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Screening the oleaginous capacity of fungi from different habitats

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Abstract: Soil encompasses a variable microbial community with different metabolic routes based on pH, temperature, moisture content, and nutrients composition. Herein, 50 soil samples collected from different localities were subjected to organic carbon and nitrogen determination as well as the isolation and purification of their fungal flora. Further, the oleaginous capacity of the isolated fungal species was estimated. The obtained results revealed that 62 isolates had the ability to accumulate lipids at a level more than 20% of their dry biomass under shaking conditions and 46 fungal isolates on static incubation. These isolates involved 7 isolates with high lipid content (≥ 40%) where *Cunninghamella echinulata* represented the highest oleaginous fungal isolate with 55.37% lipid yield, followed by *Aspergillus ochraceus* and *A. terreus* with lipid productivity of 53.78% and 48.26%, respectively. Mining for oleaginous fungi represents a promising prospect to introduce sustainable lipids and fatty acids sources available for diverse biotechnological applications.

Keywords: Oleaginous fungi, Lipids, Organic carbon, Nitrogen, C/N ratio.

1. Introduction

Microbial products are considered to be natural and have high safety profiles compared to chemical products, which produce harmful byproducts that may be hazardous to the ecosystem (Vassileva *et al*., 2022). Fungi existed in diverse habitats and associated with chemoheterotrophic pattern afford several metabolomics comprising primary and secondary metabolites boosted in numerous bioactivities and biotechnological applications (Mohamed *et al*., 2021). These metabolites encompass polysaccharides (Giavasis, 2014), lipids and fatty acids (Mohamed *et al*., 2022; Hassane *et al*., 2024), and enzymes and peptides (Al Mousa *et al*., 2022 a, b; Khalaf *et al*., 2024), as well as, low molecular weight secondary products including mainly phenolic acids, alkaloids, saponins, flavonoids, and terpenoids (Pimentel et al., 2011; Hassane *et al*., 2022 a, b; Al Mousa *et al*., 2022 c, 2024; Abdelrahem *et al*., 2023, 2024). Moreover, different mycotoxins with negative economic importance are produced by fungi (Abo Dahab *et al*., 2016; Saber *et al*., 2016).

Lipids, naturally occurring oils and fats, represent a prime group of bio-macromolecules in all organisms ordered thirdly following proteins and carbohydrates (Akpinar-Bayizit, 2014). Lipids have a biological importance including their involvement in cellular membranes structure, energy source, signaling of pathways, hormones composition, vitamins, and genetic expression (De Carvalho & Caramujo, 2018). Polyunsaturated fatty acids (PUFAs), units of lipids acquire numerous human health benefits (Kapoor *et al*., 2021). Lipids are ordinarily derived from plants and animals' sources; meanwhile oleaginous microbes can be a substitute sources where oleaginous bacteria, yeasts, algae and fungi appear convenient SFAs, MUFAs, PUFAs sources (Liang & Jiang, 2013). Microbial lipids, also known as single-cell oils (SCOs), are universal products with applications in the nutraceutical, pharmaceutical, and industrial fields. Diverse oleaginous microbes manifested as SCOs

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producers because of their capacity to accumulate more than 20% lipids (w/w) of their gross cellular dry weight (CDW) (Ratledge & Wynn, 2002).

Fungi capability to produce SCOs is attributed to their short life cycle and independence from light energy (Sun *et al*., 2021). In addition, oleaginous fungi earn the ability to grow vastly on numerous substrates as carbon sources (agro-industrial residues, lignocellulosic material, wastewater, and crude glycerol) as well as highly pure oils yield was obtained (Bellou *et al*., 2016). Also, PUFAs production by fungi seems sustainable and independent of climatic alterations (Mhlongo *et al*., 2021). Various fungal species have been known as producers as SCOs, including *Mortierella isabellina*, *M. vinacea*, *Mucor circinelloides*, *Cunninghamella echinulata*, *Rhizopus oryzae*, *Aspergillus oryzae* and *A. terreus* (Suleiman *et al*., 2018 a). In some cases, these oleaginous microorganisms can offer multiple benefits beyond their lipid productivity, such as aiding in bio-refinery, bio-remediation, decolorization, and serving as medicinally important agents (Abdel-Razek *et al*., 2020). Therefore, there is a need to continue searching for new oleaginous fungi due to the numerous advantages of microbial lipids, particularly those derived from fungi (Soliman *et al*., 2022).

Nitrogen sources are principal compositions in growth media for microorganisms and play essential roles in primary and secondary metabolites production (Matos, 2017). The impacts of nitrogen sources restriction in oleaginous fungi regarding lipid and PUFAs accumulation and approaches for enhancing the over production of storage lipids have been widely stated (Yang *et al*., 2016; Dzurendova *et al*., 2020). The carbon/nitrogen (C/N) ratio monitors the switch between protein and lipid synthesis in fermentation media; thus, the C/N ratio is considered a critical nutritional variable that affects the gross lipids yield (Liu *et al*., 2014; Gao *et al*., 2021).

The mechanism for synthesizing fatty acids in fungi is essentially similar to that in animals and plants. After acetyl-CoA carboxylation, saturated or unsaturated fatty acids are produced through chain extension and desaturation. These fatty acids then form triacylglycerols (TAGs) (Arhar *et al*., 2021). Detection of lipogenesis potential in oleaginous fungi involves both qualitative and quantitative methods. Qualitative assessment utilizes lipophilic dyes such as Sudan Black and Nile red, known for their efficiency in staining intracellular lipid droplets. Nile red, a fluorescent dye, is particularly effective and has been used to stain fungal mycelium for subsequent examination under a fluorescence microscope (Al-Tohamy *et al*., 2021). On the other hand, quantitative methods are employed to measure lipid content more precisely. Gravimetric analysis is a popular technique involving the extraction of lipids from microbial cells with various cell lysis methods employed to ensure efficient extraction. The lipid content, expressed as a percentage of cell dry weight, is then calculated (Patel *et al*., 2019). Additionally, colorimetric methods like the sulfo-phospho-vanillin (SPV) technique are used for lipid quantification due to their rapid response and straight forward handling.

