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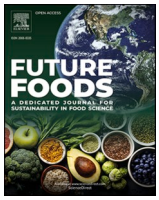

Mutagenesis treatment of *Mortierella alpina* for PUFA production enhancement for future food development

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ABSTRACT

Random mutagenesis has been identified as a key tool for improving microbial and fungal strains enabling the development of isolates with improved traits suited for industrial scale metabolite production to enhance the nutritional value of future foods. Presented here, is a random mutagenesis strategy employed to assess the effect of 5-fluorouracil (20-200 μg/ml), alone and in combination with the secondary agents octyl gallate and nocodazole, and diethyl sulfate (0.1 to 1 %) chemical mutagenic agents, on the biomass and lipid production as well as the FAME profile. Interestingly, a correlation was demonstrated between 5-fluorouracil exposure time and the arachidonic acid content, which was also influenced by the concentration used. 5-fluororuracil of 100 μg/ml treatment for 48 h resulted in the highest arachidonic acid (% TFA) content in isolates. Mutant M5F047 isolated with 5-fluororuracil (100 μg/ml) alone, proved to be most superior in terms of polyunsaturated fatty acid (PUFA) and arachidonic acid production, as compared to the *Mortierella alpina* wild type strain, with enhancements that doubled that of the parent strain. These improvements are more favorable for industrial scale production of arachidonic acid, a precursor of meaty flavour to improve plant-based meats in future food development.

1. Introduction

The production of plant-based meats that mimic animal meats, requires the creation of meaty flavours to enhance consumer acceptance. Lipid precursors, particularly triglycerides and phospholipids, play an essential role in the formation of flavour (Sun et al., 2022). Thus, future food development is contingent on the flavour profile for consumer acceptance, which is influenced by the fatty acid profile, content and degree of saturation (Duan et al., 2024). Furthermore, the increased demand for functional foods with health benefits, such as those fortified with polyunsaturated fatty acids (PUFA), which have been associated with the improvement of cardio vascular health, including peripheral artery disease, inflammation, and anticoagulation (Swanson et al., 2012), treatment of heart failure (Kelling et al., 2024; Shim et al., 2023) and in the management of neurological disorders, such as dementia and Alzheimer's disease (Alhattab et al., 2024), have rapidly amplified their demand. In 2022, the PUFA market size was valued at USD $5.9 billion and is projected to reach USD $10.9 billion by 2030, as a result of the increased health awareness which is driving the demand for nutritional supplements and functional foods (CMI, 2023). At present, the primary commercial source for PUFA are finite marine fish, which only cater to 30 % of the global demand (Glencross, 2020), and do not suit the growing vegetarian and vegan population (Vadivelan & Venkateswaran, 2014). Furthermore, the growing concerns with fish stocks as a result of overfishing, food security, and pollutants found in some fish (FAO, 2020), necessitate the need for alternative renewable and sustainable sources for PFUA production (Karageorgou et al., 2023; Patel et al., 2019). Precision fermentation, the use of microbial cells as effective cell factories for the sustainable production of various metabolites, including PUFA, has gained tremendous attention in literature due to the detrimental impacts of current resource production on the environment, such as the destruction of marine ecosystem due to overfishing (Cho et al., 2022).

Precision fermentation involves selecting microbial organisms (leveraging evolved or engineered microbes), formulating media to enhance the production of the desired metabolite, and optimising downstream processing. Precision fermentation for the industrial production of PUFA, offers numerous advantages compared to conventional sources. The first and foremost is it's a renewable environmentally sustainable feedstock, with a rapid growth rate and high lipid content.

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Furthermore, precision fermentation utilizes less water as compared conventional agriculture-based methods (Teng et al., 2021). Microbial organisms can also use various substrates, such as agricultural residues and wastewater for their growth (Srimongkol et al., 2022), reducing their release into the environment. In addition, this technique does not require arable land for cultivation, reducing our reliance on land, and can be used as a source for various nutritional value-added products, such as proteins, lipids, carbohydrates, and other bioactive molecules, that could better sustain the growing demand (Caporgno & Mathys, 2018; Trovão et al., 2022).

However, this biotechnology remains at a somewhat stand still as a result of the low levels of lipids, proteins and caroteniods produced in strains isolated from their the natural environment (Jeyachandran et al., 2023). At present, there are only a few microbial strains with the required traits suitable for industrial production (Song & Pei, 2018), which are characterized by a high growth rate, cell density, substrate to biomass yield and the affinity to produce the substrate with low nutrient requirement to minimize costs (Bajić et al., 2022). It is therefore essential to isolate and develop improved strains that could better cater to the industrial demand (Trovão et al., 2022). The fungi *Mortierella alpina* has been industrially used for the production of dietary arachidonic acid, an omega-6 fatty acid fortified in infant formula as it plays a key role in development (Chang et al., 2022). However, strain improvement techniques have proven effective in enhancing the overall PUFA content, growth rate and other industrially desirable traits.

The metabolic pathway for the biosynthesis of ARA and PUFA in *Mortierella alpina,* begins with the synthesis of palmitic acid (C16:0) by the fatty acid synthase (FAS). Next, palmitic acid is converted to stearic acid, followed by a series of elongation and Δ9, Δ6, Δ12 and Δ5 desaturation steps to form PUFA and ARA (Patel et al., 2021; Zhang et al., 2018).

Strain improvement techniques have gained momentum recently which include adaptive laboratory evolution (ALE) and genetic engineering. The latter being less desirable as it uses laboratory tools to insert foreign genetic material which results in a genetically modified strain (Ari et al., 2024; Arora et al., 2020). Genetically altered strains face scrutiny and pose biosafety concerns to the environment as they are considered unnatural (Karalis et al., 2020; Trovão et al., 2022). Furthermore, commercialization of genetically modified organisms face challenges in food applications due to consumer perceptions and the understanding of their safety risks, labelling (Rzymski & Królczyk, 2016) and regulation (Augustin et al., 2024; Pappalardo et al., 2021). As such, alternative non-genetically modifying techniques are more favourable to bring to market, as they do not pose the challenges faced with genetically modified organisms. ALE employs natural evolution principles in a controlled laboratory setting to improve strain resilience. In ALE, selection is governed by adaptation to the selected environmental pressure, as a means for improving the organism's fitness (Ferreira & Noble, 2019). ALE techniques are simple and environmentally favourable but require extended time to develop a stable strain, leading to higher processing costs as they are laborious (Trovão et al., 2022).

Random mutagenesis, on the other hand, has been employed as an effective tool for generating enhanced microbial strains suitable for industrial scale production, with improved metabolite production abilities and consequently improving the nutritional value (Jeyachandran et al., 2023). Random mutagenesis, also referred to as forward genetics, is a simple, cost-effective tool used to accelerate species evolution and genetic variability which would otherwise occur naturally and randomly in nature through for example prolonged exposure to sunlight (UV irradiation), reactive oxygen species, and chemical agents (Hlavova et al., 2015; Hu et al., 2017; Tanaka et al., 2010; Trovão et al., 2022). It is used to generate large pools of mutants with varying phenotypes, as a means for improving strain productivity to better suit industrial production processes, in a short time span (Arora et al., 2020; Parekh et al., 2000). It has been recognized as a useful tool for improving microbial strains, with regards to biomass and target compounds of interest, as well as for adapting strains to tolerate harsher environmental growth conditions such as low/high temperatures, limited nutrients, salt stress etc., without requiring extensive knowledge of the genetic alterations taking place (Kumar et al., 2018; Trovão et al., 2022).

Various studies have investigated and demonstrated the use of chemical and physical mutation methods, as well as the combination of these techniques to improve ARA and other essential fatty acid (FA) contents in microalgae and fungi organisms (Jareonkitmongkol, Kawashima, et al., 1992; Kawashima et al., 1997; Li et al., 2015; Nisha, 2009; Sakuradani et al., 2004; Shimizu et al., 1992; Zhang et al., 2018; Zhao et al., 2018), as summarized in Table 1. The greatest enhancements in ARA of more than 3 times, were obtained using chemical mutagenesis applied as a primary or secondary sequential treatment, with the exception of the UV treatment on *Nannochloropsis oculate* which also attributed to an isolate with an improvement of more than 3 times (Moha-León et al., 2019). However, UV treatment of *M. alpina* demonstrated lower improvements as compared to chemical mutagenesis (Nisha, 2009).

Thus, the aim of this study is to employ random mutagenesis on *M. alpina* to enhance the biomass, lipid production and PUFA content using the mutagenic agents 5-fluorouracil (5FLU) and diethyl sulfate (DES) at varying exposure times and concentrations. The treatment agents octyl gallate and nocodazole have also been tested both independently and in combination with 5FLU as a secondary treatment. The assumption made here, that exposure to these mutagenic agents will result in mutant derivatives. Triphenyltetrazolium chloride (TTC) stain is also employed and assessed as means to quickly identify isolates with higher PUFA content, as the colourless compound reduces down to form triphenylformazan (red pigment) with enzymatic dehydrogenase/desaturase (Chang et al., 2022), to assist in identifying isolates with enhanced lipid precursors and traits to suit the industrial development of future food precursors such as plant-based meats, improving the consumer acceptance and experience (Rout & Srivastav, 2024; Singh et al., 2021).

5-flurorouracil, an aromatic heterocyclic compound, which resembles that of pyrimidine molecules present in DNA, can be converted by organisms into fluorouracil deoxynucleotides that hinder the synthesis of DNA (Zhang et al., 2018), thus causing genetic variability. Diethyl sulphate, a commonly used alkylating agent in chemical mutagenesis, induces mutations by adding an alkyl group to the hydrogen-bonded oxygen in guanine base pairs (Trovão et al., 2022), causing breaks in the DNA double strand, giving rise to strain variation. Whereas, octyl gallate and nocozadole have been observed to impact desaturation activity in the fatty acid synthesis, enabling the selection of isolates with enhanced expression abilities (Azam et al., 2015; Zhang et al., 2018).

2. Materials and methods

2.1. Mutagenesis approach

Mutagenesis of *Mortierella alpina* strain (provided by Nourish Ingredients, Australia), were carried out and assessed following the methodology depicted in Fig. 1. Briefly, mutagenesis was performed on the harvested spores (process described below). Various chemical stresses were employed to induce mutations, and samples were taken at various time intervals and spread plated with no dilution, 10^{-1} and 10^{-2} dilutions, to obtain individual colony isolates. Isolates were selected ensuring large colonies and small ones were taken for screening as well as pigmented and non-pigmented colonies by initial transfer onto a fresh agar plate, where they were left to grow for 3 days. A minimum of 10 isolates, where possible, were taken for each treatment screening with at least 3 isolates from each agar plate prepared (no dilution, 10^{-1} and 10^{-2}dilutions), for inference on the effect. Next, a small aliquot of equal size was taken for homogenization and transferred to a proprietary

Table 1
Physical and chemical mutagenesis of various species and the impact on fatty acid content.

FA	Species	Mutagenic Treatment	FA Content (g L^{-1}) or % of TFA	Operational Parameter	Reference
ARA	*Mortierella alpina*	None	3.62 g L^{-1}	-	(Li et al., 2015)
		ARTP	4.56 g L^{-1}	195s	
		ARTP + DES	5.09 g L^{-1}	195s, 20 % (v/v) in ethanol	
		ARTP + DES + Cultivation	6.82 g L^{-1}	Feeding strategy, yeast extract: soy flour (1:2 w/w)	
ARA	*Mortierella alpina*	None	1.24 g L^{-1}	-	(Zhang et al., 2018)
		Heavy Ion	3.8 g L^{-1}	240 Gy	
		Heavy Ion + 5-fluorouracil + octyl gallate	5.26 g L^{-1}	240 Gy + 20 μg mL^{-1}	
ARA	*Mortierella alpina*	None	17.2 % (TFA)	-	(Hanna et al., 2024)
		5-fluorouracil + octyl gallate	30.0 % (TFA)	20 μg mL^{-1} +15 mg/L	
ARA	*Tetraselmis tetrathele*	None	4 %	-	(Cortez et al., 2015)
		EMS	7.7 %	0.1-0.3 M, 15-105 min	
ARA	*Mortierella alpina*	None	47 % (w/w)	-	(Jareonkitmongkol, Shimizu, et al., 1992)
		MNNG	10.6 % (w/w)	0.1 mg/ml	
ARA	*Mortierella alpina*	None	0.95 g L^{-1}	-	(Sakuradani et al., 2004)
		MNNG + Cultivation	1.48 g L^{-1}	0.1 mg/ml, 4 % glucose, soy flour, 16-20°C	
			1.77 g L^{-1}		
ARA	*Mortierella alpina*	None	0.96 g L^{-1}	-	(Nisha, 2009)
		UV	1.35 g L^{-1}	24 min	
		MNNG	1.95 g L^{-1}	25 μg mL^{-1}, 90 min	
		EMS	1.55 g L^{-1}	20 μg mL^{-1}, 120 min	
ARA	*Nannochloropsis salina*	None	2.5 %	-	(Beacham et al., 2015)
		EMS	12.1 %	0.24 mol L^{-1}	
ARA	*Nannochloropsis oculata*	None	0.63 %	-	(Moha-León et al., 2019)
		UV	2.72 %	120 min	
EPA	*Nannochloropsis oculata*	None	23.9 %	-	(Chaturvedi & Fujita, 2006)
		EMS	30.8 %	100 mM, 60 min	
DHA	*Schizochytrium sp.*	None	6 g L^{-1}	-	(Zhao et al., 2018)
		ARTP + MA	7.6 g L^{-1}	ARTP 15s, MA 6.0 mmol/L	
		ARTP - MA + ARTP -Zeocin	9.4 g L^{-1}	ARTP 15s, MA 6.0 mmol/L, Zeocin 2 mg/L	
		Cultivation Media Supplement	14 g L^{-1}	Fe2+ addition 0.5 mmol L^{-1}	
Mead Acid	Mortierella alpina	MNNG	1.18 g L^{-1}	0.1 mg/ml	(Kawashima et al., 1997)
		MNNG + MNNG	1.53 g L^{-1}	0.1 mg/ml	

FA: fatty acid; ARTP: atmospheric room temperature plasma; DES: diethyl sulfate; TFA: total fatty acid; MA: Malonic acid; EMS: ethyl methane sulphonate

minimal fermentation media consisting mainly of yeast extract and glucose (YEG), in 50 mL Erlenmeyer flasks, where they were incubated at 30°C and 180 rpm for the specified time period. At the end of the specified growth period (3-5 days), the biomass was harvested via centrifugation (3500 rpm, 5 min), washed three times with autoclaved distilled water and stored at -80°C for freeze drying. Once dried, sample analysis was carried out, which included biomass, lipid yield, and FAME profiling to determine the effect of the mutation.

