oleaginous filamentous fungi, the concentration of cerulenin was set to be 0.022 mg/L to exclude low FAS activity strains.

Meanwhile, various TTC concentrations (0 g/L, 0.5 g/L, 1 mg/L, 1.5 g/L, 2 g/L) was added to PDA plates for proper concentration. The results (Fig. 1) showed the colony color turned deeper with the TTC concentration increasing. As an indicator, TTC at the concentration of 0.5 g/L could cater the need. Therefore, the concentration of 0.5 g/L TTC was chosen in the following preliminary screening assay. Colonies with deep color were selected as potential oil producers for the second screening with shake-flask fermentation.

3.2. Isolation and identification of oleaginous filamentous fungi from soil

Based on the above results, 0.022 mg/L cerulenin and 0.5 g/L TTC were added into preliminary screening medium. According to colony color (similar and darker shade of red colonies compared with *M.alpina* ATCC32222 were selected) and diameter, 46 single fungal colonies were separated from ten soil samples located in different areas, Among them, 19 oleaginous fungi (containing lipids at > 20% of DCW) from high altitude areas were finally obtained through the secondary screening with shake-flask fermentation. However, none of oleaginous filamentous fungi was isolated from plain area soils, declaring that oleaginous microorganisms are commonly present in alpine frigid region. All of the 19 isolates in this study were assigned as *Mortierella* species through morphological comparison and gene sequencing.

3.3. Fatty acids analysis of oleaginous fungal isolates

The growth characteristics of these 19 oleaginous fungi are shown in Table 1, in which the DCW of thirteen oleaginous fungi exceed 10 g/L. Strain SL-4 (CCTCC AF 2017013) presented the most potential ability with accumulating 29.89% of its biomass weight as lipid. After evaluating the lipid production of all the fungal isolates obtained from soil, only the fungal isolates with high lipid content (> 20% of DCW) were selected for genetic identification of fungal internal transcribed spacer (ITS) regions.

FAMEs were analyzed by GC–MS and it was found that the fatty acid compositions of the 19 high lipid isolates were quite similar (Table 2). All these fungal isolates were rich in C16 and C18 fatty acids. The main lipids included myristic acid (C14: 0), palmitic acid (C16: 0), stearic acid (C18: 0), oleic acid (C18: 1), linoleic acid (C18: 2), linolenic acid (C18: 3), eicosatrienoic acid (C20: 3), arachidonic acid (C20: 4) and tetracosanoic acid (C24: 0), which amounting to about 80% of the total fatty acid production. The most abundant fatty acids produced were palmitic acid (C16: 0), oleic acid (C18: 1), linoleic acid (C18: 2), and arachidonic acid (C20: 4). Moreover, trace amounts of C17 fatty acids were also detected in these isolates, a very rare metabolite in cells. Comparing the total fatty acids (TFAs) content of the 19 isolates, 8 candidate strains (TSM-2, TSM-3 (CCTCC AF 2017012), TSM-4, TSM-5, SL-2, SL-4, LS-2 and ZS-5) were screened for further study, based on