These methods often require preliminary steps such as cell disruption and lipid extraction but provide efficient measurements, making them suitable for assessing lipid production in oleaginous microorganisms (Hashem *et al*., 2022). This study aims to isolate and identify oleaginous fungal strains with a high capacity to produce lipids from various soil samples. Also, studying the effects of natural habitat on lipid productivity concerning carbon to nitrogen ratio was studied.

2. Materials and methods

2.1. Samples Collection

Fifty samples from different sources were collected from Assiut and Sohag governorates, Egypt, then transported aseptically to the laboratory (Table 1). The samples were brought to. All samples were collected from May 2021 to February 2022.

Table 1: Sources and sites of the collected samples.

Sample codes	Source of sample	Location
1 - 5	Soil from fuel station	Assiut city
6 - 10		Al Balyana, Sohag
11 - 15	Soil from auto lubricant centers	Assiut city
16 - 20		Al Balyana, Sohag
21 - 30	Soil from popular food restaurant	Al Balyana, Sohag
31 - 40	Soil from butchery centers	Al Balyana, Sohag
41 - 45	Agricultural soil	Campus of Al-Azhar University, Assiut
46 - 50		Al Balyana, Sohag

2.2. Determination of total organic carbon in soil samples

Total organic carbon was estimated using the method of Walkley & Black (1934). Soil samples were air-dried, gently crushed, and passed through a 2 mm sieve. One gram of each soil sample was weighed into an Erlenmeyer flask, followed by adding 10 mL of 1 N potassium dichromate solution. After that, 20 mL of sulfuric acid was added, and the mixture was gently rotated for one minute before being left to stand for 30

minutes. Next, the mixture was diluted to 200 mL with deionized water followed by addition of 10 mL of phosphoric acid, 0.2 gram of ammonium fluoride, and 10 drops of diphenylamine indicator. Titration was carried out with a 0.5 N ferrous ammonium sulfate solution until the color changed from blue to green. Finally, a blank was prepared and titrated in the same manner (Okalebo *et al*., 2002).

The percentage WB carbon is given by the formula:

$$WBC = M \times \left(\frac{V1 - V2}{W}\right) \times 0.30 \times CF$$

where M is the molarity of the $FeSO_4$ solution (from blank titration), V1 is the volume (mL) of $FeSO_4$ required in blank titration, V2 is the volume (mL) of $FeSO_4$ required in actual titration, W is the weight (g) of the oven-dried soil sample, and CF is the correction factor. The CF is compensation for the incomplete oxidation and is the inverse of the recovery. This CF was set by Walkley (1947) to 1.32 (recovery of 76%).

2.3. Determination of total nitrogen in soil samples by Kjeldahl method

The Kjeldahl method for nitrogen determination involves three main steps: digestion, distillation, and titration. Initially, samples were digested by boiling in concentrated sulfuric acid, which converts the nitrogen present into ammonium sulfate. This step included the addition of catalysts like copper or selenium to speed up the reaction. After digestion, the solution was made alkaline by adding sodium hydroxide, which converted the ammonium ions into ammonia gas. During the distillation phase, ammonia was distilled off and absorbed into a known volume of standard boric acid. The amount of ammonia was determined by back-titration of the excess acid with a standard solution of a base (NaOH). The nitrogen content is calculated from the amount of acid neutralized by ammonia (Jones, 1991).

2.4. Isolation and identification of fungi

2.4.1. Isolation media

Isolation of filamentous fungi from different soil samples involved serial decimal dilutions on two different types of agar media. Potato dextrose agar (PDA) which contained 20 g dextrose, 20 g agar, and potato infusion (200 g of sliced potatoes boiled in 1 liter of water), with a final pH of 6.5±0.2 (Mislivec & Bruce, 1976). Czpek's (Cz) agar which contained 30 g sucrose, 2 g sodium nitrate, 1 g potassium dihydrogen orthophosphate, 0.5 g potassium chloride, 0.5 g magnesium sulfate, 0.002 g ferrous sulfate, 20 g agar, and 1 L of distilled water, with a final pH of 6.5±0.2 (Abildgren *et al*., 1987). The media were supplemented with an antimicrobial agent (chloramphenicol) at a concentration of 0.2 g/L to inhibit bacterial growth (Ali *et al*., 2016). The air-dried soil sample (10 g) was shaken for 15 minutes with sterilized distilled water (90 milliliter) in a 250 mL Erlenmeyer flask. After the suspension was allowed to settle for 2 minutes, serial dilutions were prepared using sterilized distilled water. The 1/1000 dilution yielded an optimal number of fungal colonies, making it suitable for isolation of fungi. One mL of the selected dilution was transferred to each sterilized Petri dish, followed by the addition of 16 mL of sterilized mildly warm medium. Adequate dispersion was achieved by rotating the dishes before the solidification of agar (Waksman, 1922).

The dishes were incubated at 28±2 °C for 5 days. Following this period, colonies were counted, purified, and identified. The purified fungal isolates were transferred to slants of potato dextrose agar (PDA) medium and stored in a refrigerator for preservation. The average colony count for each medium was divided by the dilution factor to determine the fungal count per gram of the original sample, expressed as colony forming units per gram (CFU/g) for each soil sample. The total count (TC) of each taxon was calculated as the sum of its CFUs across the 10 samples and expressed as a percentage of the total count of all taxa. The number of cases of isolation (NCI) indicated the frequency of occurrence of each taxon, with occurrence remarks (OR) rated as high (H), moderate (M), low (L), or rare (R).

2.4.2. Identification of fungal genera and species

Identification of fungi isolated was carried out based on the morphological and microscopical features according to Raper & Fennell (1965); Barnett & Hunter (1972); Moubasher (1993) and Pitt & Hocking (2009).

