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Rhizopus oryzae Mekky1907: A promising isolated fungus for the production of medically important polyunsaturated fatty acids from agricultural waste

Ibrahim A. Abozaid[1], Hesham M. Mahdy[1], Samia A. Haroun[2], Alsayed E. Mekky[1,*]

[1]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Egypt.

[2]Botany Department, Faculty of Science, Mansoura University, Egypt

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Corresponding author:

Alsayed E. Mekky, Ph. D

E-mail: alsayedessam@azhar.edu.eg

Mobile: (+2)01222806275

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ABSTRACT

Zygomycetes, particularly oleaginous fungi, are very capable of producing enormous amounts of lipids that are rich in poly-unsaturated fatty acids (PUFAs), which are crucial for medicine. *Rhizopus oryzae* Mekky1907 was the most abundant of the twelve oleaginous fungal isolates from Horse dung as environmental samples and examined for lipid accumulation. By using morphological analysis and molecular confirmation using 18S rRNA sequencing, the isolate's species identity was established and recorded on gene bank under accession number PQ182608. In order to enhance lipid accumulation, the culture conditions were improved, *R. oryzae* Mekky1907 showed the highest lipid production in the presence of 100g/L sucrose and 10 g/L yeast extract at pH, 5 after five days of incubation at 30°C under static conditions. Solid state fermentation medium was made: A 5-liter flask was filled with 40g of dry bagasse and rice straw as inexpensive carbon sources, mixture evenly moistened at a 60% level using sterile distilled water and peptone (5 g/L), autoclaved after cooling, spores added with known concentration, pH 5, temp. 15°C – 20°C and incubated for 5 days *R. oryzae* Mekky1907 showed lipid production (dray biomass 4.23 g/L giving lipid content 2.92 g/L Equivalently 69.03 %). The experiments demonstrated the effectiveness of PUFAs, an anti-cancer, antioxidant, and anti-inflammatory agents. *R. oryzae* Mekky1907 is therefore thought to be a promising oleaginous filamentous fungus that can be used in factories to produce PUFAs from agricultural waste for use in medicine.

Key words: *R. oryzae*, poly-unsaturated fatty acids, agricultural waste

1. Introduction

Rhizopus oryzae can synthesize many products, including poly-unsaturated fatty acids, volatile compounds and organic acids, and enzymes (such as cellulases, tannases, xylanases, proteases, pyruvate decarboxylases, lipases), it is widely used in industry (Sebastian et al., 2019). There are many reasons why microbial oils are preferable to vegetable oils. Unlike vegetable oils, which are subject to seasonal fluctuations, microbial oils are not subject to seasonal fluctuations. They also have shorter production times and require less space and labor. Most importantly, they are not a substitute for the food industry. In addition, microbial oils produced from many sources of carbon, which enhances the versatility and stability of a bio refinery. Microbial oils are a sustainable and renewable alternative to vegetable oils that can be used as biofuel and biochemical feedstock (Caporusso et al., 2021). Fungi are known for their rapid growth, brief life cycles, low light energy needs, scalability, and capacity to use a wide range of carbon sources, including wastewater, lignocarbon biomass, and agri-industrial wastes

(Chen et al., 2023). In certain situations, oleaginous microbes can be used for more than just producing lipids that can be used in multifunctional fields; they can also be used for decolorization, biorefinery, bioremediation and medically important agents (Ali et al., 2021). The production of lipids from fungi appears to have been primarily focused on the genera *Zygomyctes* including *Cunningghamella*, *Cucor*, *Mucor*, *Mortierella* and *Rhizopus* (Tauk-Tornisielo et al., 2009). Poly-unsaturated fatty acids (PUFAs) long-chain have 18+ carbon atoms and many carbon double bonds. The placement of the first double bond, as determined from the methyl end, is used to categorize PUFAs. For instance, based on its methyl end, an omega-3 PUFA has its initial double bond at position 3. A common usage of the symbol n is as a synonym for the symbol ω.

For instance, Δ4(7, 10,13, 16, 19)-22:6 or n-22(6ω3) can be used to represent DHA, the acronym for all-Cis-4,7, 10, 13, 16, 19-docosahemoic acid, which means that the fatty acid has 22 carbon atoms with 6 double bonds (Bharathiraja et al., 2017). On the third carbon atom (methyl end group) of the third carbon is the first double bond. Although not all of them are still in use, the following ambiguities are related to the omega-3 sequence: linolenic [18:3], stearidony [18:5], moroctic [18:4], timnodony [20:5], clupanodic [22:5], and cervonic [22:6] (Bharathiraja et al., 2017). Plant seed oils are the main commercial source of 18 Carbone atom polyunsaturated fatty acids. Because the cellular system lacks the required enzymes, plants cannot synthesis PUFAs greater than C18 (Rizzo et al., 2023). PUFAs play a significant part in brain, nerve, and eye function, neurodegenerative and psychiatric diseases, cardiovascular disease prevention infection, and cancer prevention (Djuricic and Calder, 2021).