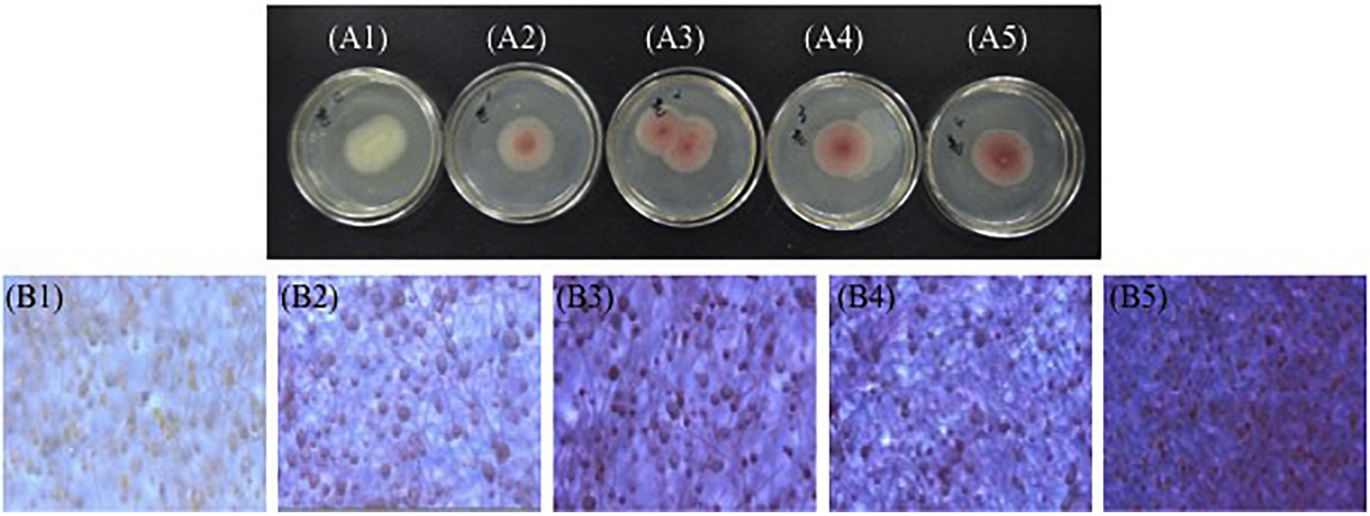

Fig. 1. The staining effect of colony and lipid droplet at different TTC concentration.
The colony was observed by the stereomicroscope, which grew on the PDA plate with TTC. The degree of staining of the lipid droplets was different at different TTC concentrations. The TTC concentrations of different PDA plates (*W/V*) were (A1 and B1): 0 g/L, (A2 and B2): 0.5 g/L, (A3 and B3): 1 g/L, (A4 and B4): 1.5 g/L, (A5 and B5): 2 g/L. No obvious difference was observed on A2, A3, A4 and A5.

Table 2
Biomass and fatty acid profile of 19 oleaginous isolates.