1.5. Screening of lipid production and cultivation conditions

To induce lipid accumulation, lipid fermentation medium was used during the screening stage, with nitrogen limitation and carbon richness. 100 µL of each fungal isolate spore suspension (10^5 spores/mL) was inoculated into 100 mL of sterilized (K & R) Kendrick and Ratledge broth medium (Kendrick & Ratledge, 1992). Kendrick and Ratledge medium composed of g/L; 30 g glucose, 3.3 g ammonium tartrate, 7 g KH_2PO_4, 2 g Na_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g yeast extract, 0.1 g $CaCl_2 \cdot 2H_2O$, 8 mg $FeCl_3 \cdot 6H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $Co(NO_3)_2 \cdot 6H_2O$, and 0.1 mg $MnSO_4 \cdot 5H_2O$. All constituents were dissolved in 1 L of distilled water with a final pH adjusted to 6±0.2. The flasks were incubated for 7 days at 30 °C with

shaking at 120 rpm (Hussain *et al*., 2019).

1.6. Determination of mycelial dry weight and quantification of total lipids

The fungal biomass was harvested by filtration, then dried in an oven at 60 °C until reaching a constant weight. One gram of grounded biomass was mixed with 40 mL of chloroform:methanol (2:1) using an orbital shaker. The mixture was agitated for 20 min at 20 °C, filtered with Whatman filter paper no. 1 followed by addition of NaCl solution (0.9%). The lipid-containing lower layer was separated and allowed to be evaporated from the remaining solvents. The lipid content was determined using the sulfo-phospho-vanillin (SPV) method following the evaporation step (Suleiman *et al*., 2018$_b$). The sample was mixed with 2 mL of concentrated sulfuric acid (98%), heated for 10 min at 100 °C, cooled in an ice bath for 5 minutes, and then combined with 5 mL of freshly prepared SPV (dissolving 0.6 g of vanillin in 10 mL of absolute ethanol and 90 mL of deionized water, followed by continuous stirring, then 400 mL of concentrated phosphoric acid was added to the mixture, and the resulting reagent was stored in the dark until use). Subsequently, the sample was incubated for 15 min at 37 °C and 200 rpm shaking speed. Absorbance was measured at 530 nm using a spectrophotometer (Janeway 7315) to quantify the lipid content within the sample in comparison to a reference standard of canola oil (Figure 1) (Hashem *et al*., 2023).

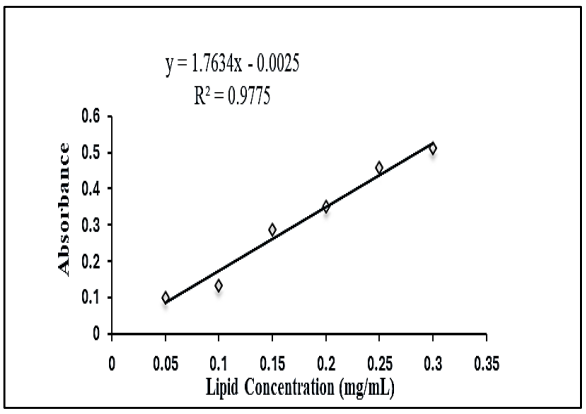

Figure 1: Standard curve of canola oil.

3. Results

3.1. Determination of carbon and nitrogen in soil samples

Table (2) demonstrated the range of total organic carbon (TOC) in soil samples, which ranged from 0.4% to 18.89%. The highest TOC level of 18.89% was found in sample 22 from popular food restaurants, followed by sample 15 at 18.09% from auto lubricant centers. The lowest TOC value of 0.4% was recorded in samples 33 and 45 from butchery centers and agricultural soil, respectively. Additionally, the total organic nitrogen (TON) in the samples was ranged from 0.73% to 0.04%. The highest TON level of 0.73% was observed in sample 30 from popular food restaurants, while the lowest value of 0.04% was recorded in sample 27, obtained from popular food restaurants.

Table 2: Determination of organic carbon and nitrogen in soil samples.

Source	No. of samples	TOC %	TON %
Soil samples from fuel station	1	1.95	0.14
	2	7.64	0.34
	3	13.27	0.45
	4	4.02	0.42
	5	12.46	0.39
	6	5.83	0.36
	7	6.23	0.28
	8	6.23	0.06
	9	5.03	0.31
	10	1.93	0.06
Soil samples from auto lubricant centers	11	9.65	0.53
	12	3.22	0.14
	13	10.45	0.31
	14	3.22	0.22
	15	18.09	0.31
	16	6.71	0.22
	17	3.78	0.64
	18	3.02	0.24
	19	6.83	0.25
	20	2.61	0.2
Soil samples from popular food restaurants	21	1.37	0.07
	22	18.89	0.25
	23	16.68	0.28
	24	6.83	0.53
	25	6.83	0.11
	26	0.6	0.14
	27	4.02	0.04
	28	4.22	0.5
	29	6.03	0.25
	30	4.02	0.73
Soil samples from butchery centers	31	5.03	0.11
	32	3.02	0.14
	33	0.4	0.06
	34	3.02	0.34
	35	3.82	0.34
	36	2.41	0.28
	37	1.81	0.08
	38	3.42	0.45
	39	0.6	0.07
	40	0.98	0.2
Soil samples from agricultural soil	41	1.56	0.08
	42	2.15	0.2
	43	1.56	0.17
	44	1.56	0.2
	45	0.4	0.06
	46	0.98	0.08
	47	1.37	0.06
	48	1.95	0.28
	49	1.37	0.34
	50	1.37	0.48

TOC: Total organic carbon and TON: Total organic nitrogen.