2.2. Spore harvest

Initially, an aliquot from *Mortierella alpina* plates, was sub-cultured onto fresh yeast, peptone and glucose (YPD) agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar), where they were allowed to grow for 3 days in an incubator maintained at 30°C. From these fresh plates an aliquot was sub-cultured onto a proprietary modified media of Czapek agar plates favouring sporulation. Spores were harvested by the addition of 5-6 ml of 20 % glycerol + 0.9 % NaCl solution onto the first sporulating plate. The spore containing suspension was pooled and poured onto the next plate (Supplementary A1). This process was continued until all plates were harvested. Finally, a 40 μm filter was fitted onto a falcon tube and the collected spores in suspension (from the last plate harvest), were poured onto the filter. The filtered suspension was centrifuged at 3000 rpm for 5 min and the supernatant was decanted leaving behind the spores settled at the bottom of the conical tube. The collected spores were resuspended in harvesting solution (noted above), and stored at -80°C in cryopreserved vials.

2.3. Random Mutagenesis treatment

Chemical random mutagenesis of *M. alpina* were carried out by adding 20 μL of spores (1×10^7 cells/ml (determined using hemocytometer counts)) in 20 ml of 0.9 % NaCl media, Fig. 1. Various mutagenic agents were added at varying concentrations to the suspension and incubated at a temperature of 25°C and orbitally mixed at 180 rpm (determined as optimal conditions for this strain). An aliquot of 100 μL was drawn at the varying exposure times and plated on YPD agar plates for screening and to determine the colony survival rate (Supplementary A2). For each exposure time interval, a plate with no dilution, and serial dilutions of 1×10^{-1} and 1×10^{-2} were prepared. An L-shaped spreader was used to distribute the spores around the entire surface of the agar plate, in order to isolate individual colonies. Plates were covered, sealed with parafilm and left to incubate at 30°C and monitored for growth. Colony counts were performed at 48 h, the point at which they were visible to count by the naked eye, and demonstrated a decline in survival rate with increased exposure time (Supplementary A2).

2.4. Screening of mutants

M. alpina mutants were screened using 2,3,5-Triphenyltetrazolium chloride (TTC) (Merck, Macquarie Park, NSW, Australia) stain as an indicator of PUFA activity (Chang et al., 2022). TTC is colorless, however, dehydrogenases/desaturases reduce it to from triphenylformazan, a red-colored compound that indicates desaturase activity, double bond formation, and consequently PUFA activity within the cell (Chang et al., 2022; Li et al., 2015). In this manner, TTC stain was used as an indicator of desaturase activity which would indicate greater PUFA production (Chang et al., 2022). TTC (0.005%) dissolved in ethanol (HPLC grade,

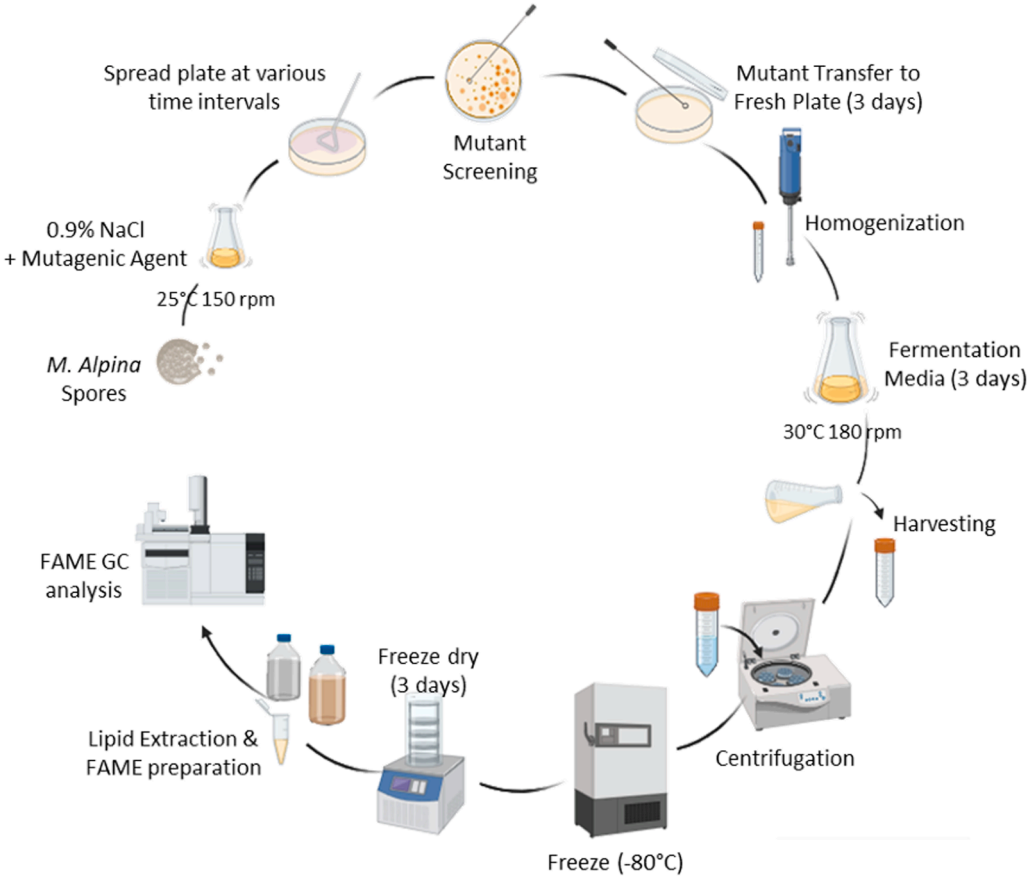

Fig. 1. Mutagenesis approach carried out for treating and screening of isolated mutants using various mutagenic agents (Created with BioRender.com).

ChemSupply, South Australia, Australia), and kept in a dark vial at 4°C was incorporated into the YPD agar plates used for screening mutants, to aid in the selection of mutants based on the degree of pigmentation. Mutants with a less intense pigmentation were also selected for comparison.

Selected isolates were screened by transferring onto a fresh YPD agar plate, also containing TTC, and left to incubate for 3 days at 30°C. The isolate naming was annotated to include the species name, mutagenetic agent and identifier number assigned in sequence (Supplementary A3). Next, a small mat of equal size was transferred to a falcon tube containing 3-4 ml of fermentation media for homogenization at 20,500 rpm for 20 seconds. The homogenized mixture was transferred into 50 mL of fermentation media (in 250 ml Erlenmeyer flask), where it was kept in incubation shaking for 3 days at 30°C and 180 rpm. Samples were harvested at the end of the experimental run in pre-weighed 50 ml falcon tubes and centrifuged (5810 R, Eppendorf, Macquarie Park, NSW, Australia) at 3500 rpm for 5 min. The supernatant is decanted and the remaining biomass is washed three times with Mili Q water, mixed and re-centrifuged. The supernatant is again decanted and the remaining biomass is kept at -80°C for at least 24 h, and freeze dried for 72 h (Beta 2–8 LSCbasic, Christ, Sciteck, NSW, Australia). Next, the samples were taken for biomass concentration determination and performing the lipid and FAME analysis, described below.

2.5. Biomass concentration determination

One of the mutant selection assessment criteria was biomass concentration, represented as grams per litre and calculated by dividing the freeze- dried biomass (grams) achieved in the respective volume of fermentation (L), Equation 1.

$$Biomass\ Concentration\ (g/L) = \frac{Biomass\ (g)_{dried}}{Volume\ (L)_{Collected}} \quad (1)$$

2.6. Screening based on lipid content and FAME profile

Each mutant isolate was also screened for fatty acid methyl ester (FAME) profile using the method described in Gupta et al. (2013). Briefly, 10 mg of freeze-dried biomass, Fig. 1, was transferred into a 1.5 ml Eppendorf tube and mixed with 600 µL of chloroform: methanol (2:1). Next, the sample was vortexed for 2 min and centrifuged for 10 min at 10,000 rpm (5420, Eppendorf, Macquarie Park, NSW, Australia). The supernatant was collected and the extraction process was repeated twice more. The collated extracts were filtered using 0.22 µm filter membranes into a pre-weighed 4 ml glass vial, and left to dry at 50°C on a heating block (DBH30, Ratek Instruments, Boronia, VIC, Australia). The dried lipid weight was recorded for lipid production quantification represented as the percentage of biomass dry cell weight (DCW).

The extract obtained is methylated and preserved by the addition of 500 µL of toluene, 200 µL of butylated hydroxytoluene, and 500 µL of 10 % acetyl chloride in methanol (prepared by the dropwise addition of acetyl chloride to methanol that is kept on ice and allowed to stir for 1 hour). The reaction is left to take place at 50°C overnight. Next, the liquid samples are transferred into a 10 ml test tube with the addition of 1 ml of sodium chloride solution (5 % w/v in Mili-Q (MQ) water), and 1 ml of hexane. The organic layer containing the fatty acid methyl esters is pipetted into another 10 ml test tube, to which 1 ml of potassium bicarbonate (2 % w/v in MQ water) is added for washing. The organic layer is again pipetted off into a third 10 ml test tube, where a small aliquot of sodium sulfate is added to further dry the mixture. Finally, the hexane layer is transferred to a 2 ml glass vial for analysis. Samples were

analysed using gas chromatography equipped with a FAMEWAX column (30 m × 0.32 mm ID (inner diameter)), and a flame ionization detector that is connected to a barrier discharge ionization unit (GC-2030, Shimadzu Scientific Instruments (Oceania)). The inlet temperature was set to 240°C with a split injection of 1/100, an injection volume of 1 µl was used with a column flow rate of 1.5 mL/min and helium (5.0) carrier gas. The oven temperature program was set to 170°C , and gradually increased to 200°C at a rate of 5°C per min, followed by an increase of 10°C per min to 240°C, where it was held for 10 min. The chromatograms were integrated using LabSolutions (Version 5.92 Shimadzu Scientific Instruments (Oceania)). The FA were identified by comparison with fatty acid methyl ester (FAME) standard (Supelco 37 component FAME Mix, CRM47885, Merck). Quantification of individual FA was reported as a percentage of total FAME.

2.7. Octyl gallate mutant screening

Octyl gallate, which has been noted to inhibit the activity of fatty acid (FA) desaturases in fungi (Zhang et al., 2018), was assessed as a primary and secondary treatment. A concentration of 14 µg/mL was adopted from the study performed by Zhang et al. (2018), and added into the YPD agar plates for screening, alongside the TTC stain. The process followed remained unchanged from that detailed in Fig. 1. For comparison, a control sample was taken from the mutation flask (time 0, 25°C, 20 ml of 0.9 % NaCl media), prior to the addition 5FLU (100 µg/mL), to determine the impact of octyl gallate alone, as it has also been demonstrated to have a cytotoxic and antiproliferative effect on tissue and cells (de Cordova et al., 2011; Jagan et al., 2008) and used as a fungicidal agent (Fujita & Kubo, 2002). The samples were drawn at 0, 6, 23, 30 and 46.5 h.

2.8. Nocodazole mutant screening

Similarly, nocodazole is an antibiotic, which was suggested to inhibit the synthesis of fatty acids (Azam et al., 2015), which may decrease the intracellular lipids. A concentration of 2.5 µM was added into the YPD agar plates for screening. TTC stain was also kept for visual desaturase activity. The process followed remained unchanged from that detailed in Fig. 1. For comparison, an initial sample was taken from the mutation flask, prior to the addition 5FLU (100 µg/mL), to determine if nocodazole alone has permanent altering effects on the PUFA, lipid and biomass production. The samples were drawn at 0, 6, 23, 30 and 46.5 h.