Isolate	Dry biomass (g/L)	TFA (% biomass)	Composition of fatty acid(% TFA)						
			C16:0	C18:0	C18:1	C18:2	C18:3	C20:3	C20:4
H-1	12.03 ± 0.08	22.52 ± 0.70	10.96 ± 0.10	9.09 ± 0.18	12.33 ± 0.44	13.41 ± 0.25	6.10 ± 0.16	7.03 ± 0.55	25.56 ± 0.30
L-1	13.09 ± 0.14	23.53 ± 2.89	18.06 ± 0.96	8.44 ± 1.21	30.42 ± 0.67	9.27 ± 0.31	4.46 ± 0.40	4.22 ± 0.13	13.55 ± 0.71
LS-2	9.36 ± 0.26	31.06 ± 0.37	16.11 ± 0.23	12.91 ± 0.81	15.48 ± 0.20	12.39 ± 0.04	6.64 ± 0.04	4.30 ± 0.45	22.95 ± 0.11
N-1	13.59 ± 0.59	21.96 ± 1.20	24.11 ± 0.18	5.91 ± 0.20	30.04 ± 0.24	6.51 ± 0.12	5.99 ± 0.04	3.14 ± 0.17	16.70 ± 0.18
N-4	7.90 ± 0.44	21.09 ± 0.58	25.94 ± 0.13	4.93 ± 0.15	29.04 ± 0.43	7.21 ± 0.06	5.78 ± 0.16	3.45 ± 0.31	16.54 ± 0.07
N-5	9.32 ± 0.32	21.15 ± 0.28	25.78 ± 0.33	5.12 ± 0.28	29.36 ± 0.45	6.95 ± 0.14	5.87 ± 0.16	3.35 ± 0.18	16.55 ± 0.40
QL-3	8.93 ± 0.32	23.67 ± 0.88	17.08 ± 0.14	6.20 ± 0.42	35.12 ± 0.15	8.25 ± 0.27	4.37 ± 0.69	4.44 ± 0.52	16.19 ± 0.20
QL-5	9.34 ± 0.33	21.73 ± 0.68	18.13 ± 0.08	5.95 ± 0.22	31.89 ± 0.17	9.16 ± 0.08	4.05 ± 0.22	3.50 ± 0.26	18.17 ± 0.0.87
QL-9	10.47 ± 0.80	22.15 ± 0.82	18.06 ± 0.45	5.67 ± 0.65	33.84 ± 0.23	8.42 ± 0.27	3.85 ± 0.14	3.49 ± 0.22	16.43 ± 0.38
QL-11	11.65 ± 0.39	21.71 ± 0.58	19.76 ± 0.79	5.11 ± 0.21	33.80 ± 0.77	9.37 ± 0.19	4.01 ± 0.20	3.32 ± 0.27	16.45 ± 0.33
QL-15	11.10 ± 0.49	21.48 ± 1.43	18.28 ± 0.34	13.68 ± 0.30	17.16 ± 0.24	9.92 ± 0.29	5.60 ± 0.37	5.09 ± 0.34	15.82 ± 0.18
QL-17	9.70 ± 0.41	23.95 ± 0.38	21.44 ± 0.42	4.48 ± 0.33	36.22 ± 0.98	6.43 ± 0.16	3.49 ± 0.29	3.33 ± 0.23	14.15 ± 0.28
SL-2	14.03 ± 0.36	25.75 ± 0.14	25.04 ± 0.11	9.49 ± 0.02	32.46 ± 0.24	7.03 ± 0.31	3.72 ± 0.01	2.19 ± 0.01	9.59 ± 0.09
SL-4	13.28 ± 0.34	29.89 ± 0.74	12.90 ± 0.23	9.06 ± 0.11	13.33 ± 0.27	12.05 ± 0.26	3.72 ± 0.11	6.22 ± 0.09	22.97 ± 0.25
TSM-2	12.51 ± 0.17	26.89 ± 1.87	14.00 ± 0.42	9.50 ± 0.16	11.20 ± 0.18	13.04 ± 0.74	3.09 ± 0.08	6.01 ± 0.40	26.20 ± 0.25
TSM-3	13.01 ± 0.60	27.72 ± 0.58	13.37 ± 0.62	10.81 ± 1.77	12.34 ± 0.04	12.77 ± 0.34	3.15 ± 0.06	6.14 ± 0.19	24.51 ± 0.04
TSM-4	11.27 ± 0.72	24.66 ± 0.66	14.44 ± 0.51	8.72 ± 0.19	11.01 ± 0.84	10.56 ± 0.19	3.04 ± 0.10	5.26 ± 0.04	27.13 ± 0.41
TSM-5	11.81 ± 0.47	27.17 ± 1.45	14.11 ± 0.45	8.66 ± 0.40	9.66 ± 0.23	11.25 ± 0.02	3.00 ± 0.01	5.02 ± 0.09	27.43 ± 0.28
ZS-5	14.27 ± 0.25	25.15 ± 0.52	14.75 ± 0.22	8.86 ± 1.00	10.55 ± 1.39	9.69 ± 0.76	3.24 ± 0.11	5.42 ± 0.19	30.12 ± 1.15

All values are mean of three replicates ± standard deviation.
QL: Qilian Mountain (Qinghai Province), SL: Liang Mountain (Sichuan Province), LS: Lhasa (Tibet Autonomous Region), TSM and ZS: Tianshui (Gansu Province), H: Hami (Xinjiang Uygur Autonomous Region), N: Huhhot (Inner Mongolia Autonomous Region), L: Lingyuan (Liaoning Province), TFA: Total fatty acid.

their ability to accumulate > 25% DCW.

3.4. Phylogenetic analysis of oleaginous fungal isolates

Based on the ITS regions sequencing results, a phylogenetic tree was built to describe the similarity of fungal isolates with high lipid content. All 19 oleaginous strains belong to the same genus of *Mortierella* (Fig. 2), even though they were screened from soil samples collected in different areas dispersed throughout China. Phylogenetic analysis indicated that the nucleotide sequence of 12 isolates (TSM-2, TSM-3, TSM-4, TSM-5, SL-4, LS-2, N-1, N-5, QL-9, QL-11, QL-15, ZS-5) closely matched with *M. alpina*. Taking morphological identification into consideration, 12 potential isolates were identified as the species of *M. alpina*. The nucleotide sequence homology of 4 strains (QL-3, QL-5, QL-17, H-1) reached up to 100% by comparing with *Mortierella verticillata.* However, the nucleotide sequences of strains (N-4, L-1, SL-2) closely identify with *Mortierella* sp., but the relation to specific species needs to be determined. Many genera of *Mortierella* have already been recognized and studied as oleaginous microorganisms (Gao et al., 2014; Ji et al., 2014; Wang et al., 2011). The identification result revealed that *Mortierella* is the dominant oleaginous filamentous fungi in soil.