All membranes and cell components, including those of the neurological system, brain, eyes, heart, adrenal system, and thyroid, are needed for lipids to function properly. Blood pressure management, arterial wall maintenance, and the synthesis of eicosanoids, a family of hormone-like substances that regulate numerous organ systems are all facilitated by lipids (Ali and Szabó, 2023). As the most energy-dense macronutrient, fats are essential for both human and animal energy requirements as well as the soluble in fat vitamins D, A, and E absorption. The health benefits of good fat, which have been connected to different kinds of dietary fats, have become more widely known in recent years. (Savarino et al., 2021). Lipids are crucial structural components of cell membranes in physiology, are utilized in medications, nutraceuticals, and food supplements, and are vital to the metabolism of energy in many organisms (Pandey, 2023). Controlling the nutritional composition of the medium and manipulating environmental conditions are popular strategies to enhance biosynthesis of lipids in lipid fermentation, based on the features of fungal lipid fermentation production. Currently, there are three common regulating approaches used for lipid fermentation. The primary variables affecting the formation of fatty acids are the incubation temperature, pH, nitrogen-to-carbon (N/C) ratio, nitrogen and carbon sources, and dissolved oxygen (Yu et al., 2019). However, other elements, such as minerals (such as phosphorus, sulfur and zinc) and vitamins (such as biotin and thiamine), are also vital to microbial activity (Dzurendova et al., 2020). Additionally, secondary metabolites such as citrate have an impact on the synthesis of lipids (Carsanba et al., 2018).

The most common conventional techniques for breaking down walls are grinding, acid treatment followed by cell autolysis followed by freezing and thawing repeatedly, ultrasonication, and enzyme treatment (Kot et al., 2020). The enzymatic treatment technique has moderate conditions and does not damage intracellular components, but it is costly and cannot be used for large-scale treatment. The autolysis method, on the other hand, has simple procedures and is low cost, but it has poor crushing result and low lipid yield. Ultrasonication is among the more often employed techniques. Researchers have found an environmentally friendly method for boosting the synthesis of designer lipids with a variety of nutritional benefits by using ultrasound to enhance the production of designer lipids (Jadhav et al., 2021). Lipid extraction is mostly carried out using organic solvents with low boiling points. Currently, the acid heat method,

Soxhlet extraction method, and supercritical CO2 extraction method are the most widely used techniques for extracting microbial lipids (Ong and Chen, 2022). Lipid can be extracted from fungus with a high extraction rate of up to 70% (w/w) content by employing a combination of microwave, acid-catalyzed predicament, and quick ultrasonic-microwave treatment. This is a revolutionary green extraction technology. (Martínez et al., 2020). The main objective of the current search was to isolate and idenificat a promising oleaginous fungus isolate *Rhizopus* spp. with a far greater capacity to generate large amounts of poly-unsaturated fatty acids, with optimization various process variables for poly-unsaturated fatty acids accumulation.

2. Materials and methods

Chemicals

The majority of the chemicals and solvents used were bought from Sigma-Aldrich, Germany.

Isolation and purification of most potent oleaginous *Zygomycetes*.

Horse dung samples were taken from various kinds of locations in Ezpet Mekky Belbies center Al-Sharqia Government, Egypt. One gram of each sample was suspended in one ml of sterilized distilled water, serially diluted 10- to 2-fold, then plated onto Oxoid (MEA) Malt Extract Agar. Plates were incubated under controlled conditions for three days at 30 °C. Single fungal colonies were isolated then transferred to new His MEA plates repeatedly until obtain pure cultures. Pure cultures then grown on slants of MEA then stored at 4°C until use (Al-dhubiea et al., 2023).

Lipid production and cultivation conditions.

The composition of the production medium (g/l): Glucose 100, yeast extract 10, adjusted to pH 5.4. A 10% mycelial suspension of the isolated culture was inoculated into a 100 ml flask containing 50 ml of broth and incubated at 30°C for 7 days (Ahmed et al., 2006).

Determination of dry weight, lipid extraction, and lipid quantification

After harvesting the cells by filtration and drying them at 55–60 °C for an overnight period or until their weight remained constant, the amount of biomass produced was calculated. To extract the lipid, 40 mL of a 2:1 chloroform-methanol combination was added to 1 g of dry biomass that had been ground. The mixture was then stirred for 20 minutes at 20 °C and filtered through Whatman paper no. 1. Lipids were identified when the solvent containing them was separated and subsequently evaporated. The extracted lipid was quantified using sulfo-phospho vanillin method (SPV) (Suleiman et al., 2018). Vanillin (6%) and phosphoric acid (85%) were combined to create phosphovanillin reagent. The specimen was created by diluting 20 µL of samples with 180 µL of sulfuric acid, incubating at 100 °C for 10 minutes, and then allowing it to cool at room temperature. Next, phosphovanillin reagent was added and the sample was left for a while to develop color before being measured at 530 nm (Hashem et al., 2022).

Methyl ester preparation and fatty acid analysis

Fatty acids in the extracted lipid were methylated to create fatty acid methyl ester (FAMEs) (Miao, 2006). Cooling caused the formation of two layers. Chloroform was used to remove the bottom layer containing fatty acid methyl esters (FAMEs), and the solution's chloroform was then evaporated to yield the final FAMEs product. Gas chromatography/mass spectrometry (GC/MS) was used to examine FAMEs (Suleiman, 2020).

Identification of most USFA producer fungal isolates.

In order to confirm the morphological characteristics of the fungal isolates, morphological identification of the chosen isolate was carried out based on the culture characteristics (color, texture appearance, and diameter of the colonies) as well as microscopic investigations using both the light and scanning electron microscope (Mekky et al., 2021). Finally, the 18S-rRNA has been used in the molecular technique to corroborate the fungal diagnosis. The PCR results were also sequenced using the Applied Biosystems Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit. Using the NCBI BLAST search software in the National Center for Biotechnology Information, the resultant sequence was aligned with similar sequences. With MEGA-x,

evolutionary analyses were carried out according to Mega (2018).

Maximization lipid production by optimizing culture conditions

The impact of environmental and nutritional factors on fungal growth and lipid production was studied under static settings in an effort to improve *Rhizopus* spp. lipid production.