2.9. Scale-up fermentation

Scale-up (200 ml and 1 L) fermentation investigations were carried out following the methodology depicted in Fig. 1, however, it differs in that an aliquot is taken from a freshly prepared plate (3 days old) and is added to 50 mL of inoculum media (in 250 mL Erlenmeyer flask), made up of YPD media. The inoculum is maintained for 48 h in an incubator shaker set to 30°C and 180 rpm. Next, it is homogenized and transferred into the fermentation media (described in section 5.3), at 8 % of the total volume (200 ml or 1 L). The mycelia are allowed to grow at conditions of 30°C and 180 rpm for the specified period which ranged from 3 to 5 days. At the end of the fermentation, the mycelia are harvested by centrifugation at 3500 rpm for 5 mins and the decantated pellet is washed with autoclaved MQ water a total of three times.

2.10. Data analysis

GC analysis of the chromatograms generated were integrated using LabSolutions (Version 5.92 Shimadzu Scientific Instruments (Oceania)). The FA were identified by comparison with a fatty acid methyl ester (FAME) standard (Supelco 37 component FAME Mix, CRM47885, Merck). Quantification of individual FA was reported as a percentage of total FAME. Data presented here is the average of 2 replicate samples.

Statistical inferences were made by two sample t-Test comparisons, using Microsoft Excel (version 2311) data analysis package. In addition, the level of significance in the FAME profile, biomass, lipid and PUFA production were performed by z-score comparison, where the *M. alpina* parent strain variation in 12 different samples fluctuated in z-scores between -1.5 and 1.5. As such, a significant improvement or variation is deemed by a z-score which is $|\text{z-score}|>1.5$. Heat maps were also employed to assess the biomass, lipid, fatty acids and PUFA content where a deep green color is used to indicate z-scores of 3 and greater, and a deep red represents z-scores which are equal to or less than -3. Computations were made using Microsoft Excel (version 2311).

3. Results and discussion

Random mutagenesis using 5-fluorouracil (5FLU) and diethyl sulfate (DES) at various concentrations and exposure times following the mutagenesis approach outlined in Fig. 1, were carried out on *Mortierella alpina* parent strain harvested spores. These agents have been documented to impair the DNA synthesis by conversion into fluorouracil deoxynucleotides (Zhang et al., 2018) that hinder the synthesis of DNA, and forming alkyl groups to the hydrogen-bonded oxygen in the guanine base pairs (Trovão et al., 2022), causing breaks in the DNA double strand, resulting in strain variation. Colonies were selected for screening based on size, where large samples are thought to have enhanced growth abilities and also 2,3,5-Triphenyltetrazolium chloride (TTC) staining degree as an indicator for desaturase activity to identify isolates with enhanced PUFA, as it is reduced down to form the pigmented compound triphenylformazan (Chang et al., 2022). It should be noted, that both intensely pigmented and less pigmented colonies were screened to identify the correlation between pigmentation and PUFA, as their production involves a series of desaturase and elongase steps. The effects of the treatment agents nocodazole and octyl gallate were also investigated, with and without undergoing mutagenesis treatment with 5FLU, which have been noted to impact the fatty acid syntheses and desaturase enzymatic activity. Isolates were screened for biomass, lipid production and FAME profiles.

3.1. 5-Fluorouracil

Varying concentrations of 5FLU (20 to 200 µg/ml) were carried out on *M. alpina* spores for bioprospecting. The colony number reduced by 70-79 % over an exposure period of 5 days for the tested concentration range (Supplementary A2).

Samples drawn at various intervals for biomass, lipid and FAME profile analysis, and those that showed alterations in the FAME lipid profile, biomass and PUFA production as compared to the *M. alpina* parent strain are depicted in Table 2. Isolates that demonstrated similar profiles, biomass and lipid contents as others were left out in order to reduce the table size.

Variations were particularly apparent in palmitic acid (C16:0), oleic acid (C18:1n9), linolenic acid (C18:3n6) and arachidonic acid (C20:4n6). The palmitic acid levels in the isolates generated using the 5FLU concentrations of 20 and 200 µg/ml were significantly different from those of the parent strain ($p<0.05$). In terms of oleic acid, linolenic acid and ARA, the contents were significantly different when compared to the parent *M. alpina* strain ($p<0.05$). Heat map color indicators were used to provide quick inference on the z-score value where a deep red, yellow and deep green signify values of at least -3, 0 and 3, respectively. It can be seen that isolate M5F009, generated with 5FLU concentration of 20 µg/ml, accumulated the highest palmitic acid content, as depicted by the green color, which attributes to a z-score value of 2.2 indicating it is more than two standard deviations away from the mean value achieved in the *M. alpina* parent strain. The other isolates generated from 5FLU of 20 and 200 µg/ml also resulted in slightly greater contents of palmitic acid, some of which were significantly greater as compared to the *M. alpina* parent strain. Interestingly, however, a 5FLU concentration

Table 2
M. alpina FAME profile (% TFA), biomass and lipid production of isolates generated from random mutagenesis using 5-fluorouracil (20 and 200 μg/ml) at various time intervals.

	5FLU Concentration (μg/ml)												
	-	20	20	20	200	200	200	200	200	200	200	200	200
FAME	*M. alpina*	M5F003	M5F008	M5F009	M5F014	M5F016	M5F018	M5F019	M5F023	M5F024	M5F030	M5F032	M5F034
C12:0	0.3	0.1	0.3	0.1	0.7	0.5	1.4	0.7	0.2		0.8	0.2	0.4
C14:0	2.7	3.5	2.5	3.1	2.6	2.4	2.9	2.4	2.9	2.2	2.2	3.3	2.9
C15:0	0.3	0.3	0.7	0.9	0.3	0.5	0.3	0.4	0.8	1.2	2	1.0	0.7
C15:1													
C16:0	24.0	28.7	26.4	31.3	28.4	26	24.8	28.2	29.1	24	26.3	30.3	27.5
C16:1	0.5	0.4	0.4	0.8	0.5	0.5	0.3	0.5	0.4	0.3	0.4	0.5	0.6
C17:0	0.3	0.2	1.2	0.7	0.3	0.4	0.4	0.2	0.5	0.6		0.4	0.4
C17:1n7													
C18:0	9.8	9.3	7.4	6.3	9.2	7.9	8.7	9	7.3	5.7	8.8	8.0	7.4
C18:1n7													
C18:1n9	26.2	21.1	19.6	18.5	24.2	21.4	20.2	25.2	20.9	25.7	22	23.7	21.9
C18:2	8.0	10.5	12.6	11.2	9.1	11	10.3	9.1	9.2	10.8	9.6	9.0	10.5
C18:3n6	4.8	5.3	9.1	5.5	5.2	7.3	7.4	5.4	6.3	8.4	6.6	5.2	7.8
C19:0													
C20:0	1.1	0.4	0.3	0.3		0.5	0.6	0.1		0.6			1.1
C20:2													
C20:3n6	2.9	3.5	1.9	2.9	2.8	4.2	2.6	2.4	3.7	3.1	3.4	2.9	2.8
C20:4n6	13.6	15.1	16	17.6	13.5	16.5	16.7	12.5	15.1	14.4	15.4	11.5	14.5
C20:3n3													
C20:5 EPA													
C22:0	1.8	0.8			1.1		1	1.5	1.6		1	1.5	
C22:5n6 DPA													
C24:0	3.8	0.9	0.6		2	0.4		1.8	2.2	1.1	1.5	2.5	1.5
C22:6 DHA													
Biomass (g/L)	15.3	20.9	3.9	3.9	16.4	9	4.6	15.9	7.6	8.5	7.5	20.9	20.9
Lipid % (DCW)	42.9	48.5	44.6	49	50	46.5	44.1	49.5	43	38	37.9	48.5	48.5
SFA (%)	44.1	44	39.4	42.7	44.5	38.5	40	44.5	44.5	35.4	42.5	47.2	41.9
MUFA (%)	25.4	21.5	20	19.3	24.7	21.9	20.5	25.7	21.3	26	22.4	24.2	22.5
PUFA (%)	30.4	34.5	39.5	37.2	30.5	38.9	37	29.4	34.3	36.7	35.1	28.6	35.7

	5FLU Concentration (μg/mL)								
	-	100	100	100	100	100	100	100	100
FAME	*M. alpina*	M5F047	M5F048	M5F051	M5F059	M5F063	M5F066	M5F069	M5F071
C12:0	0.3	0.4	0.4	0.4	0.2	0.1	0.1	0.3	
C14:0	2.7	2.2	1.9	1.9	2.1	1.9	2.3	2.1	1.9
C15:0	0.3	0.3	0.2	0.2		0.2		0.4	
C15:1									
C16:0	24.0	23.6	21.3	22.1	21.5	21.1	21.6	21.2	22.3
C16:1	0.5			0.3	0.3	0.3	0.3	0.2	0.3
C17:0	0.3			0.2	0.2	0.2			0
C17:1n7									
C18:0	9.8	10	8.6	8.7	7.8	8.8	7.4	7.8	8.1
C18:1n7									
C18:1n9	26.2	19.5	23.1	21.1	21.2	18.6	21.1	22.2	22.6
C18:2	8.0	10	9.2	10.9	10.8	12.1	11.2	10.7	10.3
C18:3n6	4.8	4.8	5.2	5.1	6.5	5.1	5.9	5	5.1
C19:0				0					
C20:0	1.1	0.8	0.8	0.6	0.4	0.6		0.8	0.7
C20:2									
C20:3n6	2.9	3.5	3.7	3.7	3.6	3.4	3.7	3.5	3.8
C20:4n6	13.6	19.3	19.5	18.9	20.4	22.2	21.7	21.1	19.5
C20:3n3									
C20:5 EPA									
C22:0	1.8	2	1.9	1.9	1.3	2	1.6	1.6	1.6
C22:5n6 DPA									
C24:0	3.8	3.6	4.4	4	3.9	3.5	3.3	3.3	3.9
C22:6 DHA									
Biomass (g/L)	15.3	21.4	14.9	19.6	11.6	18.7	14.9	20	20.8
Lipid % (DCW)	42.9	51	44.6	55.6	41.2	50.9	49	44.2	52.9
SFA (%)	44.1	42.9	39.4	40	37.3	38.4	36.2	37.3	38.5
MUFA (%)	25.4	19.5	23.1	21.4	21.4	18.9	21.4	22.4	22.9
PUFA (%)	30.4	37.6	37.5	38.6	41.3	42.8	42.4	40.3	38.7

FAME: fatty acid methyl ester; TFA: Total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; color indicator presents that the z-score values, where a deep red equates to a z-score equal to or less than -3, yellow equates to a z-score value of 0 and a deep green equates to a z-score of 3 or greater.

of 100 μg/ml resulted in lower contents of palmitic acid, which were deemed non-significant as compared with the *M. alpina* wild type parent strain ($p>0.05$). In the case of oleic acid, the content in all isolates generated from 20 to 200 μg/ml, were significantly less ($p<0.05$) than that of the *M. alpina* parent strain (z-score -2.2 to -0.3).

In contrast, significant linolenic acid improvements ($p<0.05$) were observed across various isolates reaching enrichments of 46 % with prolonged exposures in M5F008, attributing to a z-score of 5. Other

notable improvements were observed in M5F016, M5F018, M5F024 and M5F034 which all resulted in z-scores of greater than 2.9 as compared to the *M. alpina* parent strain. These isolates were generated using 5FLU concentrations of 20 and 200 μg/ml. While a concentration of 100 μg/ml resulted in isolates with linolenic acid contents which were not significantly different from the parent strain ($p>0.05$), with the exception of M5F059 which resulted in a z-score of 1.9, attributing to an enrichment of 35 % in comparison with the *M. alpina* parent strain.

The lipid production in the isolates generated from 5FLU concentration of 20 to 200 μg/ml, did not vary significantly from the *M. alpina* parent strain ($p>0.05$), however, isolate M5F051 resulted in a z-score of 1.6, corresponding to an improvement of 30 %. In terms of PUFA content, M5F008, M5F009, M5F016 and M5F018 isolated from 20 and 200 μg/ml of 5FLU, resulted in significant improvement ($p<0.05$) amounts with z-score values above 2.3. However, these isolates resulted in low biomass concentrations, lowering the overall production amounts. A 5FLU concentration of 100 μg/ml resulted in significant ($p<0.05$) PUFA contents (z-score>2.4) in most isolates, with similar biomass content as that of the *M. alpina* parent strain ($p>0.05$). The PUFA contents improved by 24 to 41 % in M5F047, M5F051, M5F063, M5F066 and M5F071 as compared to the *M. alpina* parent strain.

Isolates M5F016 and M5F030 were selected for reproducibility testing, as a result of the greater arachidonic acid content achieved in the isolates generated from the 20 and 200 μg/ml. The results from 4 replicates, demonstrating a variation of less than 10 % among the replicates for the biomass, lipid and FAME profile, not shown here, demonstrated better biomass production as compared to the initial screening, and greater arachidonic acid content improvements as compared to the *M. alpina* wild type. Despite this improvement, the biomass still remained lower than that of the *M. alpina* parent strain and varied by 8 and 3 % in M5F016 and M5F030, respectively, with an arachidonic acid content (% TFA) enrichment of 24 %. Furthermore, the PUFA content in M5F030 improved by 6 % as compared to the original parent strain, however, M5F016 did not demonstrate any improvements.

Arachidonic acid (ARA) which is the main fatty acid this fungal organism is known for producing, resulted in significant improvements in the isolates $p<0.05$, (z-scores>1.5), with improvements of 26 to 39 % observed in M5F008 (z-score 2.1), M5F009 (z-score 3.5), M5F016 (z-score 2.6) and M5F018 (z-score 2.7), Table 2. Interestingly, the higher improvements achieved in these isolates corresponded to the least biomass accumulated. It should be noted that the lower biomass accumulation was observed in flasks where a single clumped colony was observed, which maybe attributed to the presence of agar during transfer.