3.5. Effect of carbon and nitrogen sources on physiological property and fatty acids production

The yields of total fatty acids and ARA are closely correlated with carbon sources and nitrogen sources. To determine the relationship between fatty acids and carbon or nitrogen sources, different organic carbon and nitrogen sources were utilized in order to further explore the effects on physiological lipids profile in flask cultures (the results are shown in Table 3).

High lipid productivity did not always correspond to high biomass yield. Taking lipid productivity as standard, when yeast extract was used as nitrogen source, the best carbon source for TSM-3 was corn flour, and the average ARA production accounts for 31.77% of the TFA with accumulating to 3.58 g/L. However, SL-4 produced the highest lipids content (4.34 g/L) in glucose as carbon source and the highest ARA levels in corn flour, which mean the production reached up to 1000 mg/L. Using glucose as the sole carbon source and comparing three different nitrogen sources, the results showed that soybean meal was the best nitrogen source for TSM-3 (which accumulated 3.80 g/L of TFA, 980 mg/L ARA) and yeast extract was the best nitrogen source for SL-4 (4.64 g/L lipid, 950 mg/L ARA).

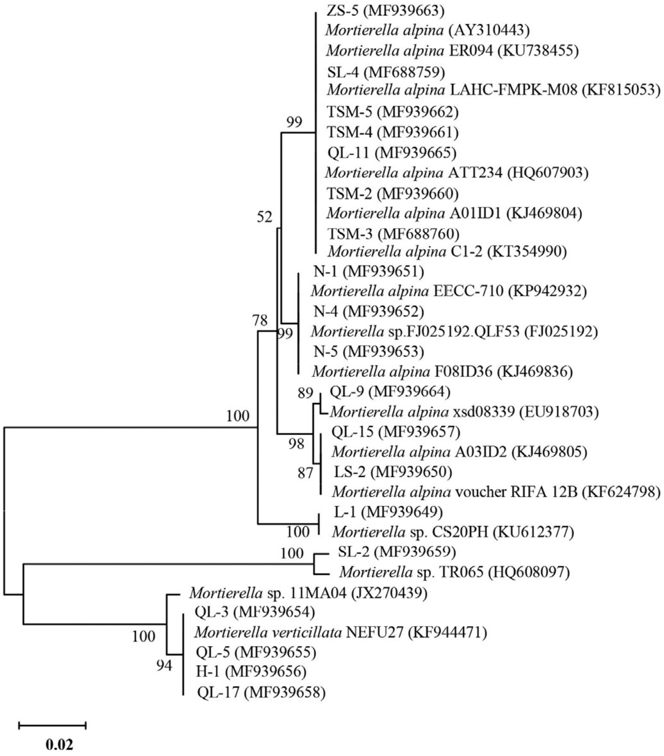

Fig. 2. Phylogenetic analysis of fungal isolates aligned with similar strains.The representative ITS sequences were compared to the entries in GenBank using Blastn (http://www.ncbi.nlm.nih.gov/blast/), and those displaying similarities > 99% with known species were identified as corresponding species. These sequences were aligned using Clustal X. Bootstrapping included 1000 replicates for neighbor-joining-based trees. Phylogenetic trees were constructed by the neighbor-joining (NJ) method using software MEGA (Molecular Evolutionary Genetics Analysis; version 5.05).

The specific products of filamentous fungi in submerged

Table 3
Effect of carbon and nitrogen sources on biomass, lipid, TFA and ARA yield in TSM-3 and SL-4 strains.