3.2. Total isolated filamentous fungi

3.2.1. Total filamentous fungi isolated on Cz agar medium

A total of 67.64 × 10^4 CFU were isolated from 50 samples investigated on Cz agar medium. They were identified as 29 fungal species and 2 varieties belonging to 20 genera. The most common genus was *Aspergillus*, with a high frequency of occurrence. In contrast, *Penicillium*, *Alternaria*, *Cochliobolus*, and *Giberella* were recorded with a low frequency of occurrence, and other genera were recorded rarely. *Aspergillus* was the most abundant genus accounting for 78.99% of total filamentous fungi. It was represented by 7 species; among them, *A. flavus* was the most abundant species contributing to 35.52% of total filamentous fungi and 45% of the genus *Aspergillus* (Figure 2a). After *Aspergillus*, *Giberella* ordered secondly making up 5.07% of all other filamentous fungi with a low frequency of occurrence and was represented by 1 species, *Giberella fujikuroi*. *Penicillium* occupied the third representation after *Aspergillus* and *Giberella* constituting 4.72% of total filamentous fungi. It was represented by 3 species and 1 variety; among them, *P. chrysogenum* was the most abundant species forming 1.92% of total filamentous fungi and 41% of the *Penicillium* genus (Figure 2b). *Cochliobolus* represented 3.52% of total filamentous fungi and was recorded by 3 species; among them, *C. australiensis*, the most plentiful species, constituting 1.54% of total filamentous fungi and 44% of the *Cochliobolus* genus (Figure 2c). Other genera were isolated with a rare frequency of occurrence.

3.2.2. Total filamentous fungi isolated on PDA medium

A total of 110.08 × 10^4 CFU were isolated from 50 samples surveyed on PDA medium. They were identified as 33 fungal species and 1 variety belonging to 19 genera. *Aspergillus* was the most distributed genera; their frequency of occurrence was high. In contrast, *penicillium*, *Cochliobolus*, *Giberella*, and *Rhizopus* were recorded with low frequency of occurrence, and other genera were recorded rarely. *Aspergillus* was the most abundant genus giving rise to 64.72% of total filamentous fungi. This genus was reproduced by 9 species; among them, *A. niger* was the most abundant species contributing to 30.04% of total filamentous fungi and 46% of this genus (Figure 3a). *Penicillium* occupied the second rank after *Aspergillus* constituting 8.42% of total filamentous fungi. This genus was represented by 3 species; among them, *P. corylophilum* was the most abundant species forming 4.15% of total filamentous fungi and 49% of this genus (Figure 3_b).

Cochliobolus came in the third rank after *Aspergillus* and *Penicillium* constituting 5.52% of total filamentous fungi. This genus was represented by 3 species; among them, *C. lunatus* was the most abundant species, forming 2.04% of total filamentous fungi and 37% of this genus (Figure 3c). *Giberella* and *Rhizopus* accounted for 6.07% and 3.74% of total filamentous fungi, respectively, and recorded with a low frequency of occurrence. *Rhizopus* was represented by 3 species; among them, *R. arrhizus* was the most abundant species forming 3.63% of total filamentous fungi and 97% of this genus (Figure 3d). Other genera were isolated with rare frequency of occurrence.

3.3. Screening of filamentous fungi for lipid production

Screening of lipid production was conducted for all filamentous fungal species isolated from five different soil sources on K & R medium under static and shaking conditions. Lipid content was estimated by colorimetric methods with sulfo-phospho-vanillin reagent. The lipid contents of fungal isolates obtained from fuel station under both shaking and static conditions declared that *Absidia corymbifera*, under shaking conditions, had the highest lipid content at 41.4%, while *A. ochraceus* and *A. terreus* had moderate lipid contents of 38.94% and 35.37%, respectively. Under static conditions, *G. fujikuroi* and *A. flavus* presented moderate lipid contents of 36.11% and 32.39%, respectively. These isolates were derived from samples no. 2 and 5, while other fungal isolates produced less than 30% lipid content under shaking and static conditions.

The fungal isolates obtained from auto-lubricant centers revealed that *A. terreus*, isolated from sample no. 17, recorded the highest lipid production of 46.91%. *Rhizopus oryzae*, *A. fumigatus*, and *Lichtheimia ramose*, isolated from samples no. 11, 15, and 16, exhibited moderate lipid content of 31.99%, 33.21%, and 31.41%, respectively. *Aspergillus flavus*, isolated from samples no. 16, 17, 18, and 20, produced moderate lipids content of 35.25%, 33.59%, 33.2%, and 30.74%, respectively. On the other hand, *Rhizopus oryzae*, isolated from sample no. 14, recorded moderate lipid content of 35.64% under static conditions. The other fungal isolates produced less than 30% lipid content under shaking and static conditions.