However, using 5FLU concentration of 100 μg/ml resulted in isolates with significant ARA content ($p<0.05$) in all mutant isolates (z-scores>4.7) as compared to the *M. alpina* parent strain, and the biomass were not significantly different from the *M. alpina* parent strain. The highest ARA content of 22.2 % was achieved in M5F063 which resulted in a z-score of 7.6. M5F066 also resulted in a z-score of 7.2, however, the biomass was not significantly different $p>0.05$ (z-score of -0.1) from the *M. alpina* parent strain. M5F047, on the hand, resulted in a significant ARA content improvement (z-score of 5.0) and biomass (z-score>1.5), as compared to the *M. alpina* parent strain. M5F071 demonstrated good potential with elevated lipid content (z-score of 1.2) and biomass (z-score of 1.37), despite these being deemed insignificant as compared to the *M. alpina* parent strain.

Interestingly, a trend existed between the 5FLU concentration and exposure time on the arachidonic acid content (%TFA) attained in the isolates, Fig. 2. A 5FLU concentration of 20 μg/ml demonstrated an increase in arachidonic acid content from 13.9 to 17.2 % as the exposure time increased from 24 to 120 h, respectively, after which a decrease to 14.7 % was observed at 148 h. A similar trend also existed using a 5FLU concentration of 100 and 200 μg/ml, however, the peak point occurred earlier than that of the lower concentration of 20 μg/ml. Furthermore, the ARA content achieved in isolates using a 5FLU concentration of 200 μg/ml (ARA 12.1-14.8 %) were less than those generated from 20 μg/ml (ARA 13.8-17.2 %), however those achieved from a concentration of 100 μg/ml (ARA 15.6-21.3 %) were greater.

The highest average ARA content of 21.3 % (% of TFA) was achieved here using a 5FLU concentration of 100 μg/ml and 48 h of exposure, Fig. 2. These results suggest that an optimal concentration and exposure period exist for maximum arachidonic acid production.

It is thought that 5FLU stimulates the FA synthesis of the cells as was observed in the non-optimized concentrations of 5FLU (20 and 200 μg/ml), which resulted in significantly greater palmitic acid (C16:0) contents in the isolates. Whereas, in the optimized concentration of 5FLU of 100 μg/ml, the palmitic acid remained similar to the *M. alpina* parent strain, with a lower oleic acid content as it is being actively used in the elongase and desaturase synthesis pathway to produce ARA. This is in agreement with the finding of Hanna et al. (2024), that also noted that *M. alpina* ITS 5FLU mutagenesis of 20 μg/ml coupled with octyl gallate containing agar plates, had optimal exposure time where the palmitic acid and oleic acid levels were lower and increased ARA levels were achieved. They also noted that greater exposures to 5FLU impaired the ARA due to increased inhibition of the DNA, as observed in this study. The differences between the studies being the lower exposure period

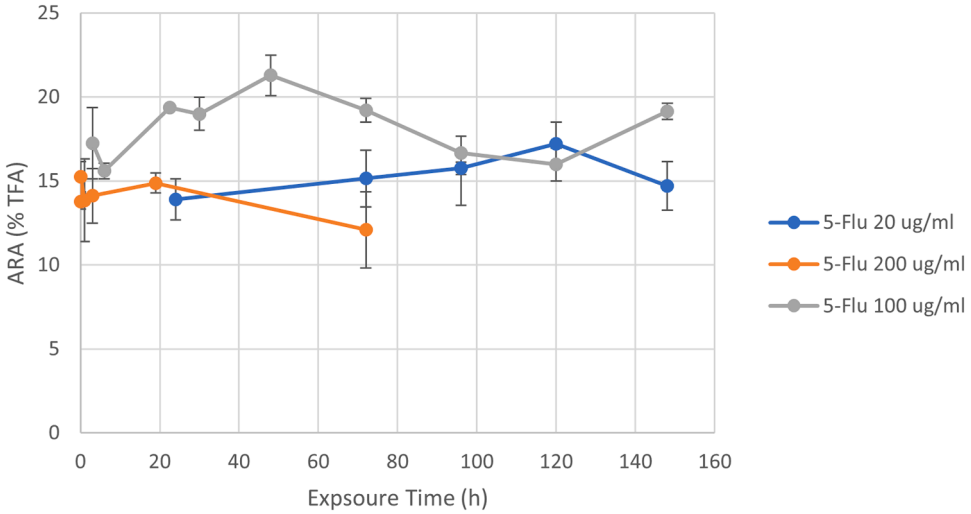

Fig. 2. Impact of 5-fluorouracil concentration and exposure time on ARA (%TFA) content of *M. alpina* isolates. Averages based on at minimum 2 samples.

investigated as the study does not investigate 5FLU treatment alone, but couples it with the secondary octyl gallate treatment.

Zhang et al. (2018), demonstrated a 12% improvement in biomass concentration using 5FLU (20 μg/ml) mutagenesis of *M .alpina* combined with exposure to octyl gallate containing plates. This differs from the present study, as the authors did not investigate 5FLU exposure alone before octyl gallate treatment. The higher biomass improvements observed here maybe attributed to the greater 5FLU concentration (100 μg/ml), as the concentration of 20 μg/ml only resulted in a minor improvement of up to 2.9 %, compared to the *M. alpina* wild type strain. While higher concentrations of the mutagenic agent are thought to induce significant variations compared to the parent strain, excessive concentration may disrupt DNA synthesis, potentially impeding growth, as observed in this study. This is further supported by the colony survival over the tested concentration range, which demonstrated increased colony survival with decreasing agent concentration (Supplementary A2). The lower concentration of 5FLU (20 μg/ml) which was also tested, was perhaps too low to cause significant DNA changes. In the study of Zhang et al. (2018) using 5FLU concentration of 20 μg/ml, they coupled it with the screening agent octyl gallate to limit the isolates to those able to overcome the fatty acid desaturase inhibition.

The FAME lipid classes in the isolates generated from 5FLU at 20 and 200 μg/ml appeared to favor the accumulation of palmitic acid and linolenic acid in some isolates as compared to the parent strain. However, 5FLU at 100 μg/ml resulted in slightly lower saturated fatty acid content, with slightly higher monounsaturated and polyunsaturated fatty acid content in the isolates. Notably, 5FLU at100 μg/ml demonstrated significant improvements in the ARA content of up to 63 %, as compared to the parent strain, which was greater than those achieved with 20 and 200 μg/ml concentrations, Fig. 2. In terms of polyunsaturated fatty acids improvements as compared to the *M. alpina* parent strain, isolates obtained from 100 μg/ml of 5FLU exhibited an average improvement of 23 %, while those obtained from 5FLU of 20 and 200 μg/ml showed an average improvement of only 11 and 8 %, respectively. Similarly, the average arachidonic acid production improvements observed from 5FLU (100 μg/ml) isolates were 40 % as compared to the parent strain, however, 20 and 200 μg/ml of 5FLU exhibited very little average variation in the isolates.

These results suggest that an optimal concentration exists for enhancing PUFA contents, and in particular ARA which is the primary fatty acid *M. alpina* is known for synthesizing. This is also evident in Fig. 2, where increasing the exposure time generally resulted in greater average ARA content in the respective isolates at all tested 5FLU concentrations, until a maximum peak point was reached. However, the peak point reached varied with 5FLU concentration and reached the highest maximum peak at 48 h using a concentration of 100 μg/ml.

In this study we report that the concentration of 5FLU impacted the maximum ARA content attained in mutant isolates, and that a less aggressive concentration (5FLU 100 μg/ml) and slow exposure (48 h) resulted in the greatest ARA content. The recent work of Hanna et al. (2024) also reported that 5FLU coupled with octyl gallate resulted in increased ARA as a function of exposure time, where 3 hours of exposure resulted in the greatest ARA content of 30 %, and prolonged exposure to 18 h resulted in decreased ARA content to 12 %. The higher ARA content achieved as compared to that of this study, maybe attributed to the fermentation duration of 7 days as compared to only 3 days used in this study. However, random mutagenesis studies have selected mutagenic agent concentrations and exposure time on the basis of achieving 70 to 80 % cell death, as this reduces the majority of the cells and therefore only the fittest with enhanced tolerance adaptability survive (Guo et al., 2019; Li et al., 2015; Ou et al., 2023; Zhang et al., 2018). Jeyachandran et al. (2023) also supported that the ideal mutagenic agent dosage in random mutagenesis is the most efficient in the functioning of random screening. Furthermore, the work of Tillich et al. (2012) supported that an optimal concentration of methyl methanesulphonate of 0.1 or 1 %, over the tested range of 0 to 5 %, resulted in the maximum rate of non-lethal point mutations which increased the thermotolerance of *Synechocystis* sp, but increasing the concentration beyond this resulted in rapid cell death.

3.2. TTC stain screening

Isolates were screened using 2,3,5-Triphenyltetrazolium chloride (TTC), a colourless compound that serves as an indicator of desaturase activity. Enzymatic dehydrogenases/desaturases reduce TTC to form triphenylformazan, a red pigment, signaling desaturase activity (Chang et al., 2022). Particularly, this compound was cited in the literature noting pigmentation was used an indicator of ARA and EPA content (Li et al., 2015; Ryan et al., 2010; Zhu et al., 2004). As such, it has been employed in an attempt to quickly identify mutant isolates by visualization, with greater PUFA content in this study. All *M. alpina* mutant isolates and parent strain demonstrated a red pigment in the presence of the compound in 3 days of growth, however, to a varying degree (Supplementary A4). *M. alpina* parent strain (also referred to as "control") demonstrated a light red centre and yellow surrounding. In this study, M5F071 resulted in the greatest PUFA content of 4.3 g/L, followed by M5F051, M5F047 and M5F063 with 4.2, 4.1 and 4.1 g/L, respectively, which all displayed varying degrees of pigmentation. As such, no clear correlation could be drawn at this stage in this study, due to the deep red pigmentation observed in isolates that demonstrated both higher and lower PUFA contents, suggesting the TTC is being reduced, however, it was not an indication of PUFA production ability.

This finding differs from previous studies (Li et al., 2015; Ryan et al., 2010; Zhu et al., 2004), which reported a strong correlation between TTC dye and enhanced ARA content. This variation maybe attributed to the extraction and UV detection methods employed in these studies, whereas, in this study, TTC was trialed as a quick visualization marker. Notably, some isolates appeared entirely red, while others exhibited a central red pigment. However, no conclusive links were evident between this phenomenon and the FAME profile.

3.3. Octyl gallate treatment

Octyl gallate, which has been noted to inhibit the activity of FA desaturases in fungi (Zhang et al., 2018), was used to determine its impact on the isolates as a primary and secondary treatment. A concentration of 14 μg/mL was adopted from the study performed by Zhang et al. (2018), and added into the YPD agar plates, along with the TTC stain. The process followed remained unchanged from that detailed in Fig. 1. For comparison, an initial sample was taken from the mutation flask (prior to the addition 5FLU (100 μg/mL)), to determine the impact of octyl gallate alone as a primary treatment, as it has also been demonstrated to have a cytotoxic and antiproliferative effect on tissue and cells (de Cordova et al., 2011; Jagan et al., 2008) and has been used as a fungicidal agent (Fujita & Kubo, 2002). Following this, 5FLU (100 μg/mL) was added to the mutation flask and samples were drawn at 0, 6, 23, 30 and 46.5 h.

The effect of octyl gallate (14 μg/ml) alone (prior to the exposure to 5FLU) on the biomass, lipid and FAME profile was determined. Interestingly, the isolates did not grow as large as those in the absence of octyl gallate (only grew to half the size based on visual comparison (Supplementary A5), and isolates without exposure to 5FLU grew faster on the octyl gallate agar plates as compared to those exposed to 5FLU for 6 h. It should be noted that the presence of octyl gallate in the agar plates resulted in 23 colonies at time zero (prior to the addition of 5FLU), which is a 70 % reduction in colony number as compared to that without exposure to any agents. Isolates from octyl gallate exposure only, were transferred to fresh agar YPD plates and screened as per the process depicted in Fig. 1.

The FAME profile of octyl gallate treated isolates showed non-significant variation in palmitic acid and linolenic acid, and significantly less oleic acid ($p<0.05$) as compared to the *M. alpina* parent

strain, $p>0.05$ (z-score>1.5), Supplementary A6. On the other hand, arachidonic acid content achieved between 16 to 18 % in all isolates were significantly greater than the *M. alpina* parent strain of 13.6 % ($p<0.05$), with improvements of up to 32 %. Isolate MOG005 demonstrated the highest ARA content of 18 %. All of these isolates exhibited significantly greater PUFA content production with improvements ranging from 16 to 22 %, in comparison with the *M. alpina* parent strain ($p<0.05$). In particular, MOG002 resulted in the greatest PUFA concentration of 1.6 g/L, and also the highest ARA production ability when taking into account the biomass and lipid contents achieved. For these reasons it was selected for reproducibility testing, which resulted (not shown here) in a PUFA content of 1.44 g/L (based on 4 replicates demonstrating less than 10 % variation) as compared to the *M. alpina* parent strain, corresponding to an improvement of 45 %. The biomass ($p<0.05$) and lipid production ($p>0.05$) in the octyl gallate isolates showed no improvements as compared to the *M. alpina* parent strain.