Effect of carbon sources and nitrogen sources on production of lipid

We measured the impact of various carbon sources on lipid synthesis by adding 50g/L of Eight carbon sources (fructose, lactose, glucose, sucrose and maltose) to the basal medium and were analyzed in an equimolar amount. In terms of the nitrogen supply, the six nitrogen sources: yeast extract, ammonium nitrate, sodium nitrate, peptone, ammonium acetate, and casein were used at 0.5g/L (Youssef et al., 2021).

Effect of pH and incubation period on lipid production

Before autoclaving the culture media to values between 2 and 8 under static circumstances for seven days, the original pH of the medium was adjusted using 1 N HCl or 1 N NaOH. Several incubation times (between 1 and 7 days) were investigated for their impact on lipid synthesis (Youssef et al., 2021).

Effect of incubation temperature on lipid production

In this experiment, which was conducted under static conditions, the impact of various incubation temperatures (20°, 25°, 30°, 35°, and 40°C) on lipid synthesis was assessed (Youssef et al., 2021).

Solid- State Fermentation

In this work, we employed sugarcane bagasse and rice straw as inexpensive carbon sources to promote the growth of oleaginous fungi. We gathered the sun-dried sugarcane bagasse and rice straw from the local market in our region, which is located in the Ezpet Mekky, Belbies center, Al-Sharqia Government, Egypt.

Bagasse pretreatment

The bagasse and Rice Straw were autoclaved after treating by distilled water and at 121º C for 15 minutes (Aguiar et al., 2010).

Media preparation

This is how the medium of solid-state fermentation made: A 5-liter flask was filled with 40g of dry bagasse and rice straw, mixture evenly moistened at a 60% level using sterile distilled water and peptone (5 g/L) and it was autoclaved for 15 minutes at 121ºC. and let to cool (Yafetto, 2022).

Inoculum preparation

In this study, the inoculum for solid-state fermentation was made by fungus was growing on the MEA slants for 7 days at the optimal temperature. sterile Tween-80 was added after that, 2-3 ml of to the fully sporulated fungal slant, using sterile needle with gently scraping spores removed from the sporangiophores. Prior to inoculation, spore count was carried out using a hemocytometer (Yafetto, 2022).

Gas chromatography/mass spectrometry examination

Using an Agilent Technologies 6890N (Net Work GC system) in the USFAS, gas chromatographic analysis of fatty acids methyl esters (FAMEs) for the high producer isolate *Rhizopus oryzae* Mekky1907 was carried out in Central Laboratories of the National Research Centre.

Biological activity

Cytotoxicity assay for PUSFAs to evaluate toxicity by using tissue culture using MTT protocol was described according to Riss and Moravec (2004). Antitumor activity of USFAs against human colon tumor cells (Caco-2) was assessed by Kamiloglu et al. (2020). The antioxidant capacity of PUFAs was assessed using the 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging method (Baliyan et al., 2022). Human red blood cell membrane stabilization (HRBC method) has been used as a method in estimating the anti-inflammatory property (Aidoo et al., 2021).

Statistical analysis

Mini-Tab software (version 19) was used for statistical analyses, and all experiments were run in triplicate. Standard deviation, or mean ± SD, is used to express values. Unless otherwise indicated, a significance level of $p< 0.05$ was taken into account.

3. Results

Isolation and screening of fungal isolates

Using dilution agar plating, a total of twelve *Rhizopus* spp isolates were isolated from the dung sample. Using lipid extraction, lipid measurement, and dry weight determination, the twelve *Rhizopus* spp isolates were evaluated for accumulation of lipid. mekky1907 was the most potent *Rhizopus* spp. for lipid production. *Rhizopus* spp. mekky1907 was the highest for total lipid production and lipid percentage among other *Rhizopus* spp., where this isolate produced (dray biomass 5.69 g/L giving lipid content 3.95 g/l Equivalently 69.44 %) in the presence of 100g/L sucrose and 10 g/L yeast extract at pH, 5 after five days of incubation at 30°C under static conditions (Fig.1).

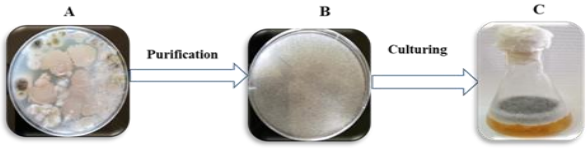

Fig. 1. Different fungal and bacterial isolated from Hors dung samples (A), *Rhizopus* spp. after purification Plates were incubated at 30 °C for 3 days (B) and culturing isolate on broth media incubated at 30 °C for 5 days (C).

Identification of the most potent fungi

The microscopic and macroscopic characteristics of mekky1907 *Rhizopus* spp. isolate, when it was demonstrated that the 3-day-old culture had moderate growth (Fig. 2).

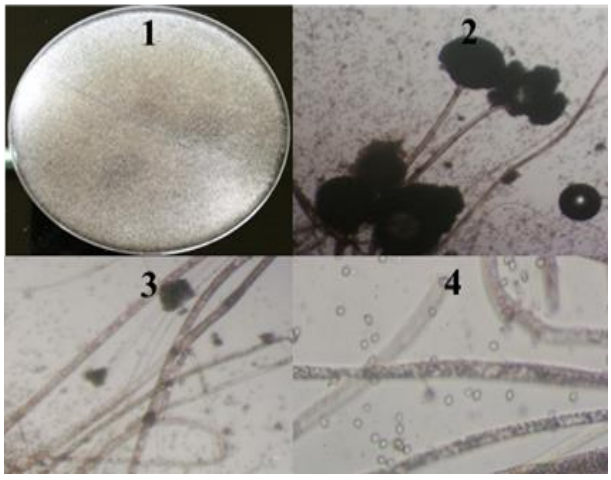

Fig. 2. *Rhizopus* sp. Mekky1907, growth on surface of MEA (1). sporangiophore and Sporangium under light microscope (2). Rhizoids under light microscope (3). Spores and hyphae under light microscope (4).