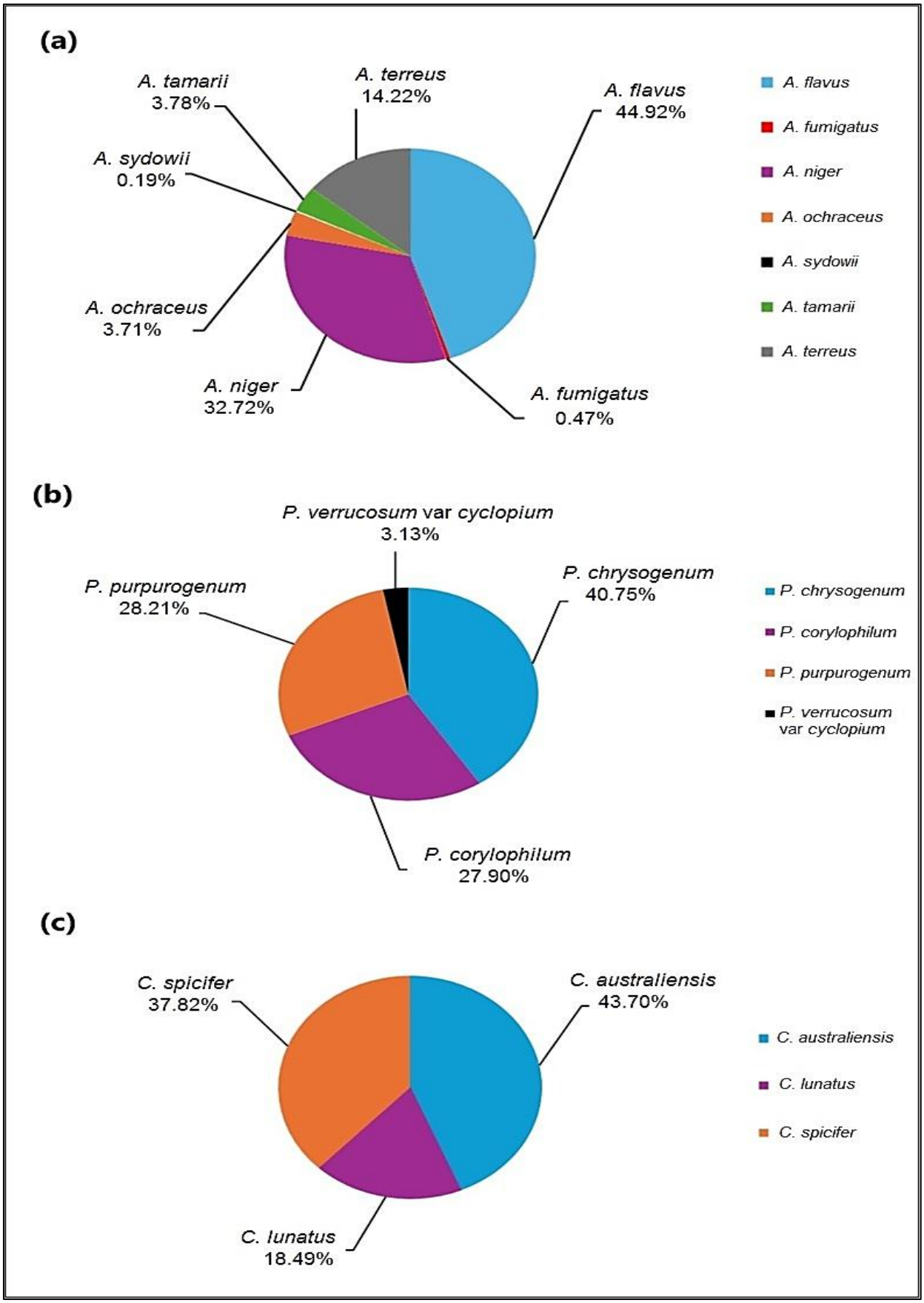

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Figure 2: Genera with a high frequency of occurrence **(a)** *Aspergillus* spp., **(b)** *Penicillium* spp., and **(c)** *Cochliobolus* spp. isolated on Cz agar medium.

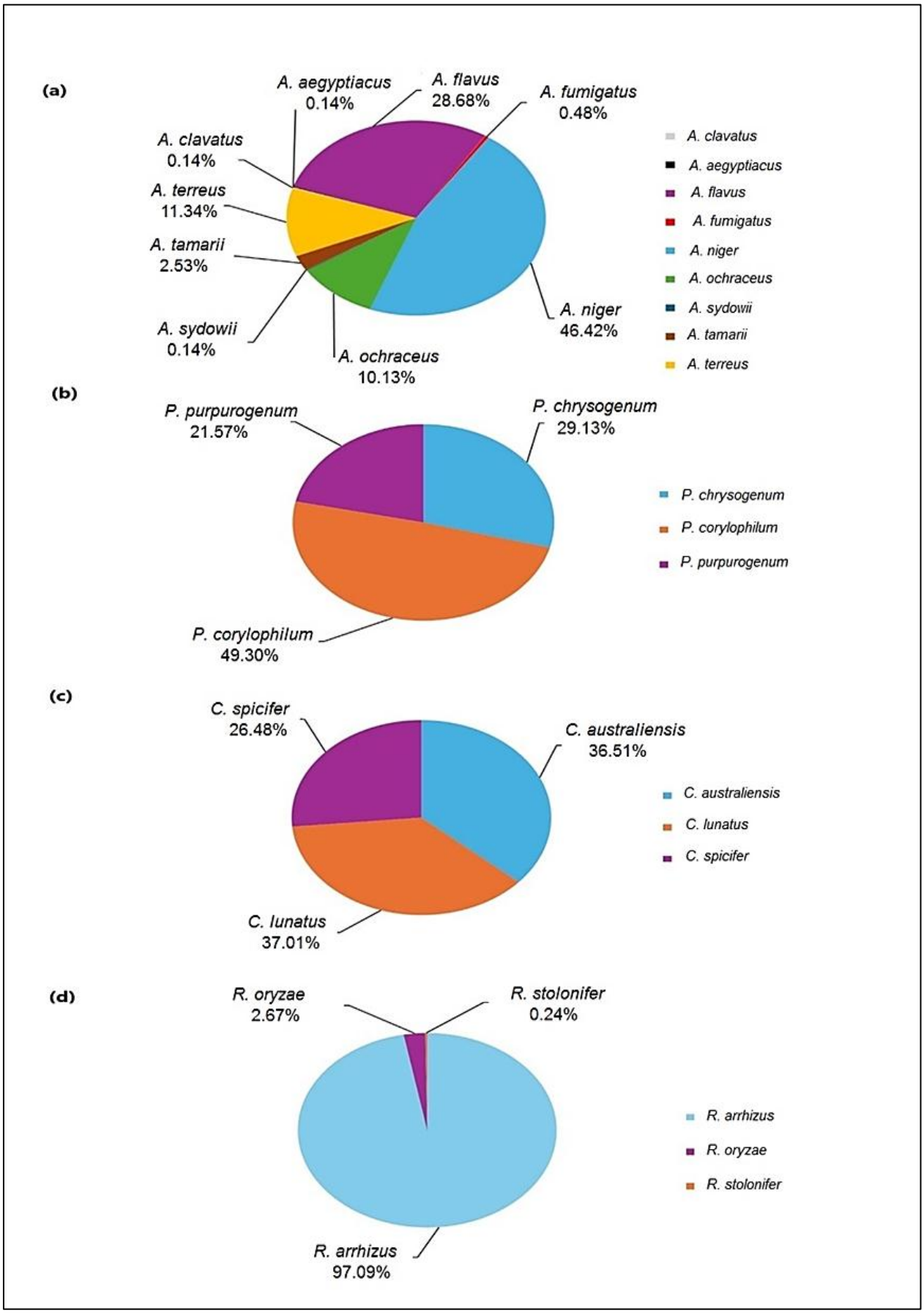

Figure 3: Genera with a high frequency of occurrence **(a)** *Aspergillus* spp., **(b)** *Penicillium* spp., **(c)** *Cochliobolus* spp., and **(d)** *Rhizopus* spp. isolated on PDA medium.