The impact of the antioxidant agent octyl gallate, which has been reported as a strong inhibitor of the Δ6- fatty acid desaturase activity (Kawashima et al., 1996; Zhang et al., 2018), with a cytotoxic and antiproliferative effect on cells (de Cordova et al., 2011; Jagan et al., 2008), showed improvements in arachidonic and polyunsaturated fatty acids as high as 32 and 22 %, respectively, as compared to the *M. alpina* parent strain. This may be attributed to the upregulated activity of the Δ9, Δ12 and Δ6 fatty acid desaturase enzymes, which reduce stearic acid (C18:0) to form oleic acid (C18:1), then to linolenic (C18:2) and linolenic acid (C18:3n6), respectively, as result of the elevated linolenic acid content observed in the octyl gallate isolates as compared to the parent *M. alpina* strain (Supplementary A6). This is also in agreement with the results observed by Li et al. (2015) and Zhang et al. (2018), which noted that octyl gallate has resulted in isolates with upregulated desaturase enzymatic activity. However, no significant improvements were observed in biomass and lipid production as compared to the *M. alpina* parent strain.

The initial exposure of 5FLU (100 μg/ml), followed by a secondary exposure using octyl gallate (14 μg/ml) contained in agar plates resulted in 14 isolates, which were selected for screening, Table 3. Interestingly, the ARA content demonstrated significant improvement (13 to 19 %), as compared to the wild type, in isolates M5F075 to M5F078 ($p<0.05$), which were exposed to 6 hours of 5FLU as a primary treatment followed by plating on octyl gallate containing agar plates (z-score>1.6). The biomass was significantly less ($p<0.05$) as compared to the *M. alpina* parent strain. However, 5FLU exposure of 22.5 h and higher resulted in non-significant ARA contents in isolates as compared to the *M. alpina* parent strain ($p>0.05$), and the biomass was significantly less for these isolates, $p<0.05$ (z-score<-1.5), with the exception of M5F089 which resulted in a non-significant difference. There were no notable improvements in the other fatty acids as compared to the *M. alpina* wild type. In terms of PUFA content, isolate M5F076 is the only one which resulted in a significantly higher PUFA content (z-score of 1.7) as compared with the *M. alpina* parent strain. Other isolates did not demonstrate a notable variation ($p>0.05$).

Interestingly, the addition of 5FLU (100 μg/ml) did not demonstrate further enhancements in ARA, with isolate M5F076 showing the greatest enhancement in ARA and PUFA of 19 and 16 %, respectively, as compared to the *M. alpina* parent strain. The other fatty acid contents remained similar to those achieved with octyl gallate alone, however, the arachidonic acid content achieved from mutant isolates generated from 5FLU (100 μg/ml) alone, were higher than those achieved with the secondary treatment with octyl gallate. The work of Zhang et al. (2018) noted an increase in ARA content (% TFA) in *M. alpina* treated with heavy ion mutagenesis followed by 5FLU (20 μg/ml) and octyl gallate treatment from 39 to 49 %. However, no mention of the impact of 5FLU treatment alone was made. The differences may be attributed to the low concentration of 5FLU used which would limit the DNA interference and perhaps allow more tolerance for secondary octyl gallate treatment. The higher concentration of 5FLU used in this study of 100 μg/ml, resulted in only a handful of isolates available for prospecting with octyl gallate. Future studies testing higher concentrations should consider sampling at

Table 3
M. alpina 5-fluorouracil (100 μg/mL) primary mutagenesis followed by a secondary octyl gallate (14 μg/mL) treatment contained in agar plates.

FAME	*M. alpina*	M5F075	M5F076	M5F077	M5F078	M5F079	M5F080	M5F083	M5F084	M5F085	M5F086	M5F089	M5F090
C12:0	0.3	0.2	0.1	0.2	0.2	0.3	0.1	0.4	0.3	0.2	0.3	0.2	0.4
C14:0	2.7	3	3.1	2.8	2.9	3.1	3.1	3.1	3.3	3	3.2	3.2	3.5
C15:0	0.3	0.2	0.2	0.3	0.2	0.3	0.4	0.3	0.4	0.3	0.2	0.3	0.3
C15:1													
C16:0	24.0	23	23	23.1	23.6	24.4	24.2	23.5	24.1	23.7	24.5	23.8	24.3
C16:1	0.5	0.8	0.5	0.4	0.9	0.6	0.5	0.5	0.5	0.5	0.6	0.6	0.6
C17:0	0.3	0.3	0.4	0.3	0.3	0.3	0.4	0.3	0.3	0.4	0.3	0.4	0.4
C17:1n7													
C18:0	9.8	10.2	9.8	10.3	9.3	9.2	9.3	9.8	9.6	10	9.6	10.2	9.2
C18:1n7													
C18:1n9	26.2	21	20.9	21.8	21.7	24.2	23.9	23	24.3	23.1	21.7	20.7	25.3
C18:2	8.0	10.7	10.7	10.2	10.2	9.4	9.4	9.7	9.6	9.4	10.3	11	9.1
C18:3n6	4.8	4.9	4.8	4.8	4.8	5.1	5.3	5.1	5.1	4.8	4.9	5	5
C19:0													
C20:0	1.1	1	1	1.1	1.1	1.2	1.1	1	0.9	1.2	0.9	1.1	1.1
C20:2													
C20:3n6	2.9	2.9	3.6	3.2	3.4	3.2	3.2	3.6	3.1	3.3	3.2	3.1	2.8
C20:4n6	13.6	15.6	16.2	15.4	15.6	13.6	13.5	14.5	13	14.3	14.6	14.9	13.2
C20:3n3													
C20:5 EPA													
C22:0	1.8	2.2	1.8	2.1	2	1.5	1.7	1.6	1.4	2	1.8	2.2	1.5
C22:5n6 DPA													
C24:0	3.8	4	4.1	4	3.9	3.7	4	3.6	4.2	3.8	4	3.5	3.5
C22:6 DHA													
Biomass (g/L)	15.3	9.9	9.9	10.3	9.6	9.9	8.8	8.8	8.3	8.9	9.2	10.4	7.9
Lipid % (DCW)	42.9	43.4	51	41.4	42.4	36.5	40.2	43.5	41.5	48.4	45.2	43.2	45.2
SFA (%)	44.1	44.2	43.4	44.2	43.5	43.9	44.3	43.6	44.4	44.6	44.8	44.8	44.1
MUFA (%)	25.4	21.7	21.3	22.2	22.5	24.9	24.4	23.5	24.8	23.6	22.3	21.3	25.8
PUFA (%)	30.4	34.1	35.3	33.6	34	31.2	31.3	32.9	30.8	31.8	32.9	34	30.1

FAME: fatty acid methyl ester; TFA: Total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; color indicator presents that the z-score values, where a deep red equates to a z-score equal to or less than -3, yellow equates to a z-score value of 0 and a deep green equates to a z-score of 3 or greater.

lower time intervals, here samples were taken at 6 h periods. It was demonstrated here, that increasing the concentration of the mutagenic agent resulted in a maximum ARA content in isolates much quicker, Fig. 2. Zhang et al. (2018) also noted that secondary mutagenesis with 5FLU (20 μg/ml) resulted in 93% cell death at 36 h, whereas in this study 5FLU (20 μg/ml) exposure of 148 h only achieved 70% cell death (Supplementary A2). Further suggesting that coupling of the treatments significantly limits the colony survival which was observed here to a much quicker extent as a result of the increased concentration.

3.4. Nocodazole treatment

Similarly, nocodazole is an antibiotic known to inhibit the fatty acid synthase thereby altering the fatty acids contents produced (Azam et al., 2015). Additionally, antibiotics have been reported to induce mutagenesis in bacterial populations (Revitt-Mills & Robinson, 2020). As such, not only was nocodazole used to narrow the number of isolates with over active fatty acid synthase (Azam et al., 2015) capable of overcoming the hinderance using the optimized concentration of 5FLU (100 μg/ml), it was also used to assess its impairment on the biomass, lipid and fatty acid production as compared to the parent *M. alpina* strain.

The effect of nocodazole treatment was assessed individually by drawing a sample at time zero from the *M. alpina* parent strain spore containing flask (prior to the addition of 5FLU (100 μg/ml) and spread onto nocodazole containing plates (2.5 μM). The biomass, lipid and FAME profiles achieved from the isolates exposed to nocodazole (2.5 μM) only (Supplementary A7), showed no significant improvements (z-score<1.5) as compared to the *M. alpina* parent strain.

However, the isolates resulted in significant improvements in ARA as compared to the *M. alpina* parent strain with the added exposure to 5FLU ($p<0.05$) in M5F108, M5F112-M5F115 which attributed to z-scores ranging from 1.8 to 2.3, with the highest improvement noted in M5F114, Table 4. No significant (z-score<1.5) improvements were observed in biomass, lipid and other fatty acids, with the exception of M5F108 which demonstrated a significant improvement in PUFA of 15 % (z-score of 1.54). M5F106 demonstrated the greatest lipid content value as compared to the other isolates, despite its non-significance when compared to the *M. alpina* parent strain, thus it was selected for reproducibility testing along with M5F108.

Reproducibly testing based on 2 replicates, demonstrated a variation of less than 10% among the replicates (results not shown here), showed good improvement in ARA, biomass and lipid production of 14, 15 and 19 %, respectively, in M5F106 as compared to the parent strain. However, M5F108 resulted in decreased ARA, lipid and biomass production as compared to the *M. alpina* parent strain.

The antibiotic nocodazole (2.5 μM), capable of inhibiting the fatty acid synthesis pathway (Azam et al., 2015), like that of octyl gallate, was also employed, to narrow down the isolates to those with an over-expression of fatty acid synthesis. As a primary treatment (prior to the addition of 5FLU) isolates demonstrated no significant improvements in biomass, lipid, PFUA and FAME profiles as compared to the *M. alpina* parent strain. This is similar to the results of Azam et al. (2015) which noted a nocodazole concentration of 2 μM resulted in a 40 % biomass reduction as compared to non-exposed biomass. In this study however, the effect was much more pronounced, this maybe attributed to the incorporation method which was on agar plates in this study and in the case of Azam et al. (2015) it was in broth media. Furthermore, when coupled with 5FLU (100 μg/ml) mutagenesis as a secondary treatment, significant improvements (z-score>1.5) as high as 19 % in ARA were achieved. The biomass and lipid production were not significantly different from that of the *M. alpina* parent strain in either the primary or secondary treatment with nocodazole.

Although there was an improvement in ARA content observed with 5FLU and nocodazole, which was not noted with nocodazole alone, the enhancements remained less than those achieved with octyl gallate treatments. Furthermore, these improvements were even less than those achieved with 5FLU mutagenesis alone. The study of Azam et al. (2015), demonstrated that nocodazole (1-10 μM) was an effective inhibitor of saturated fatty acid syntheses in *M. alpina*, where the effect was more

Table 4
M. alpina 5-fluorouracil (100 μg/mL) mutagenesis coupled with nocodazole (2.5 μM) treatment in containing age plates.

FAME	*M. alpina*	M5F104	M5F105	M5F106	M5F107	M5F108	M5F109	M5F110	M5F111	M5F112	M5F113	M5F114	M5F115
C12:0	0.3	0.2	0.5	0.3	0.3	0.2	0.3	0.3	0.2	0.3	0.3	0.2	0.2
C14:0	2.7	2.9	3.4	3.1	3.1	3	3	3	2.9	2.9	3	2.8	2.7
C15:0	0.3	0.3	0.4	0.3	0.2	0.2	0.3	0.2	0.3	0.3	0.2	0.3	0.3
C15:1													
C16:0	24.0	23.4	25.4	23.3	23.5	22.6	23.4	22.4	23.1	22.9	22.9	23	22.8
C16:1	0.5	0.7	0.5	0.5	0.4	0.4	0.5	0.4	0.4	0.5			0.6
C17:0	0.3	0.3	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.3
C17:1n7													
C18:0	9.8	9.6	9.9	9.9	9.4	10	9.3	10.1	9.9	9.9	9.8	9.4	9.1
C18:1n7													
C18:1n9	26.2	21.8	28.5	23	24.4	21.6	24.5	22.4	22.2	22.2	23.3	21.5	23.5
C18:2	8.0	10.6	0.1	10.3	9.9	10.8	10	10.2	10.9	9.6	9.6	10.9	9.7
C18:3n6	4.8	4.8	5.8	4.9	5	4.8	5.1	5.1	5	5	5	5.2	5.3
C19:0													
C20:0	1.1	0.9	0.9	0.9	1	0.9	0.9	1	1	0.9	0.9	1	1
C20:2													
C20:3n6	2.9	3.2	2.6	2.9	2.9	3.3	3	3.2	3.1	3.3	3.4	3.2	3.5
C20:4n6	13.6	15	15.1	14.7	14	16	13.7	15.1	15.1	16	15.6	16.2	15.7
C20:3n3													
C20:5 EPA													
C22:0	1.8	1.8	1.8	1.8	1.8	1.8	1.7	1.9	1.9	1.9	1.8	1.8	1.6
C22:5n6 DPA													
C24:0	3.8	4.3	4.6	3.9	3.8	4.1	3.9	4.5	3.8	4	3.9	4.1	3.7
C22:6 DHA													
Biomass (g/L)	15.3	9.3	7.6	9.4	8	9.6	8.9	9.3	9.8	9.6	9.4	9.4	9.1
Lipid % (DCW)	42.9	41.5	41.5	47.4	40	43.4	43.2	42.6	38	41.2	37.8	42.9	41.5
SFA (%)	44.1	43.9	47.4	43.8	43.5	43.1	43.1	43.6	43.4	43.5	66.1	64.1	41.8
MUFA (%)	25.4	22.6	29	23.5	24.8	22	25.1	22.8	22.6	22.7	9.9	11.3	24.1
PUFA (%)	30.4	33.5	23.6	32.7	31.8	34.9	31.9	33.6	34	33.9	24	24.6	34.1

FAME: fatty acid methyl ester; TFA: Total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; color indicator presents that the z-score values, where a deep red equates to a z-score equal to or less than -3, yellow equates to a z-score value of 0 and a deep green equates to a z-score of 3 or greater.

pronounced with higher concentrations. The authors noted that a concentration of 10 μM resulted in a biomass reduction of more than 50 %. However, in this study a concentration of nocodazole of 5 μM was trialed, but no colony growth was observed, thus half the concentration was investigated. The differences in concentrations maybe attributed to the primary treatment with 5FLU which limited the colony survival prior to nocodazole treatment. The results shown here demonstrate that 5FLU mutagenesis alone proved to be most effective in identifying higher PUFA producing organisms as compared to coupling with the secondary nocodazole agent.