The top hit displayed 98% exact identity with at the molecular level of *R. oryzae* strains. Fungal isolate *R. oryzae* Mekky1907 was identified as *R. oryzae* and recorded in Gene-Bank under accession number PQ182608.

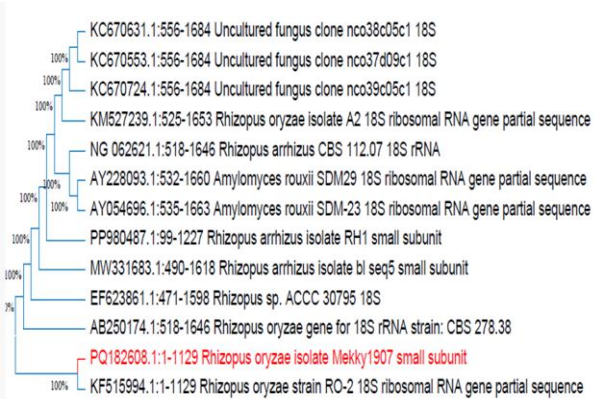

Fig. 3. Phylogenetic tree of gene sequences of the *R. oryzae* Mekky1907 isolate with the sequences retrieved from NCB Gene Bank site, with accession number PQ182608.

Different carbon sources like starch, lactose, maltose, glucose, sucrose, bagasse and cellulose, all sources were ordered gradually from high to low lipid production, glucose is the highest for lipid production and carried symbol (a) was 1.47 ± 0.08 g/L. However, a low carbon source of starch showed the lowest production of lipids was 0.31 ± 0.03 g/L. Dry biomass and lipid content were 2.95 ± 0.04 g/L and 49.87 ± 1.91 % respectively. Starch levels were 1.51 ± 0.06 g/L and 20.29 ± 0.89 % respectively (Fig. 4 A). Yeast extract gives the maximum values 3.95 ± 0.04 g/L. However, a minimum nitrogen source of asparagine showed the lowest production of lipids was 0.52 ± 0.04 g/L. Dry biomass and lipid content for yeast extract were 5.69 ± 0.03 g/L and 69.44 ± 0.4 %, respectively, but asparagine were 2.74 ± 0.06 g/L and 23.13 ± 1.85 %, respectively **(**Fig. 4 B**)**. The current results suggested that optimum lipid production by *R. oryzae* Mekky1907 was observed with glucose as a carbon source and yeast extract as a nitrogen source.

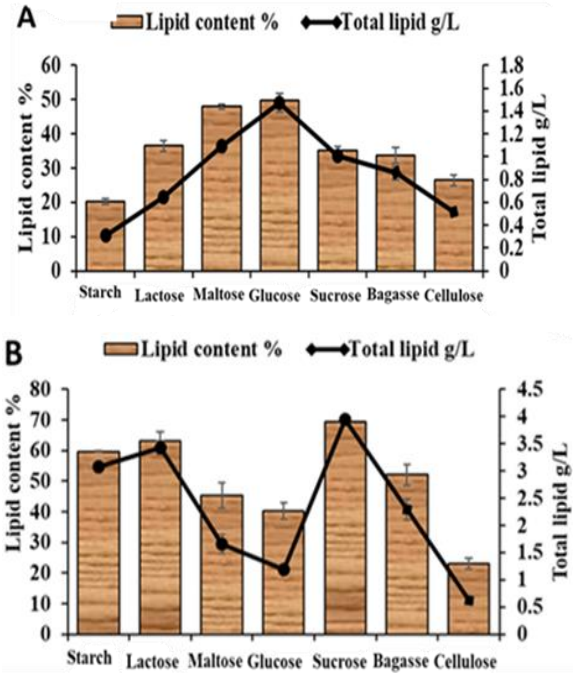

Fig. 4. Effect of different carbon sources (A) and effect of different nitrogen sources on lipid production by *R. oryzae* Mekky1907.

R. oryzae Mekky1907 was incubated at various temperature ranges from 5 º to 50 ºC. The results proved that the better temperature to produce lipids by *R. oryzae* Mekky1907 was 2.59 ± 0.06 g/L at 30°C. However, a high temperature of 50ºC showed the lowest production of lipids was 0.65 ± 0.02 g/L. Also, dry biomass and lipid content at 30°C were 5.25 ± 0.06 g/L and 49.20 ± 0.53 % respectively, but at 50ºC were 3.04 ± 0.59 g/L and 21.93 ± 5.25 %, respectively (Fig. 5 A).

Thus, from the present study, it was found that there is an increase in the production of lipids at 30 ºC and a further decrease in the production of lipids by *R. oryzae* Mekky1907 was observed in higher temperatures. Seven levels of pH (2 and 8) were used in production of lipids by *R. oryzae* Mekky1907 at pH 5 high lipid produced was 2.15 ± 0.06 g/L and low at pH 8 was 0.46 ± 0.02 g/L. Also, dry biomass and lipid content at pH 5 were 4.87 ± 0.02 g/L and 44.05 ± 0.99 %, respectively, but at pH 8 were 2.09 ± 0.02 g/L and 21.78 ± 0.55 %, respectively. Therefore, the center point of pH 5 is better than others as shown in (Fig. 5 B). Different incubation periods were selected for lipid production from *R. oryzae* Mekky1907. For main optimization, 2, 3, 4, 5, 6, 7, and 8 days were used to detect the center point of incubation period. The optimum incubation period of *R. oryzae* Mekky1907 isolated for the production of lipids was found at 5 days with the higher maximum incubation periods and incubation conditions for production of lipids by *R. oryzae* Mekky1907 under static condition was 1.58 ± 0.03 g/L. However, a low incubation period of 1 day showed the lowest production of lipids was 0.42 ± 0.04 g/L. Also, dry biomass and lipid content at 5 days were 3.89 ± 0.04 g/L and 40.63 ± 0.51 %, respectively, but at 1 day were 1.82 ± 0.04 g/L and 23.09 ± 1.53 %, respectively (Fig. 5 C).