The fungal isolates obtained from popular food restaurants, *A. terreus* and *A. ochraceus*, under shaking conditions, recorded high lipid content of more than 40% of total cell biomass. While *A. fumigatus*, *F. solani*, and *S. racemosum* had moderate lipid contents of 31.27%, 31.32%, and 30.73%, respectively. *Aspergillus flavus* isolated from samples no. 21 and 22 with moderate lipid content, meanwhile other isolates produced lipid contents less than 30%. Under static conditions, all fungal isolates produced less than 30% lipid content.

The lipid constituent of fungal isolates derived from butchery centers explored that *A. flavus*, isolated from samples no. 31, 33, and 34, recorded moderate lipid content during shaking incubation with values of 36.91%, 37.39%, and 35.9%, respectively. In contrast, other fungal isolates showed low and rare lipid contents under shaking conditions. All fungal isolates produced less than 30% lipid content under static conditions.

The lipid production of fungal isolates from agricultural soil involving *C. echinulata* isolated from sample no. 42, that showed the highest lipid production in both shaking and static incubations, with a lipid content of 55.37% and 41.06%, respectively. While the other fungal isolates indicated low and rare lipid content under shaking incubation. On the other hand, under static conditions, *R. arrhizus*, isolated from samples 47, 48, 49, and 50, showed moderate lipid contents ranging from 32.85% to 35.82%. Meanwhile, other isolates exhibited less than 30% lipid content.

3.4. Selection of filamentous fungi for lipid production

Fungal isolates (108 isolates) were capable of accumulating lipids of more than 20% within their tissues under shaking and static conditions (Figure 4). A total of 62 isolates were obtained under shaking conditions, including 7 isolates with high lipid content belonging to two genera of fungi, 17 isolates with moderate lipid content belonging to three genera, and 38 isolates with low yields. On the other hand, 46 fungal isolates, under static incubation, comprising only one isolate with high lipid content, 7 isolates belonging to 3 genera with moderate lipid content, and 38 isolates with low lipid content (Table 3).

3.5. Relationship between carbon to nitrogen ratio (C/N ratio) in soil samples and lipids accumulation

The relationship between the C/N ratio in soil samples and lipid production can be determined by conducting multiple linear regression analysis. The regression model used in this study is formulated as y = 0.0957x + 26.708

Table 3: Lipid contents of the selected isolates.

Genera and species	OR (Shaking)			OR (Static)		
	H	M	L	H	M	L
Absidia corymbifera	1	-	2	-	-	2
Aspergillus aegyptiacus	-	-	1	-	-	-
A. clavatus	-	-	1	-	-	-
A. flavus	-	9	10	-	1	7
A. fumigatus	-	2	-	-	-	1
A. ochraceus	2	1	-	-	-	3
A. sydowii	-	-	1	-	-	1
A. tamarii	-	-	3	-	-	1
A. terreus	3	1	8	-	-	13
Cladosporium cladosporioides	-	-	-	-	-	1
C. oxysporum	-	-	1	-	-	-
Cochliobolus spicifer	-	-	-	-	-	1
Cunninghamella echinulata	1	-	-	1	-	-
Fusarium solani	-	1	-	-	-	-
Gibberella fujikuroi	-	-	2	-	1	2
Lichtheimia ramosa	-	1	-	-	-	1
Mucor racemosus	-	-	2	-	-	1
Penicillium chrysogenum	-	-	-	-	-	1
Rhizopus arrhizus	-	-	5	-	4	-
Rhizopus oryzae	-	1	-	-	1	1
Rhizopus stolonifer	-	-	1	-	-	1
Syncephalastrum racemosum	-	1	-	-	-	1
Ulocladium atrum	-	-	1	-	-	-
Total count = 108 isolates	7	17	38	1	7	38
	62			46		

OR: Occurrence remarks, H: High lipid content (40≤ H <56 %), M: Moderate lipid content (30≤ M <40 %), L: Low lipid content (20< L<30 %).

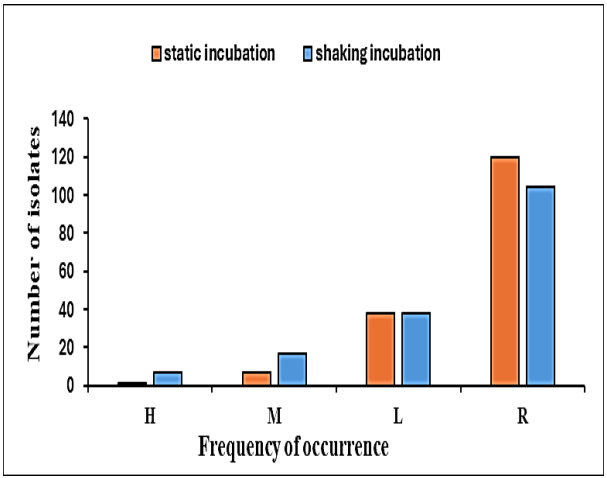

Figure 4: Frequency of occurrence for lipid accumulation under static and shaking condition.

The carbon-to-nitrogen ratio ranged from 2.9 to 103.8. The highest ratio of 103.8 was observed in sample

Table 4: Positive screening of lipid production with C/N ratio in soil samples.