3.5. Diethyl sulfate (DES)

Diethyl sulfate over the concentration range of 0.1 to 1 % was also trailed as a mutagenic agent for bioprospecting of *M. alpina* isolates, which resulted in a colony survival rate of 27 to 0 %, respectively, in 1 hour of exposure, supplementary A2. A 5 % working solution of DES was prepared in ethanol for mutagenic experiments. The methodology followed for mutagenesis is depicted in Fig. 1. Isolates were screened for biomass, lipid production and FAME profile, Table 5.

Diethyl sulfate (DES) was added to 20 mL of 0.9 % NaCl containing 20 μL of *M. alpina* spores to achieve the desired final concentration of 0.1 to 1 %. Samples were taken for plating at initially, 5 min of exposure and every 10 min thereafter for a total exposure time of 1 hour. Plates were left to incubate for 3 days prior to colony counts in an incubator set to 30°C. Complete cell death was achieved within 15 mins of exposure using the higher concentration of 1 %. The colonies formed were isolated by transferring to a fresh YPD plate and kept for 3 d of incubation. Naming annotation provided followed that depicted in supplementary A3.

ARA improvements (z-score>1.5) were achieved in isolates MDS001, MDS010, MDS020, MDS036 and MDS046 as compared to the *M. alpina* parent strain. Significant enhancements (z-score>1.5) were also achieved in linolenic acid in MDS007, MDS012, MDS015, and MDS032 as compared to the *M. alpina* wild type ($p<0.05$). However, no significant enhancements were observed in other FAME. Similarly, the biomass and lipid contents also showed no improvements, with the biomass being significantly less as compared to the *M. alpina* parent strain ($p<0.05$). Interestingly however, the PUFA content was significantly greater (z-score>1.5) as compared to the *M. alpina* parent strain ($p<0.05$) in MDS002, MDS004, MDS015, MDS020, and MDS032.

The improvements observed in ARA with DES treatment at various concentrations maybe attributed to the lower biomass concentrations achieved. The higher lipid concentrations were also noted in isolates which resulted in low biomass. This maybe attributed to the well-known trade-off that exists between lipid and biomass production in the fungi and microbial organisms, in that the cells are either using their energy to produce biomass or convert it into storage lipids (Miranda et al., 2015; Munch et al., 2015; Papanikolaou & Aggelis, 2011). Despite lowering the concentration of DES treatment to 0.1 %, no significant improvements in the biomass or lipid production were achieved.

Reproducibility testing was performed on MDS010 (2 replicates which demonstrated a variation of less than 10%), and improvements were observed in biomass of 10.4 g/L as compared with the initial screening of 4.4 g/L. This corresponded to a decrease in ARA content to 14.3 % (% of TFA) and a decrease in lipid amounts to 43 % (results not shown here). Despite the improvement, the biomass and lipid production values remained less than that of the parent strain *M. alpina*, and the ARA content was not significantly different from the *M. alpina* parent strain.

DES is an alkylating agent, which works by adding an alkyl group to the base pair that hinders the DNA double helix binding (Li et al., 2015), causing a breakage in the strands and results in gene mutation (Guo et al., 2019). In this study, the alterations caused by DES did not demonstrate enhancements in ARA, biomass and lipid as compared to the 5FLU treatment.

The FAME showed greatest improvements in stearic acid in isolates generated from DES of 0.5 % as compared to other concentrations and

Table 5
M. alpina random mutagenesis using diethyl sulfate (0.1 to 1 %), FAME profile (% TFA), biomass and lipid production of isolates.

	DES Concentration (%)												
	-	1	1	1	1	1	1	1	0.5	0.5	0.1	0.1	0.1
FAME	*M. alpina*	MDS001	MDS002	MDS007	MDS010	MDS012	MDS014	MDS015	MDS020	MDS021	MDS032	MDS036	MDS046
C12:0	0.3	1.3	2.1	1.4	0.9	1.6	0.3	0.2	0.1	0.1	0.2	0.1	0.1
C14:0	2.7	3.2	2.7	3	2.7	2.3	2.9	2.5	3	3.2	3.5	3.5	3.5
C15:0	0.3	0.4	0.6	0.6	0.4	1.6	0.4	0.3	0.4	0.4	0.4	0.3	0.3
C15:1													
C16:0	24.0	25.9	26	26.1	26.9	26.7	26	26.1	23.8	23.8	25	23.8	23.2
C16:1	0.5	0.5	0.4	0.4	0.4	0.4	0.5	0.3	0.4	0.5	0.8	0.8	0.5
C17:0	0.3	0.2	0.6	0.6	0.4	1.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
C17:1n7													
C18:0	9.8	10.2	8.8	8.6	10.2	8.8	8.8	9.9	9.7	9.3	8.9	10.4	10.3
C18:1n7													
C18:1n9	26.2	18.8	19.8	21.1	19.4	19.6	20.1	19.8	21	22.8	23	19.6	20
C18:2	8.0	8.2	10.9	10.8	8.5	10.9	9.8	12.2	9.5	9.8	9.3	10.5	10.4
C18:3n6	4.8	4.2	6	6.4	5	6.5	6.3	7	6	6	6.2	5.3	5.2
C19:0													
C20:0	1.1	1.1	0.6	1.3	1.1	0.5	0.7		0.3	0.6	0.8	0.9	1.1
C20:2													
C20:3n6	2.9	3.7	3	2.1	3.5	2.5	2.9	2.9	3.1	3.2	3.5	3.3	3.2
C20:4n6	13.6	15.7	15.3	15.4	16.7	12.6	15.1	14.1	16.8	15.1	13.5	15.4	15.4
C20:3n3													
C20:5 EPA													
C22:0	1.8	1.7	0.3	0.9	1.4		1.1	0.8	1.7	1.1	1.7	1.9	2.7
C22:5n6 DPA													
C24:0	3.8	4.1	1.6	0.7	2	2.7	4.4	2.4	4.1	4.1	2.9	4	3.7
C22:6 DHA													
Biomass (g/L)	15.3	5.2	4.7	7.7	4.4	10.7	7.5	8.9	7.2	6.6	10.2	12	12.3
Lipid % (DCW)	42.9	46.7	46.2	40.6	51.5	51	45.7	47	42.4	26.3	33.3	39.1	39.4
SFA (%)	44.1	48.1	43.3	43.1	45.9	45.5	45	42.7	43.4	42.8	43.7	45.2	45.3
MUFA (%)	25.4	19.3	20.2	21.4	19.8	20	20.5	20.1	21.3	23.2	23.8	20.3	20.4
PUFA (%)	30.4	31.7	35.2	34.7	33.7	32.6	34	36.2	35.3	34	32.6	34.5	34.3

FAME: fatty acid methyl ester; TFA: Total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; color indicator presents that the z-score values, where a deep red equates to a z-score equal to or less than -3, yellow equates to a z-score value of 0 and a deep green equates to a z-score of 3 or greater.

5FLU treatment. The greatest arachidonic acid improvement of 23 % achieved from the various DES concentrations tested, was attained in MDS020 isolated at a concentration of 0.5 %, in comparison with the *M. alpina* original strain. Interestingly, both the ARA and PUFA acid yields showed improvements using a DES concentration of 0.5 % as compared to the other concentrations which showed little to no improvements in the isolates. Despite this, the PUFA and ARA content improvements were greatest in the isolates generated from 5FLU at a concentration of 100 μg/ml. These results are in agreement with the results found by Li et al. (2015), in that the *M. alpina* ARTP generated isolates improved the ARA from 4.56 g/L to 5.09 g/L (10.4 %) with further treatment using DES of 1 %. While Zhang et al. (2018) demonstrated that 5FLU (20 μg/ml), screened with octyl gallate, resulted in an increase in ARA in *M. alpina* mutant isolate generated from heavy ion from 3.8 to 5.26 g/L (38 %).

3.6. Top isolate selection

The three top isolates from the various treatments mentioned above, which exhibited high ARA production potential (M5F047, MOG002 and M5F106), were tested in 200 mL of fermentation setup following the procedure shown in Fig. 1. This step aimed to identify best-performing isolate for further scale-up to 1 L.

The FAME profile achieved in 200 mL fermentation, Fig. 3, for these isolates demonstrated no significant improvement in palmitic, oleic and linolenic acid, despite MOG002 which showed an enhancement of 11 % in palmitic acid, that was deemed non-significant ($p>0.05$) when compared to the *M. alpina* parent strain. However, ARA levels were significant (z-score>2.2) for both M5F047 and M5F106 signifying enrichments of 21 and 17 %, respectively, as compared to the *M. alpina* wild type (Supplementary A8).

All three isolates resulted in lipid production improvements ($p<0.05$) which ranged from 18 to 35 % (z-score>3), as compared to the wild type strain. The highest lipid improvement achieved in MOG002, which was the only isolate that resulted in a significant improvement in PUFA content as compared to the parent strain. However, the biomass production for MOG002 was the lowest achieved as compared to M5F047 and M5F106.

The highest ARA content of 15.2% was achieved in isolate M5F047, which attributed to an improvement of 22 % as compared to the *M. alpina* parent strain. The lower improvement observed at this volume may be ascribed to the significantly lower biomass attained in the *M. alpina* parent strain of 6.9 g/L which resulted in a greater ARA content of 12.9 %, as compared to that achieved by M5F047 of 10 g/L. The lipid content of M5F047 was also 20 % greater as compared to the *M. alpina* parent strain. M5F106 isolated from 5FLU coupled with secondary nocodazole exposure, resulted in the second highest ARA content of 14.7 % with a lipid content of 24.6 %, however, the biomass achieved was only 8.3 g/L. Isolate MOG002, extracted from octyl gallate exposure alone, resulted in the lowest ARA content of 12.8 %, but with the highest lipid content of 25.5 %, which maybe attributed to the lower biomass attained of only 6.9 g/L as compared to the other isolates and the *M. alpina* wild type strain.

In terms of ARA concentration, the greater content observed in M5F047 coupled with the higher biomass and good lipid production, boasts a potential ARA concentration of 0.34 g/L as compared to *M. alpina* wild type, M5F106 and MOG002 which resulted in 0.16, 0.30 and 0.23 g/L, respectively. As such, isolate M5F047 was selected as the top isolate for further scale-up study.

In this study, isolate M5F047 obtained from 5FLU (100 μg/ml) demonstrated superior PUFA and ARA production compared to isolates MOG002 and M5F106, which were the top-performing strains from other treatments. M5F047 achieved ARA and PUFA concentrations of 0.34 and 0.59 g/L, respectively (supplementary A8), representing 2.1 fold and 1.7 fold increases over the the *M. alpina* parent strain in 200 ml of fermentation (supplementary A8). Consequently, isolate M5F047 was selected for further scale-up testing in 1 L of fermentation.