Strain			Dry biomass (g/L)	TFA (% dry biomass)	TFA (g/L)	ARA (%TFA)	ARA(mg/L)
TSM-3	Carbon source	Glucose	12.35 ± 0.60	25.47 ± 0.49	3.15 ± 0.21	24.61 ± 1.05	770 ± 0.02
		Soluble starch	17.57 ± 0.13	15.77 ± 0.21	2.77 ± 0.06	29.61 ± 0.49	820 ± 0.02
		Glycerol	9.78 ± 0.77	19.74 ± 0.89	1.93 ± 0.24	21.46 ± 1.01	420 ± 0.07
		Corn flour	16.26 ± 0.14	22.02 ± 0.74	3.58 ± 0.14	31.77 ± 0.40	1140 ± 0.04
	Nitrogen source	Yeast extract	12.35 ± 0.60	25.47 ± 0.49	3.15 ± 0.21	24.61 ± 1.05	770 ± 0.02
		Peptone	12.31 ± 0.21	22.91 ± 1.20	2.91 ± 0.02	25.99 ± 1.31	760 ± 0.03
		Soybean meal	16.60 ± 0.24	23.68 ± 0.53	3.80 ± 0.21	25.66 ± 0.07	980 ± 0.05
SL-4	Carbon source	Glucose	14.34 ± 0.35	30.29 ± 1.08	4.34 ± 0.26	21.84 ± 0.56	950 ± 0.04
		Soluble starch	15.46 ± 0.47	16.72 ± 0.39	2.58 ± 0.09	31.71 ± 0.54	820 ± 0.04
		Glycerol	9.10 ± 0.05	22.64 ± 0.81	2.06 ± 0.07	25.36 ± 1.17	520 ± 0.03
		Corn flour	14.73 ± 0.22	24.06 ± 0.21	3.54 ± 0.08	28.12 ± 0.45	1000 ± 0.02
	Nitrogen source	Yeast extract	14.34 ± 0.35	30.29 ± 1.08	4.34 ± 0.26	21.84 ± 0.56	950 ± 0.04
		Peptone	10.52 ± 0.69	28.20 ± 0.73	2.96 ± 0.12	24.67 ± 0.41	730 ± 0.04
		Soybean meal	16.43 ± 0.67	19.87 ± 0.68	3.26 ± 0.13	27.63 ± 0.61	900 ± 0.04

Data are expressed as means ± standard deviations. All values are mean of three replicates ± standard deviation.
SL: Liang Mountain (Sichuan Province), TSM and ZS: Tianshui (Gansu Province).

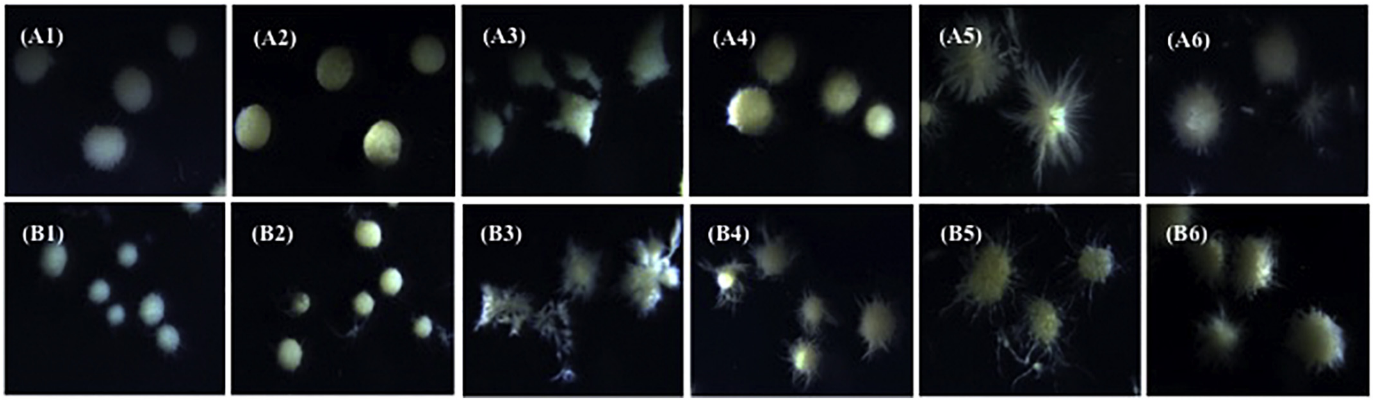

Fig. 3. The effect of different carbon or nitrogen sources on mycelium morphology.
Different fermentation substrates formed different cell morphology, which was related to the biomass and lipid accumulation. Dispersed mycelium was the favored form for biomass accumulation and the pellet morphological form was beneficial to lipid accumulation. The results showed that the cells formed dispersed mycelium when the nitrogen source was soybean meal. When glucose was used as the carbon source and yeast extract used as the nitrogen source, the cells were pellet morphological form. ((A1)–(A6): TSM-3; (B1)–(B6): SL-4; (A1), (B1): Soluble starch; (A2), (B2): Glycerol; (A3), (B3): Corn flour; (A4), (B4): Glucose and Yeast extract; (A5), (B5): Soybean meal; (A6), (B6): Peptone).

fermentation are generally connected with morphological form. Our results also showed that dispersed mycelium was the favored form for biomass accumulation and the pellet morphological form was beneficial to lipid accumulation (Fig. 3, Table 2).