Source	No. soil of sample	Isolates	Lipid content (%)	C/N ratio
Soil samples from fuel station	1	*Aspergillus flavus*	25.22±2.22	13.9
		Rhizopus stolonifer	24.36±1.47	
	2	*A. terreus*	21.95±0.64	22.5
		Cladosporium oxysporum	21.09±2.47	
		Ulocladium atrum	23.76±0.76	
	3	*A. sydowii*	24.39±0.39	29.5
		A. terreus	27.64±1.98	
	4	*A. ochraceus*	38.94±3.94	9.6
		A. terreus	35.37±1.65	
	5	*Absidia corymbifera*	23.59±1.59	31.9
	8	*Mucor racemosus*	22.46±0.46	103.8
	9	*Absidia corymbifera*	41.40±0.40	16.2
		A. flavus	21.23±0.23	
Soil samples from auto lubricant centers	11	*Rhizopus oryzae*	31.99±1.99	18.2
	13	*A. aegyptiacus*	21.93±0.28	33.7
		Gibberella fujikuroi	22.35±1.35	
	14	*A. terreus*	21.85±0.49	14.6
		Rhizopus oryzae	26.35±1.35	
	15	*A. fumigatus*	33.21±0.32	58.4
		A. terreus	27.95±0.09	
	16	*Lichtheimia ramosa*	30.41±1.41	30.5
		A. flavus	35.25±0.48	
	17	*A. flavus*	33.59±1.59	5.9
		A. terreus	46.91±1.09	
	18	*Absidia corymbifera*	24.01±1.01	12.6
		A. flavus	33.20±1.20	
		A. terreus	26.35±0.69	
	19	*A. clavatus*	29.48±1.48	27.3
		A. tamarii	27.25±0.75	
	20	*A. flavus*	30.74±0.90	13.1
Soil samples from popular food restaurants	21	*A. flavus*	32.94±0.59	19.6
		A. terreus	48.26±0.74	
	22	*A. flavus*	36.40±0.40	75.6
		A. tamarii	27.66±0.47	
		A. terreus	48.20±0.57	
		M. racemosus	24.09±0.31	
	23	*A. fumigatus*	31.27±0.27	59.6
	24	*A. tamarii*	21.92±0.08	12.9
	25	*A. ochraceus*	49.82±2.17	62.1
	26	*Fusarium solani*	31.32±1.02	4.3
	27	*A. ochraceus*	53.78±1.09	100.5
	30	*A. flavus*	20.25±0.21	5.5
		Syncephalastrum racemosum	30.73±0.73	
Soil samples from butchery centers	31	*A. flavus*	36.91±1.09	45.7
	32	*A. flavus*	20.11±0.49	21.6
	33	*A. flavus*	37.39±1.66	6.7
	34	*A. flavus*	35.90±0.10	8.9
	35	*A. flavus*	24.40±1.27	11.2
	36	*A. flavus*	20.58±0.42	8.6
	37	*A. flavus*	23.59±1.59	22.6
		A. terreus	24.17±1.51	
	38	*A. flavus*	22.93±1.93	7.6
	39	*A. flavus*	22.24±0.52	8.6
Soil samples from agricultural soil	42	*Cunninghamella echinulata*	55.37±0.37	10.8
	43	*A. terreus*	25.47±4.47	9.2
	45	*G. fujikuroi*	20.43±1.88	6.7
	47	*A. terreus*	21.97±0.53	22.8
		Rhizopus arrhizus	20.34±2.26	
	48	*A. flavus*	20.63±1.17	7
		R. arrhizus	22.64±1.64	
	49	*R. arrhizus*	24.01±1.01	4
	50	*R. arrhizus*	25.36±0.36	2.9

number 8 from the fuel station source, while the lowest ratio of 2.9 was found in sample number 50 from an agricultural soil source (Table 4). Lipid productivity ranged from 55.37% to 20.11%. The highest lipid content of 55.37% was recorded in *C. echinulata*, which was isolated from sample number 42 and had a low C/N ratio of 10.8. On the other hand, the lowest lipid content of 20.11% was obtained from *Aspergillus flavus*, isolated from sample number 32, with a C/N ratio of 21.6. The correlation factor between the C/N ratio in soil samples and lipid production is weakly positive (0.07). This is because the highest C/N ratio of 103.8 was recorded with a low lipid content of 22.46%, while the highest lipid content of 55.37% was obtained with a low C/N ratio of 10.8.

4. Discussion

Filamentous fungi and yeasts have been considered favorable oleaginous microorganisms. Several species from the Zygomycetes and Ascomycetes groups are important for their ability to accumulate significant amounts of lipids in their biomass (Mohamed *et al*., 2020). The main objective of this study was to isolate fungi from diesel-contaminated soil, samples polluted with fats and oils, and agricultural soil to produce lipids. The samples were cultured on Cz and PDA media and the total count, number of isolated cases, and remarks of the occurrence of fungal genera and species were determined. Twenty-nine species and two species varieties belonging to 20 genera were isolated and identified on Cz medium. Meanwhile, thirty-three species and one species variety belonging to 19 genera were isolated and identified on PDA medium. The most commonly occurring genus was *Aspergillus*, while *Penicillium*, *Alternaria*, *Cochliobolus*, and *Giberella* were recorded with low frequency. Other genera were rarely recorded.

Aspergillus accounted for 78.99 and 64.72% of total filamentous fungi on Cz and PDA media, respectively, and was represented by 7 species among them, *A. flavus* was the most abundant species. Gherbawy *et al.* (2016) isolated *Aspergillus flavus, A. fumigatus, A. niger, A. sydowii*, and *A. versicolor* from Brazilian fuels, while *A. flavus*, *A. fumigatus*, *A. niger*, *A. sydowii* and *A. terreus* were isolated from soil samples gathered by Chandra & Kehri (2006) from a petroleum activity sit in Indonesia. Meanwhile *A. flavus*, *A. niger*, and *P. chrysogenum* were obtained from petroleum-contaminated soil samples by Al-Dhabaan (2021) collected from Saudi Arabia. In addition, several *Penicillium* species, from oil contaminated soil samples from different localities around the world, were isolated, as well as other species isolated in low to moderate frequency namely; *Cladosporium*

cladosporioides, *C. echinulata*, *Scopulariopsis brumptii*, and *Rhizopus* sp. (Adeyemo *et al*., 2013).