3.7. Scale-up fermentation (1 L)

Scale-up studies were performed on the top performing isolate M5F047, obtained from 5FLU (100 μg/mL), following the methodology depicted in Fig. 1. Interestingly, isolate M5F047 appeared to grow in a spherical “ball” like appearance, while the *M. alpina* parent strain resembled a “mushy” like appearance (Supplementary A9). The total lipid, FAME profile and biomass, presented in Table 6, showed a remarkable ARA content improvement in M5F047 of 67 % as compared to the *M. alpina* wild type, $p<0.05$ (z-score of 7.4). The lipid content also

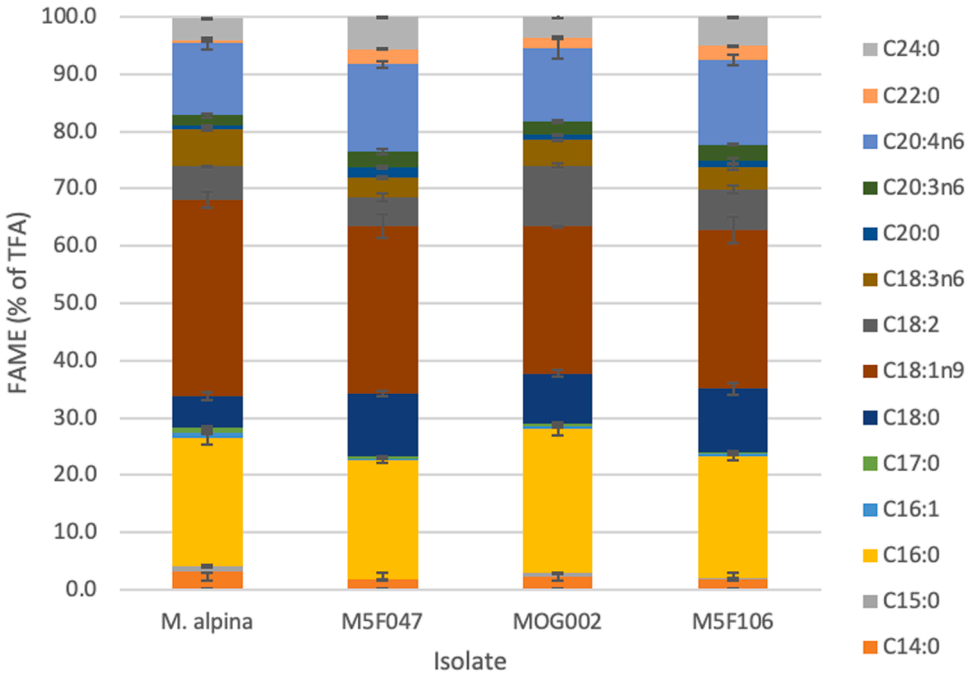

Fig. 3. FAME profile for the top three selected isolates grown in 200 ml fermentation media for 3 days.

Table 6
Scale-up (1 L) fermentation (3 days) of *M. alpina* and M5F047 isolate obtained using 5-fluorouracil (100 μg/mL).

FAME	*M. alpina*	M5F047
C16:0	28.7±0.5	23.1±0.3
C18:0	4.9±0.5	11.0±0.2
C18:1n9	39.2±0.2	26.6±0.2
C18:2	2.3±0.03	8.4±0.02
C18:3n6	3.0±0.4	3.3±0.1
C20:0	1.1±0.1	1.7±0.1
C20:4n6	7.7±0.7	12.9±0.1
C22:0	0.6±0.2	2.5±0.1
C24:0	3.4±0.1	5.3±0.1
Biomass (g/L)	9.9±0.9	8.4±1.1
Lipid % (DCW)	31.1±2.9	40.4±5.6
ARA concentration (g/L)	0.24±0.1	0.44±0.1
SFA (%)	44.9±0.9	46.1±0.1
MUFA (%)	41.5±0.2	27.2±0.3
PUFA (%)	13.6±1.1	26.7±0.3

FAME: fatty acid methyl ester; TFA: Total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; color indicator presents that the z-score values, where a deep red equates to a z-score equal to or less than -3, yellow equates to a z-score value of 0 and a deep green equates to a z-score of 3 or greater.

significantly improved by 29 % (z-score>3.2) as compared to the *M. alpina* wild strain ($p<0.05$). However, the biomass concentration decreased by 14 % compared to parent strain ($p<0.05$), but the greater ARA content and lipid amounts compensate for this as suggested by the potential ARA concentration (0.44 g/L) which nearly doubles that of the parent strain (0.24 g/L).

Furthermore, isolate M5F047 exhibited strong stability over 8 generations of re-growth, consistently producing higher ARA levels compared to the *M. alpina* wild type, with an enhancement of 1.5 to 2 times greater, across different fermentation volumes. The final generation, tested at a larger scale (1 L fermentation), demonstrated a 67 % enhancement in ARA production compared to the parent strain, highlighting its stability and regenerative capacity- key attributes for industrial applications. Similalry, the study by Zhang et al. (2018) reported good genetic stability in *M. alpina* showing a fluctuation of only 11% in ARA content over 10 generations.

In this study, the enhanced PUFA content, nearly doubles that of the *M. alpina* parent strain in the 1 L fermentation, further supports its potential for sustainable PUFA production as an alternative to conventional fish-derived sources. These PUFA could play a crucial role in enhancing plant-based meat flavours, improving consumer acceptance, as lipid precursors are key drivers of flavour development (Sun et al., 2022).

The results presented here are significant as they demonstrate an isolate with an enhanced ARA to biomass ratio compared to the parent strain, which is one of the key characteristics for industrially viable strains. Although, Zhang et al. (2018) reported a mutant isolate with an arachidonic acid yield nearly 4 times higher than *M. alpina* parent strain, which is considerably greater than the yields achieved in this study, this difference maybe attributed to the longer fermentation period of 7 days used by the authors, whereas this study employed a shorter 3-day fermentation.

Nevertheless, further scale-up fermentation testing at 30 L and pilot scale levels are necessary to better understand the strain's physiology and its interactions within the bioreactor environment, ultimately assessing its economic viability (Burgstaller et al., 2023). However, these preliminary findings highlight the strain's enhanced ARA production potential compared to its *M. alpina* parent strain.

The production of plant-based meats requires replicating meaty flavours to enhance consumer acceptance and experience (Rout & Srivastav, 2024; Singh et al., 2021). Lipid precursors, particularly triglycerides and phospholipids, play a crucial role in flavour formation (Shahidi & Hossain, 2022). Arachidonic acid has been linked to the rich umami flavour of chicken (Takahashi) and pork (Duan et al., 2024). The enhanced arachidonic acid production observed in isolates treated with 100 μg/ml of 5FLU, demonstrates strong potential for improving the sensory appeal of future plant-based meats. *M. alpina* ARA- rich oil received the generally recognized as safe (GRAS) approval from the FDA in 2001 (Jovanovic et al., 2021; Li et al., 2023). Furthermore, as random mutagenesis techniques are not classified as genetic modification, the isolate M5F047 generated from 5FLU mutagenesis is not subject to the regulatory challenges and scrutiny faced by genetically modified organisms, facilitating its potential commercialisation (Augustin et al., 2024).

4. Conclusions

In comparing various random mutagenesis treatments and approaches on *M. alpina*, chemical mutagenesis demonstrated greater enhancements as these agents directly induce changes in the nucleic acid of the cell as compared to physical techniques such as UV radiation. The chemical random mutagenesis treatments investigated in this study, revealed that 5-fluorouracil concentration and exposure time significantly influenced arachidonic acid content. The optimal conditions were identified as 100 μg/ml conentration and 48 h of exposure. Increasing the exposure time resulted in increased arachidonic acid content until a maximum point was reached, with further exposures decreasing the content. The maximum point reached appeared sooner with increased concentrations. 5-fluorouracil (100 μg/ml) treatment alone was most superior in generating an isolate with enhanced lipid, PUFA and ARA contents as compared to diethyl sulfate and coupling with octyl gallate and nocodazole agents. Isolate M5F047 and *M. alpina* (parent strain) exhibited an arachidonic acid and polyunsaturated fatty acid content of 12.9 and 26.7 %, and 13.6 and 7.7 %, respectively, which nearly doubled that of the parent strain for PUFA and were more than 1.5 times for ARA in 1 L fermentation kept for 3 days. This was achieved with the

8th re-generation of the isolate, demonstrating its stability and potential as an additive in plant-based meats to improve the meaty flavour and consumer acceptance and experience. *M. alpina* ARA oil has gained the generally regarded as safe (GRAS) status, and the isolate enhancements random mutagenesis approach employed here, steers away from the commercialization hurdles associated with genetically modified organisms. Nevertheless, future work is needed to better understand the impact of these agents on fatty acid synthase activity and DNA alterations through gene expression analysis and whole genome sequencing to confirm mutation. Further stability testing is required to ensure consistent expression of the desired fatty acid profile, along with scale-up and pilot scale trials to assess commercialization feasibility. Additionally, fermentation optimization strategies will be explored to further enhance the ARA and PUFA production.

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Ethical Statement

The authors declare that an ethical statement is not required as the work presented here is not related to human or animal studies.

CRediT authorship contribution statement

Mariam Alhattab: Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Juliana Lebeau:** Writing – review & editing, Visualization, Supervision, Data curation. **Surinder Singh:** Writing – review & editing, Supervision. **Munish Puri:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2025.100605.

Data availability

The data is attached the data in supplementary file.

References

Alhattab, M., Moorthy, L.S., Patel, D., Franco, C.M., Puri, M., 2024. Oleaginous microbial lipids' Potential in the prevention and treatment of neurological disorders. Mar. Drugs 22 (2), 80.

Ari, M., Marzie, Dadgar, L., Elahi, Z., Ghanavati, R., Taheri, B., 2024. Genetically engineered microorganisms and their impact on human health. Int. J. Clin. Pract. 2024 (1), 6638269.

Arora, N., Yen, H.-W., Philippidis, G.P., 2020. Harnessing the power of mutagenesis and adaptive laboratory evolution for high lipid production by oleaginous microalgae and yeasts. Sustainability. (12), 12. https://doi.org/10.3390/su12125125.

Augustin, M.A., Hartley, C.J., Maloney, G., Tyndall, S., 2024. Innovation in precision fermentation for food ingredients. Crit. Rev. Food Sci. Nutr. 64 (18), 6218–6238. https://doi.org/10.1080/10408398.2023.2166014.

Azam, M.S., Gu, Z., Chen, H., Chen, Y.Q., 2015. Using Mortierella alpina as a novel platform for lipid synthesis inhibitor screening. Adv. Biosci. Bioeng 3 (1), 1.

Bajić, B., Vučurović, D., Vasić, Đ., Jevtić-Mučibabić, R., Dodić, S., 2022. Biotechnological production of sustainable microbial proteins from agro-industrial residues and by-products. Foods. 12 (1), 107.

Beacham, T.A., Macia, V.M., Rooks, P., White, D.A., Ali, S.T., 2015. Altered lipid accumulation in *Nannochloropsis salina* CCAP849/3 following EMS and UV induced mutagenesis. Biotechnol. Rep. (Amst) 7, 87–94. https://doi.org/10.1016/j.btre.2015.05.007.

Burgstaller, L., Oliver, L., Dietrich, T., Neureiter, M., 2023. Pilot scale production of single cell oil by Apiotrichum brassicae and Pichia kudriavzevii from acetic acid and propionic acid. Appl. Sci. 13 (8), 4674.

Caporgno, M.P., Mathys, A., 2018. Trends in microalgae incorporation into innovative food products with potential health benefits. Front. Nutr. 5, 58.

Chang, L., Lu, H., Chen, H., Tang, X., Zhao, J., Zhang, H., Chen, Y.Q., Chen, W., 2022. Lipid metabolism research in oleaginous fungus mortierella alpina: current progress and future prospects. Biotechnol. Adv. 54, 107794.

Chaturvedi, R., Fujita, Y., 2006. Isolation of enhanced eicosapentaenoic acid producing mutants of *nannochloropsis oculata* ST-6 using ethyl methane sulfonate induced mutagenesis techniques and their characterization at mRNA transcript level. Phycol. Res 54 (3), 208–219.

Cho, J.S., Kim, G.B., Eun, H., Moon, C.W., Lee, S.Y., 2022. Designing microbial cell factories for the production of chemicals. JACS. Au 2 (8), 1781–1799.

CMI, (2023). *Polyunsaturated fatty acids market analysis and forecast 2032.* Coherent Market Insights. Retrieved 2/03/2025 from https://www.coherentmarketinsights.com/market-insight/polyunsaturated-fatty-acids-market-3986.

Cortez, R., Guevara, M., Bauza, R., Freites, L., Brito, D., Rosales, N., Lodeiros, C., 2015. Incremento del contenido de lípidos y de ácidos grasos poliinsaturados de una cepa de *Tetraselmis tetrathele* a través de mutación-selección. Translation: increase of the content of lipids and polyunsaturated fatty acids of a strain of *Tetraselmis tetrathele* through mutation-selection. Interciencia 40 (3), 204–209.

de Cordova, C.A., Locatelli, C., Assunção, L.S., Mattei, B., Mascarello, A., Winter, E., Nunes, R.J., Yunes, R.A., Creczynski-Pasa, T.B., 2011. Octyl and dodecyl gallates induce oxidative stress and apoptosis in a melanoma cell line. Toxicol. Vitro 25 (8), 2025–2034.

Duan, S., Tian, Z., Zheng, X., Tang, X., Li, W., Huang, X., 2024. Characterization of flavour components and identification of lipid flavour precursors in different cuts of pork by phospholipidomics. Food Chem. 458, 139422. https://doi.org/10.1016/j.foodchem.2024.139422.

FAO. (2020). *The State of World fisheries and aquaculture 2020. Sustainability in action.* Rome: FAO.

Ferreira, D.J.M., Noble, J., 2019. Yeast strain optimization for enological applications. Advances in Grape and Wine Biotechnology. IntechOpen.

Fujita, K.-i., Kubo, I., 2002. Antifungal activity of octyl gallate. Int. J. Food Microbiol. 79 (3), 193–201.

Glencross, B.D., 2020. Mind the (supply) gap. Nat. Food 1 (1), 26. https://doi.org/10.1038/s43016-019-0022-0. -26.

Guo, J., Liu, C., Zhang, C., Wang, J., Wang, S., Liu, X., Gao, X., Wu, X., 2019. Nitrous acid and diethyl sulfate, as chemical mutagenic agents, to improve the biomass, metabolites, enzyme activity, and antioxidant activity of wild *Phellinus igniarius*. Food Sci. Technol. Res 25 (3), 459–466. https://doi.org/10.3136/fstr.25.459.