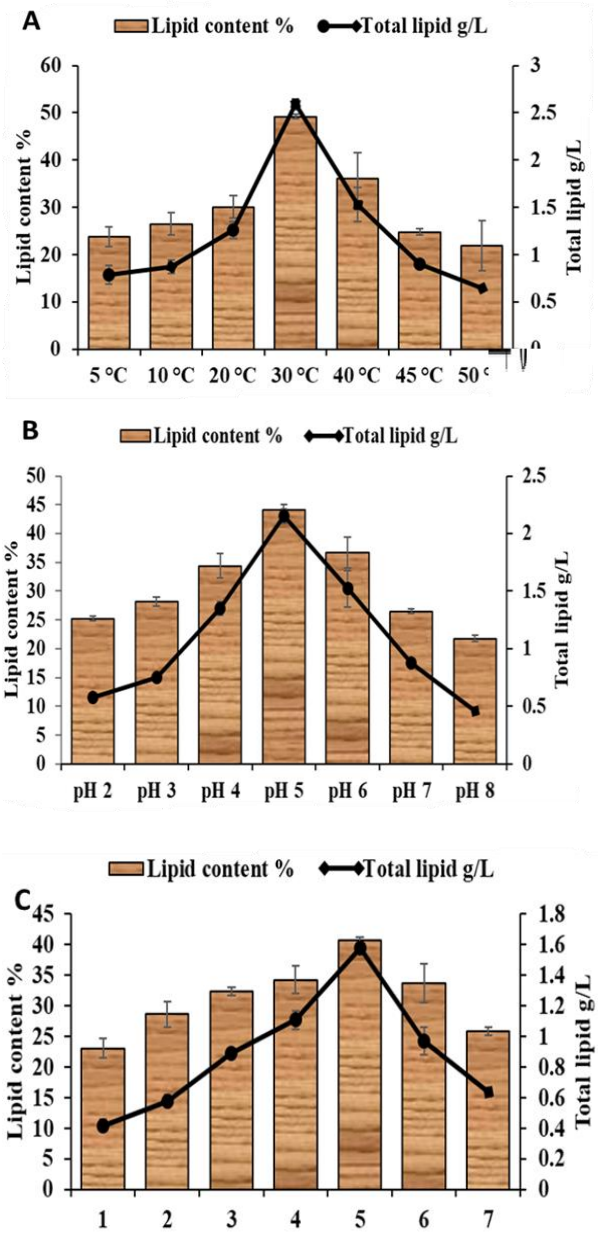

Fig. 5. Effect of different temperature (A), different pH levels (B) and effect of different incubation periods (days) (C) on lipid production by *R. oryzae* Mekky1907.

Solid state fermentation medium

A 5-liter flask was filled with 40g of dry bagasse and rice straw as inexpensive carbon sources, mixture evenly moistened at a 60% level using sterile distilled water and peptone (5 g/1), autoclaved after cooling, spores added with known concentration, pH 5, temp. 15°C – 20°C and incubated for 5 days *R. oryzae* Mekky1907 showed lipid production (dray biomass 4.23 g/l giving lipid content 2.92 g/l Equivalently 69.03 %) (Fig. 6). The process of biodegradation of agricultural waste using *R. oryzae* Mekky1907 fungi (Fig. 9). Sugar cane bagasse residue (1), sugar cane bagasse residue after cutting (3), rice straw (4), rice straw after cutting (5), adding the mixture into a 5-liter flask, fungal growth on agricultural waste (6). A close-up picture of fungal growth on agricultural waste (7).

Fig. 6. The process of biodegradation of agricultural waste using *R. oryzae* Mekky1907 fungi.

Identification of polyunsaturated fatty acids of *R. oryzae* Mekky 1907 which production using rice straw and sugar cane bagasse residue at 15°C – 20°C temperature by gas chromatography mass spectroscopy

The components present in *R. oryzae* Mekky 1907 which are produced using rice straw and sugar cane bagasse residue at 15°C – 20°C temperature was identified by GC-MS analysis. The active principles with their retention time (RT), fatty acid name, IUPAC name, compound structure, and concentration (%) in *R. oryzae* Mekky 1907. Seven compounds were detected in *R. oryzae* Mekky 1907 is produced using rice straw and sugar cane bagasse residue at 15°C – 20°C temperature. The results revealed that, oleic acid (14.21%) was found as major component followed by stearidonic acid (12.17%), nervonic acid (10.05%), docosadienoic acid (7.44%), eleostearic acid (1.66%), eicosatrienoic acid (1.08%), and gamma-Linolenic acid (0.19%) as indicated in Table.1 and Fig. 7.