4. Discussion

It has only been three decades since various yeasts and filamentous fungi were first used for industrial production of oils. Although many SCOs sources have been proposed, only six of them have been scaled up and only DHA oil from *Crypthecodinium cohnii* and ARA oil from *M. alpina* have reached a significant commerial realization (Cohen and Ratledge, 2010). Oils from these oleaginous microorganisms had been successfully applied to infant formula.

Oleaginous filamentous fungi accumulate large amount lipids and have unique advantages in producing long-chain PUFAs compared with other oleaginous microorganisms. Therefore, developing effective screening methods for exploring superior microbes is necessary and urgent. Previous plating screening methods of oleaginous microorganisms, especially filamentous fungi, were based on low pH, low temperature stress or microscopic observation after staining with liposoluble dyes (Warcup, 1950; Warcup, 1955; Zhu et al., 2004) which was time-consuming. Yang et al. screened oleaginous fungal isolates from the soybean plant and all the strains growing on plates had to undergo flask cultivation in the preliminary screening process, whatever they produced oil or not (Yang et al., 2014). Consequently, more efficient screening methods are in need.

The oleaginous fungi isolated in the present study suggested that TTC was positively correlated with the content of PUFAs in the mycelia lipids. And when combined with cerulenin, synergy effects were shown to remain goal strains visually. In contrast to previous strategies (Yang et al., 2014), a majority of non-oleaginous fungus was excluded by observing colony color and size in the preliminary screening process (eliminating light-red and other color colonies). Besides, we used TTC together with cerulenin simultaneously instead of successive steps. Plenty of time and materials are saved. The results indicate that our new strategy is rapid and effective to isolate high- PUFAs-yield strains from soil, which could be utilized to explore more potential strains for PUFAs production. Besides, it is the first time to apply this strategy in isolation of oleaginous microorganisms directly. Nineteen isolates with over 20% lipid content of their cell dry weigh (Table 1) are isolated by using this TTC-cerulenin screening strategy.

In addition, the wide adaption to various carbon and nitrogen sources shows the ability of the fungal isolates to utilize a variety of materials as feedstock. Our results show that both soluble starch and soybean meal contribute to cell growth, which is consistent with previous research results (Park et al., 1999; Jang et al., 2005). However, strains obtained higher lipid accumulation in the presence of glucose and yeast extract. Taking the lipid and ARA production into consideration, corn flour was suitable carbon source and soybean meal was the most suitable nitrogen source for TSM-3, while glucose and yeast extract were the most suitable carbon and nitrogen sources for SL-4. This study provides the possibility for strains to utilize cheap raw material, such as soybean meal. And it also would be interesting to further investigate the effect of carbon and nitrogen sources on cell growth, ARA and lipid production of these fungi.

The present study also indicates that morphological forms had a significant impact on cell growth and lipid production when different carbon, nitrogen sources or microparticles were used, which is consistent with previous ones (Park et al., 1999; Gao et al., 2014; Park et al., 2002; Yu et al., 2011). Mycelia morphology is considered as key parameters in fungal ARA-rich oil fermentation. We therefore conclude that different carbon and nitrogen sources result in various fungal morphologies, and different morphologies are tightly connected with the biomass and lipid accumulation so that optimizing the lipid production process demands a well-balanced morphological form with an ideal form between pellet and dispersed mycelium. Besides, based on the bioreactor platform, aeration and agitation could influence the mycelia morphology, which were beneficial for oxygen transfer and thus would be highly suitable for biomass accumulation (Wu et al., 2017). Thus, it is especially important for follow-up studies to explore the relationship between lipid synthesis and morphological development.

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Ethical approval

This article does not contain any studies with animals performed by any of the authors.

Declarations of interest

None.

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