The ability of fungal isolates to produce lipids was assessed under both static and shaking conditions to determine their oleaginous capacity. Among 108 identified isolates, 62 isolates accumulated more than 20% lipids in their biomass where 7 isolates were found to have high lipid content (over 40%), 17 isolates with moderate content (between 30% and 40%), and 38 isolates with low yields (between 20% and 30%) under shaking condition. In comparison, 46 isolates were examined under static incubation, where one isolate exhibited high lipid content, 7 isolates with moderate levels, and 38 isolates with low content. This indicated that shaking incubation was more effective than static incubation for lipid production.

The high lipid content, detected in the isolates, varied between 41.4% and 55.37%. *Cunninghamella echinulata*, isolated from agricultural soil, exhibited the highest lipid content (55.37%). Silva *et al*. (2014) isolated *C. echinulata* from soil with lipid productiviy of 40%w/w, while Firoozabad & Nasr (2022) reported lipid yield of 34.2% by *C. echinulata*. *Cunninghamella echinulata* was followed by *A. ochraceus* (2 isolates), isolated from popular food restaurants soil samples, with lipid contents of 53.78% and 49.82%. Jathanna *et al.* (2020) reported lipid content of *A. ochraceus* as 28.93% using the acid hydrolysate of seed cake. *Aspergillus terreus*, isolated from auto lubricant centers and popular food restaurants soil samples, recorded lipid contents ranging from 46.91 to 48.26%,. Al-Zaban & Abd El-Aziz (2024) observed that *A. terreus* KC462061 produced lipids over 40% of its dry biomass.

Absidia corymbifera, isolated from fuel station soil sample, had a lipid content of 41.4%. this result was in agreement with Hashem *et al*. (2020a) who detected a lipid content of 39.56% in *L. corymbifera*. Productions of fungal SCOs have been obviously targeted to Zygomycetes especially of the genera *Mucor*, *Cunninghamella*,, *Mortierella*, *Rhizopus* and *Zygorhynchus* (Gardeli *et al*., 2017). Batrakov *et al*. (2004) used *Absidia corymbifera* which very close to *Lichtheimia corymbifera* in biodiesel production. On the other hand, moderate lipid contents ranged from 30.4% to 38.94% were obtained from *Lichtheimia ramose* and *A. ochraceus*. The cultivation of *Lichtheimia hyalospora* UCP 1266 in glucose medium produced 28.57% lipid content (de Souza *et al*., 2022).

Our results revealed that *A. flavus* (9 isolates) contained lipids contents ranged from 30.74% to 37.39%. Similarly, *Aspergillus flavus* and *A. niger* were estimated to accumulate 25.21% and 24.34% (w/w) of their dried biomass as lipids, respectively (Merheb *et al*., 2022). *Aspergillus terreus*, *R. oryzae*, *F. solani*, and *S. racemosum* accumulated lipids at 35.37%, 31.99%, 31.32%, and 30.73%, respectively. Hashem *et al*. (2020_b) showed lipid content of 27.64% by *S. racemosum* at pH 5. Hashem *et al*. (2022) assessed lipid contents of 25.79% and 20.78% in *L. corymbifera* and *R. oryzae*, respectively. *Aspergillus fumigatus*, two isolates, offered lipid contents of 33.21 % and 31.27%. Kadhim & AL-Rubayae (2023) reported that *A. fumigatus* produced 26% lipids in a nitrogen-limited medium, while *A. terreus* yielded 24.9% lipids. However, Khot et al. (2012) revealed that some *Aspergillus* species can accumulate up to 51% (w/w) of their cell mass as lipids.

When examining the relationship between the carbon-to-nitrogen (C/N) ratio in soil samples and lipid production using multiple linear regression, the model suggests that lipid production is influenced by the C/N ratio. The C/N ratio in the tested samples ranged from 2.9 to 103.8, with the highest ratio of 103.8 observed in sample no. 8 obtained from a fuel station source, and the lowest ratio of 2.9 in sample no. 50 derived from agricultural soil. Lipid productivity of the fungal isolates varied between 55.37% and 20.11% where the highest lipid content of 55.37% was recorded in *C. echinulata*, isolated from sample no. 42 with a C/N ratio of 10.8. Conversely, the lowest lipid content of 20.11% was found in *A. flavus*, isolated from sample no. 32, which had a C/N ratio of 21.6.

The correlation factor between the C/N ratio and lipid production (0.07) was weakly positive. This weak correlation is evident as the highest C/N ratio of 103.8 corresponded with a low lipid content of 22.46%, while the highest lipid content of 55.37% was associated with a lower C/N ratio of 10.8. These results indicated that lipid productivity is not solely affected by carbon and nitrogen in the natural soil. Other factors, such as soil pH, moisture content, and microbial community composition, may provide a more comprehensive understanding of the factors influencing lipid production. The carbon-to-nitrogen (C/N) ratio in soil represents a significant factor for determining the composition of soil microbial community where elevated soil C/N ratios are proportionally associated with the plentifully of fungal biomarkers and conversely correlated to bacterial lipid biomarkers (Wan *et al*., 2015). Oleaginous microorganisms have the ability to accumulate lipids upon exposure to elevated ratios of C/N such as *Rhodotorula toruloides*, which offered high lipid content at increased C/N ratios up to 120 (Lopes *et al*., 2020). Generally, C/N ratio higher than 20% represent ideal demand for lipid accumulation in fungi (Hashem

et al., 2020b).

5. Conclusion

Soil represents a valuable pool encompasses a variety of microbial species with diverse metabolic routes. During the screening of the fungal composition of soil samples from different localities, seven isolates were proved to accumulate lipids at high levels under shaking incubation. These isolates can be further enhanced to produce higher amounts of lipids via optimizing physical and chemical conditions. The produced lipids can be involved in diverse applications, including biodiesel, nutraceuticals, and pharmaceuticals.

Conflict of interests

The *authors declare that there are no conflicts of interest.*

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Ethical approval

None applicable.

Authors' Contributions

Hany A. A. Abdou: Methodology, Investigation, and Writing original draft; **Abdallah M. A. Hassane**: Conceptualization, Supervision, Writing, Review & Editing original draft, and Formal analysis; **Mohamed A. Abdel-Sater and Abdel-Rehim A. El-Shanawany**: Supervision, and reviewing final draft.

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