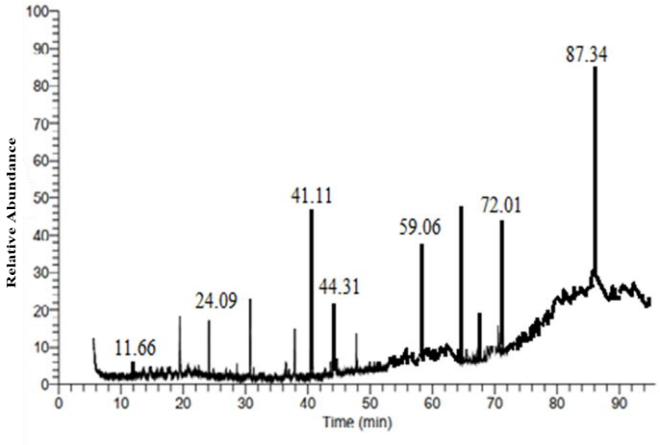

Fig.7. GC-MS spectral chromatogram of polyunsaturated fatty acids obtained from R. oryzae Mekky 1907, is produced using rice straw and sugar cane bagasse residue at 15°C – 20°C temperature

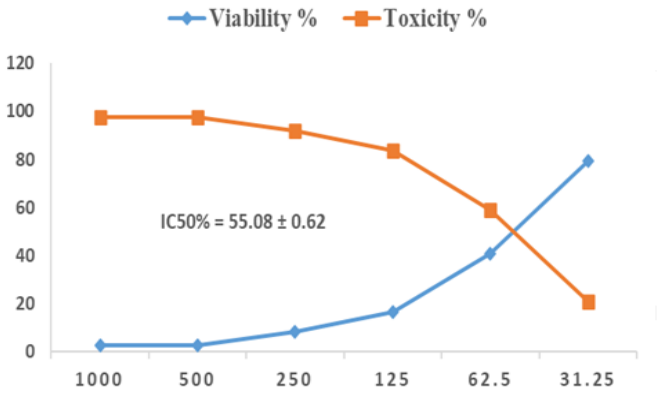

Fig. 8. Effect of PUSFAs on WI-38 cells at different concentrations

Table (1): GC-MS profile of unsaturated fatty acids obtained from *R. oryzae* Mekky 1907, which is produced using rice straw and sugar cane bagasse residue at 15°C – 20°C temperature.

No.	R.T (min)	Fatty acid name	PA (%)	Molecular formula	M.W (g/mol)	Compound structure
1	11.66	Gamma-Linolenic acid (C18:3n6)	0.19	$C_{18}H_{30}O_2$	278.4	
2	24.09	Eicosatrienoic acid (C20:3n3)	1.08	$C_{20}H_{34}O_2$	306.5	
3	41.11	Oleic acid (C18:1n9c)	14.21	$C_{18}H_{34}O_2$	282.5	
4	44.31	Docosadienoic acid (C22:2n6)	7.44	$C_{22}H_{40}O_2$	336.6	
5	59.06	Nervonic acid C24:1n9c	10.05	$C_{24}H_{46}O_2$	336.6	
6	72.01	Eleostearic acid (C18:3n5)	1.66	$C_{18}H_{30}O_2$	278.4	
7	87.34	Stearidonic acid (C18:4n3)	12.17	$C_{18}H_{28}O_2$	276.4	
8	11.66	Gamma-Linolenic acid (C18:3n6)	0.19	$C_{18}H_{30}O_2$	278.4	
9	24.09	Eicosatrienoic acid (C20:3n3)	1.08	$C_{20}H_{34}O_2$	306.5	

Cytotoxicity assay of PUSFAs on WI-38 cell line

The effect PUSFAs have toxic effect on WI-38 cells as normal cells were isolated from the lung tissue of a 3-month-old, female, embryo and the percentage of cell viability was confirmed by MTT assay. At the end of incubation period at different concentrations 1000 – 31.25 µg/mL. Fig. (8) showed healthy cell death by significantly dose-dependent also when subjected to various quantities of PUSFAs. In specifically, our IC_{50} of vero cell was 55.08 µg/mL for PUSFAs. The change in cell shape after incubation of WI-38 cell with concentrations 1000 – 31.25 µg/mL imaged by inverted light microscope Fig. (9).

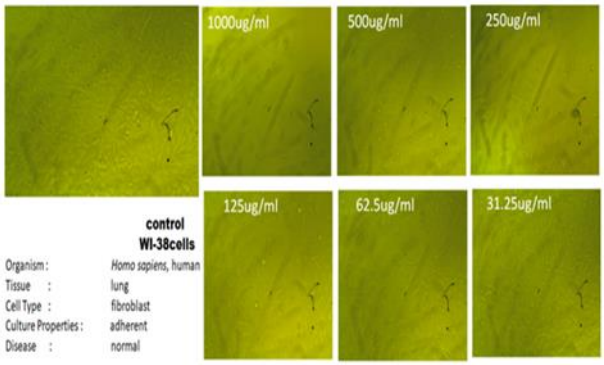

Fig. 9. Morphological characteristic of WI-38 cells treated with PUSFAs

Antitumor activity of PUSFAs against Caco-2 cells.

Data represented in Fig. (10) showed that the inhibitory effect PUSFAs against human colon tumor cells (Caco-2). At the end of incubation period at different concentrations 1000 – 31.25 µg/mL. In specifically, our IC50 for cancer cell (Caco-2) was 208.15 µg/mL for PUSFAs. Fig. (11) showed cell morphology and alteration in cell shape in a monolayer culture is the first and most readily noticeable showed after exposure of cells to PUSFAs imaged by inverted light microscope.