Gupta, A., Singh, D., Barrow, C.J., Puri, M., 2013. Exploring potential use of Australian thraustochytrids for the bioconversion of glycerol to omega-3 and carotenoids production. Biochem. Eng. J. 78, 11–17. https://doi.org/10.1016/j.bej.2013.04.028.

Hanna, V.S., Abd El-Ghany, M.N., Ibrahim, M.I., Abdel-Rahman, T.M., Tallima, H., 2024. Novel approaches to mortierella alpina identification and arachidonic acid production optimization. ACS. Omega 9 (32), 34456–34463.

Hlavova, M., Turoczy, Z., Bisova, K., 2015. Improving microalgae for biotechnology—From genetics to synthetic biology. Biotechnol. Adv. 33 (6), 1194–1203.

Hu, W., Li, W., Chen, J., 2017. Recent advances of microbial breeding via heavy-ion mutagenesis at IMP. Lett. Appl. Microbiol. 65 (4), 274–280. https://doi.org/10.1111/lam.12780.

Jagan, S., Ramakrishnan, G., Anandakumar, P., Kamaraj, S., Devaki, T., 2008. Antiproliferative potential of gallic acid against diethylnitrosamine-induced rat hepatocellular carcinoma. Mol. Cell Biochem. 319, 51–59.

Jareonkitmongkol, S., Kawashima, H., Shirasaka, N., Shimizu, S., Yamada, H., 1992. Production of dihomo-γ-linolenic acid by a Δ5-desaturase-defective mutant of *Mortierella alpina* 1S-4. Appl. Environ. Microbiol. 58 (7), 2196–2200.

Jareonkitmongkol, S., Shimizu, S., Yamada, H., 1992. Fatty acid desaturation-defective mutants of an arachidonic-acid-producing fungus, *Mortierella alpina* 1S-4. Microbiology (N. Y) 138 (5), 997–1002.

Jeyachandran, S., Vibhute, P., Kumar, D., Ragavendran, C., 2023. Random mutagenesis as a tool for industrial strain improvement for enhanced production of antibiotics: a review. Mol. Biol. Rep. 51 (1), 19. https://doi.org/10.1007/s11033-023-08975-4.

Jovanovic, S., Dietrich, D., Becker, J., Kohlstedt, M., Wittmann, C., 2021. Microbial production of polyunsaturated fatty acids — High-value ingredients for aquafeed, superfoods, and pharmaceuticals. Curr. Opin. Biotechnol. 69, 199–211. https://doi.org/10.1016/j.copbio.2021.01.009.

Karageorgou, D., Rova, U., Christakopoulos, P., Katapodis, P., Matsakas, L., Patel, A., 2023. Benefits of supplementation with microbial omega-3 fatty acids on human health and the current market scenario for fish-free omega-3 fatty acid. Trends. Food Sci. Technol. 136, 169–180. https://doi.org/10.1016/j.tifs.2023.04.018.

Karalis, D.T., Karalis, T., Karalis, S., Kleisiari, A.S., 2020. Genetically modified products, perspectives and challenges. Cureus. 12 (3), e7306. https://doi.org/10.7759/cureus.7306.

Kawashima, H., Akimoto, K., Shirasaka, N., Shimizu, S., 1996. Inhibitory effects of alkyl gallate and its derivatives on fatty acid desaturation. Biochimica et Biophysica Acta (BBA)-Lipids Lipid Metabol 1299 (1), 34–38.
Kawashima, H., Nishihara, M., Hirano, Y., Kamada, N., Akimoto, K., Konishi, K., Shimizu, S., 1997. Production of 5, 8, 11-eicosatrienoic acid (Mead acid) by a (Delta) 6 desaturation activity-enhanced mutant derived from a (Delta) 12 desaturase-defective mutant of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4. Appl. Environ. Microbiol. 63 (5), 1820–1825.
Kelling, M., Dimza, M., Bartlett, A., Traktuev, D.O., Duarte, J.D., Keeley, E.C., 2024. Omega-3 fatty acids in the treatment of heart failure. Curr. Probl. Cardiol., 102730
Kumar, S.D., Sojin, K., Santhanam, P., Dhanalakshmi, B., Latha, S., Park, M.S., Kim, M.-K., 2018. Triggering of fatty acids on *Tetraselmis* sp. by ethyl methanesulfonate mutagenic treatment. Bioresour. Technol. Rep. 2, 21–28.
Li, J., Chen, H., Chang, L., Wu, C., Zhang, H., Chen, Y.Q., Chen, W., 2023. Functions and substrate selectivity of diacylglycerol acyltransferases from Mortierella alpina. Appl. Microbiol. Biotechnol. 107 (18), 5761–5774. https://doi.org/10.1007/s00253-023-12694-y.
Li, X., Liu, R., Li, J., Chang, M., Liu, Y., Jin, Q., Wang, X., 2015. Enhanced arachidonic acid production from *Mortierella alpina* combining atmospheric and room temperature plasma (ARTP) and diethyl sulfate treatments. Bioresour. Technol. 177, 134–140. https://doi.org/10.1016/j.biortech.2014.11.051.
Miranda, A.F., Taha, M., Wrede, D., Morrison, P., Ball, A.S., Stevenson, T., Mouradov, A., 2015. Lipid production in association of filamentous fungi with genetically modified cyanobacterial cells. Biotechnol. Biofuels. 8 (1), 179. https://doi.org/10.1186/s13068-015-0364-2.
Moha-León, J.D., Pérez-Legaspi, I.A., Ortega-Clemente, L.A., Rubio-Franchini, I., Ríos-Leal, E., 2019. Improving the lipid content of *Nannochloropsis oculata* by a mutation-selection program using UV radiation and quizalofop. J. Appl. Phycol. 31 (1), 191–199. https://doi.org/10.1007/s10811-018-1568-1.
Munch, G., Sestric, R., Sparling, R., Levin, D.B., Cicek, N., 2015. Lipid production in the under-characterized oleaginous yeasts, rhodosporidium babjevae and rhodosporidium diobovatum, from biodiesel-derived waste glycerol. Bioresour. Technol. 185, 49–55. https://doi.org/10.1016/j.biortech.2015.02.051.
Nisha, A., 2009. Biotechnological Studies for the Production of Arachidonic Acid from Mortierella Alpina. University of Mysore.
Ou, Y., Li, Y., Feng, S., Wang, Q., Yang, H., 2023. Transcriptome analysis reveals an eicosapentaenoic acid accumulation mechanism in a schizochytrium sp. Mutant. Microbiol. Spectr. 11 (3), e00130. -00123.
Papanikolaou, S., Aggelis, G., 2011. Lipids of oleaginous yeasts. Part I: biochemistry of single cell oil production. Eur. J. Lipid Sci. Technol 113 (8), 1031–1051.
Pappalardo, G., D'Amico, M., Lusk, J.L., 2021. Comparing the views of the Italian general public and scientists on GMOs. Int. J. Food Sci. Technol. 56 (7), 3641–3650.
Parekh, S., Vinci, V.A., Strobel, R.J., 2000. Improvement of microbial strains and fermentation processes. Appl. Microbiol. Biotechnol. 54 (3), 287–301. https://doi.org/10.1007/s002530000403.
Patel, A., Karageorgou, D., Katapodis, P., Sharma, A., Rova, U., Christakopoulos, P., Matsakas, L., 2021. Bioprospecting of thraustochytrids for omega-3 fatty acids: A sustainable approach to reduce dependency on animal sources. Trends. Food Sci. Technol. 115, 433–444. https://doi.org/10.1016/j.tifs.2021.06.044.
Patel, A., Matsakas, L., Hrůzová, K., Rova, U., Christakopoulos, P., 2019. Biosynthesis of nutraceutical fatty acids by the oleaginous marine microalgae phaeodactylum tricornutum utilizing hydrolysates from organosolv-pretreated birch and spruce biomass. Mar. Drugs 17 (2), 119.
Revitt-Mills, S.A., Robinson, A., 2020. Antibiotic-induced mutagenesis: under the microscope. Front. Microbiol. 11, 2611.
Rout, S., Srivastav, P.P., 2024. A review on development of plant-based meat analogues as future sustainable food. Int. J. Food Sci. Technol. 59 (1), 481–487.
Ryan, J., Farr, H., Visnovsky, S., Vyssotski, M., Visnovsky, G., 2010. A rapid method for the isolation of eicosapentaenoic acid-producing marine bacteria. J. Microbiol. Methods 82 (1), 49–53. https://doi.org/10.1016/j.mimet.2010.04.001.
Rzymski, P., Królczyk, A., 2016. Attitudes toward genetically modified organisms in Poland: to GMO or not to GMO? Food Secur. 8 (3), 689–697. https://doi.org/10.1007/s12571-016-0572-z.
Sakuradani, E., Hirano, Y., Kamada, N., Nojiri, M., Ogawa, J., Shimizu, S., 2004. Improvement of arachidonic acid production by mutants with lower n-3 desaturation activity derived from *Mortierella alpina* 1S-4. Appl. Microbiol. Biotechnol. 66 (3), 243–248.
Shahidi, F., Hossain, A., 2022. Role of lipids in food flavor generation. Molecules. (15), 27. https://doi.org/10.3390/molecules27155014.
Shim, J.E., Lee, Y., Song, S., 2023. Trends in dietary intake and food sources of long-chain polyunsaturated fatty acids among Korean adults between 2007 and 2018. Epidemiol. Health 45.
Shimizu, S., Jareonkitmongkol, S., Kawashima, H., Akimoto, K., Yamada, H., 1992. Inhibitory effect of curcumin on fatty acid desaturation in *mortierella alpina* 1S-4 and rat liver microsomes. Lipids 27 (7), 509–512.
Singh, M., Trivedi, N., Enamala, M.K., Kuppam, C., Parikh, P., Nikolova, M.P., Chavali, M., 2021. Plant-based meat analogue (PBMA) as a sustainable food: a concise review. Eur. Food Res. Technol 247 (10), 2499–2526. https://doi.org/10.1007/s00217-021-03810-1.
Song, M., Pei, H., 2018. The growth and lipid accumulation of *scenedesmus quadricauda* during batch mixotrophic/heterotrophic cultivation using xylose as a carbon source. Bioresour. Technol. 263, 525–531.
Srimongkol, P., Sangtanoo, P., Songserm, P., Watsuntorn, W., Karnchanatat, A., 2022. Microalgae-based wastewater treatment for developing economic and environmental sustainability: current status and future prospects. Front. Bioeng. Biotechnol. 10.
Sun, A., Wu, W., Soladoye, O.P., Aluko, R.E., Bak, K.H., Fu, Y., Zhang, Y., 2022. Maillard reaction of food-derived peptides as a potential route to generate meat flavor compounds: a review. Food Res. Int 151, 110823. https://doi.org/10.1016/j.foodres.2021.110823.
Swanson, D., Block, R., Mousa, S.A., 2012. Omega-3 fatty acids EPA and DHA: health benefits throughout life. Adv. Nutr. 3 (1), 1–7. https://doi.org/10.3945/an.111.000893.
Tanaka, A., Shikazono, N., Hase, Y., 2010. Studies on biological effects of ion beams on lethality, molecular nature of mutation, mutation rate, and spectrum of mutation phenotype for mutation breeding in higher plants. J. Radiat. Res. 51 (3), 223–233. https://doi.org/10.1269/jrr.09143.
Teng, T.S., Chin, Y.L., Chai, K.F., Chen, W.N., 2021. Fermentation for future food systems: precision fermentation can complement the scope and applications of traditional fermentation. EMBo Rep. 22 (5), e52680.
Tillich, U.M., Lehmann, S., Schulze, K., Dühring, U., Frohme, M., 2012. The optimal mutagen dosage to induce point-mutations in synechocystis sp. PCC6803 and its application to promote temperature tolerance. PLoS. One 7 (11), e49467. https://doi.org/10.1371/journal.pone.0049467.
Trovão, M., Schüler, L.M., Machado, A., Bombo, G., Navalho, S., Barros, A., Pereira, H., Silva, J., Freitas, F., Varela, J., 2022. Random mutagenesis as a promising tool for microalgal strain improvement towards industrial production. Mar. Drugs 20 (7), 440. https://www.mdpi.com/1660-3397/20/7/440.
Vadivelan, G., Venkateswaran, G., 2014. Production and enhancement of omega-3 fatty acid from *Mortierella alpina* CFR-GV15: its food and therapeutic application. Biomed. Res. Int. *2014*.
Zhang, H., Lu, D., Li, X., Feng, Y., Cui, Q., Song, X., 2018. Heavy ion mutagenesis combined with triclosan screening provides a new strategy for improving the arachidonic acid yield in *Mortierella alpina*. BMC. Biotechnol. 18 (1), 23. https://doi.org/10.1186/s12896-018-0437-y.
Zhao, B., Li, Y., Li, C., Yang, H., Wang, W., 2018. Enhancement of *schizochytrium* DHA synthesis by plasma mutagenesis aided with malonic acid and zeocin screening. Appl. Microbiol. Biotechnol. 102 (5), 2351–2361. https://doi.org/10.1007/s00253-018-8756-4.
Zhu, M., Yu, L., Liu, Z., Xu, H., 2004. Isolating *Mortierella alpina* strains of high yield of arachidonic acid. Lett. Appl. Microbiol. 39 (4), 332–335.