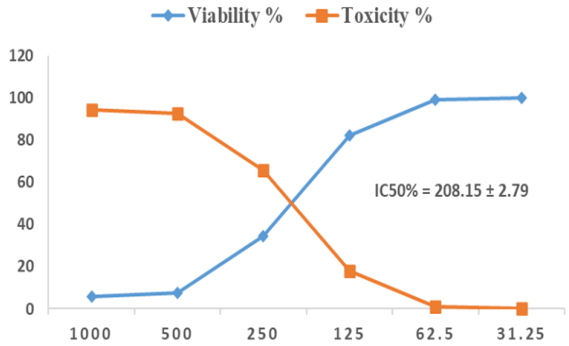

Fig. 10. Antitumor activity of PUSFAs with different concentrations

Fig. 11. Morphological characteristic of cancer cell (Caco-2) treated with PUSFAs

Antioxidant activity of PUSFAs using DPPH scavenging activity

The antioxidant activity showed considerable DPPH scavenging activity. The results of PUSFAs were found to be significant (P <0.05). In the current study, antioxidant properties of ascorbic acid, PUSFAs was evaluated at different concentrations from 1000 to1.95 µg/mL as shown in Fig. (12).

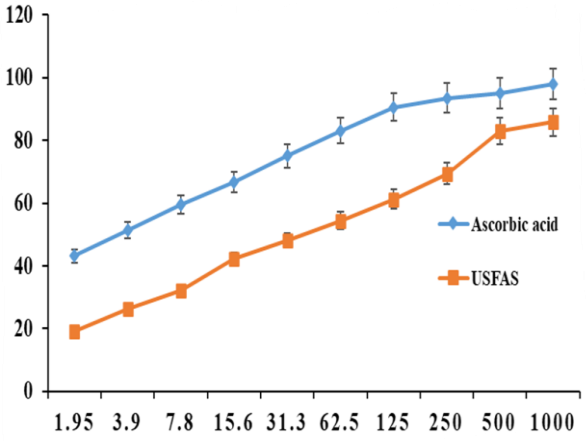

Fig. 12. DPPH radical scavenging activity of ascorbic acid and PUSFAs The average of the three replications + the standard deviation makes up the values.

Anti-inflammatory activity of PUSFAs on HRBC hemolytic and membrane stabilization

Anti-inflammatory activity of PUSFAs and extract was assessed based on their ability to hypotonicity induced hemolysis assay *in vitro.* In the current study, anti-inflammatory activity of PUSFAs was evaluated at different concentrations from 1000 to100 µg/mL as shown in (Fig. 13).

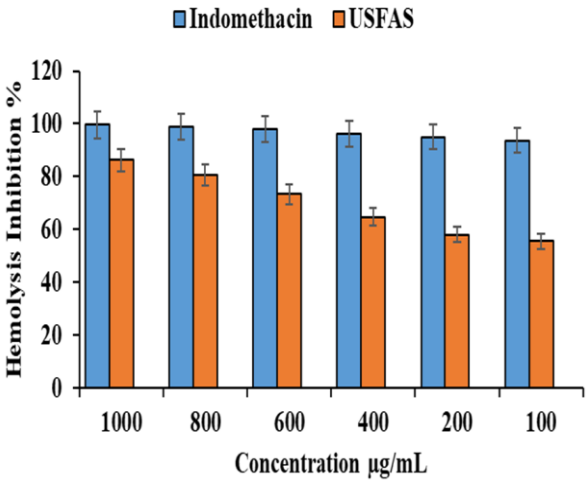

Fig. 13. Effect of PUSFAs on HRBC hemolytic and membrane stabilization

4. Discussion

The function of the brain, nerves, and eyes as well as the prevention of neurodegenerative and mental disorders, infections, and cancer are all significantly impacted by poly unsaturated fatty acids. (Djuricic and Calder, 2021). The ability of *zygomycete*-related fungal isolates, which include those from the genera *Mortierella, Cunninghamella, Rhizopus, and Mucor*, to accumulate lipids, led to their selection (Suleiman et al., 2018). It seems that the genera *Zygomyctes*, which include *Cunningghamella, Cucor, Mucor, Mortierella*, and *Rhizopus*, have been the main focus of lipid production from fungi (Tauk-Tornisielo et al., 2009). To produce lignocellulose biomass biofuels on an industrial scale, it is required to identify appropriate microbial strains with fermentation capabilities (Tsegaye et al., 2019).

Molecular and classical characterization and identification of the higher producible *Rhizopus* spp. isolates, mekky1907, were performed. One of the most used methods for identifying fungus is routine identification (Benny 2008). The top hit displayed 98% exact identity with at the molecular level of *R. oryzae* strains. Fungal isolate *R. oryzae* Mekky1907 was identified as *R. oryzae* and recorded in Gene-Bank under accession number PQ182608, our results were in agree with the results recorded by another researchers in wich isolates of *Rhizopus sp. JSK6, R. oryzae-JSK3, Rhizopus sp. JSKp and Rhizopus sp. JSK8,* were identified and recorded (Sukrutha et al., 2014).

In the current study, glucose is the highest for lipid production and carried symbol (a) was 1.47 ± 0.08 g/L. However, a low carbon source of starch showed the lowest production of lipids was 0.31 ± 0.03 g/L. Also yeast extract gives the maximum values 3.95 ± 0.04 g/L. However, a

minimum nitrogen source of asparagine showed the lowest production of lipids was 0.52 ± 0.04 g/L, the majority of earlier research revealed that glucose and yeast extract are the most effective for lipid synthesis by numerous mucorales species, including *Cunninghamella* sp., *Mucor* sp., and *Mortierella* sp. **(**Ling et al., 2016). Incubation temperature, starting pH, incubation duration, carbon source, and nitrogen source were identified as the most critical variables influencing lipid formation in recent studies (Enshaeieh et al., 2014). *R. oryzae* Mekky1907 was incubated at various temperature ranges from 5 º to 50 ºC. The results proved that the better temperature to produce lipids by *R. oryzae* Mekky1907 was 2.59 ± 0.06 g/L at 30°C. Also, pH factor is considered as an important factor for lipid production (Enshaeieh et al., 2014). Therefore, seven levels of pH (2 and 8) were used in production of lipids by *R. oryzae* Mekky1907 at pH 5 high lipid produced was 2.15 ± 0.06 g/L and low at 8 was 0.46 ± 0.02 g/L. Incubation period also is important to accumulate lipid inside cell wall of fungi. The optimum incubation period of *R. oryzae* Mekky1907 isolated for the production of lipids was found at 5 days with the higher maximum incubation periods and incubation conditions for production of lipids by *R. oryzae* Mekky1907 under static condition was 1.58 ± 0.03 g/L. These results are consistent with the results reported by Suleiman et al. (2018).

Fungi are known for their rapid growth, brief life cycles, low light energy needs, scalability, and capacity to use a wide range of carbon sources, including wastewater, lignocarbon biomass, and agri-industrial wastes (Chen et al., 2023). Solid state fermentation is typically used in the industrial manufacture of amylases, although solid-state fermentation has drawn more attention recently because of its higher productivity, lower energy need, and easier fermentation media. Furthermore, a number of studies have documented the ideal growth circumstances for fungal amylases, including pH, the presence of various inhibitors, temperature, and other factors, as well as the substrate that is utilized to produce biomass (Corbu et al., 2023). Fungi are important in agriculture for a variety of reasons, including plant development and protection. For instance, mycorrhizal fungi form a mutualistic relationship with plant roots to increase the surface area of the root system, which enhances the plant's ability to absorb nutrients. This connection facilitates the plant's uptake of nutrients that are not easily found in the soil, such as nitrogen and phosphorus (Şesan et al., 2010). The endophytic fungi that grow on plant tissue are another important type of fungal species used in agriculture. Endophytic fungi and plant tissue have a complicated connection that involves regulating the plant's defense mechanism to prevent phytopathogens and promote plant growth even in the face of biotic and abiotic stress (Galindo-Solís and Fernández, 2022). The effective role of fungi remains in breaking down agricultural waste, and they are aided in this by the effective enzymatic system they possess, which enables them to transform agricultural waste into good materials that can be relied upon in many fields (Corbu et al., 2023). The filamentous growth pattern and the secretion capabilities of proteins and primary and secondary metabolites assist fungal proliferation in nature. These characteristics are used by industry to make tiny molecule chemicals, proteins, and, more recently, mycelium materials. These bio-based goods could be packaged and utilized as acoustic and thermal insulation (Jones et al., 2017). Either the whole substrate is completely broken down into fungal components, or the fungal skin is removed from the substrate's surface.

The type of fungus, growing circumstances, and substrate affect the mycelium's characteristics (Appels et al., 2018). SFAs were more prevalent than USFAs based on the GC/MS profile, which also revealed that Caproic, Undecanoic, Tridecanoic, Palmitoleic, Heptadecanoic, Heneicosanoic, and Behenic were the most prevalent SFAs. Moreover, PUSFAs as Linolenic, Cis-11, 14, 17-Eicosatrienoic, Gamma-Linolenic, Cis-8, 11, 16-Docosadienoic and 14-Eicosatrienoic and Cis-13n were present in small quantities (El Zanaty et al., 2022; Al-dhubiea et al., 2023).

IC_{50} of vero cell was 55.08 µg/mL for PUSFAs.. In the current study, the results consistent with Kwak et al. (2008) findings, who demonstrated the effectiveness of saturated and unsaturated fats extracted from mushrooms and their use as

anti-cancer agents. Results showed that, as compared to AA, $PUSFA_S$ had considerable antioxidant activity. The IC_{50} of $PUSFA_S$ exhibited good antioxidant activity (5.21 µg/mL) compared to standard drug was 1.47 µg/mL. As the most energy-dense macronutrient, fats are essential for both human and animal energy requirements as well as the soluble in fat vitamins D, A, and E absorption. The health benefits of good fat, which have been connected to different kinds of dietary fats, have become more widely known in recent years (Savarino et al., 2021). Hemolysis is the most commonly utilized initial toxicity assessment method. For example, blood is frequently in touch with a hydrogel wound dressing used in biomedical applications. Therefore, the right amounts of hemolytic activity are required in any biomaterial that can hasten the healing of skin wounds (Luo et al., 2008). The results showed that, as compared to indomethacin (standard drug), $PUSFA_S$ had considerable anti-inflammatory activity percent hemolysis inhibition was 86.29 % at concentration 1000 µg/mL, while indomethacin showed 99.63% activity at the same concentration. Finally, the experiments conducted also demonstrated the effectiveness of polyunsaturated fatty acids as an anti-cancer, antioxidant, and anti-inflammatory agent. *R. oryzae* Mekky1907 is therefore thought to be a promising oleaginous filamentous fungus that can be used to produce PUFAs from agricultural waste.

5. Conclusion

The current study produced lipids and showed that temperature, pH, carbon supply, and nitrogen have a major impact on lipid formation. By using morphological and molecular confirmation, the isolated species identity was established and recorded on Gene Bank under accession number PQ182608. *R. oryzae* Mekky1907 showed the highest lipid production. The experiments conducted also demonstrated the effectiveness of polyunsaturated fatty acids as an anti-cancer, antioxidant, and anti-inflammatory agents. *R. oryzae* Mekky1907 is therefore thought to be a promising oleaginous filamentous fungus that can be used to produce PUFAs from agricultural waste.

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