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UNSATURATED FATTY ACIDS PRODUCTION BY OLEAGINOUS YEAST OBTAINED FROM DIFFERENT MARINE SOURCES

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) play critical roles in human diets as well as in the prevention and control of several chronic diseases and for improvement of heart and brain functions, so its demand is growing. A ten yeast isolates were isolated from different marine habitats. The ability of these isolates to accumulate intracellular PUFAs was primary screened using Nile red and further screened using Triphenyltetrazolium chloride (TTC) stain at an absorbance of 485 nm. Amongst these isolates, four yeast isolates (namely D1, E2, G2, and W6) were exhibited the best results for PUFAs production. The E2 and W6 isolates showed higher lipid accumulation than G2 and D1 isolates with percent between 25 to more than 35% of the cell area with the Nile red with staining degree of 1.43, 1.30, 1.20, and 1.12, with the TTC dye, respectively. When the isolates were cultured on YPD medium at 25°C, isolate E2 yielded 29.3% lipid per dry biomass, while isolates W6 yielded 26.2%, G2 yielded 25.6%, and D1 yielded 23.4%. These isolates were identified based on phenotypic and genotypic characteristics as *Candida parapsilosis* E2, *Candida parapsilosis* D1, *Pichia kuriavzevii* W6, and *Meyerozyma guilliermondii* G2. Production of PUFAs was conducted under nitrogen-limited conditions using a shake flask technique. The fatty acids profile analysis showed that the lipid extracted from these strains contained oleic acid (18:1), linoleic acid (18:2), and palmitic acid (C16:0) as the major intracellular fatty acids produced that accounted for 90% of total lipids. Also, these strains displayed (27-32%) saturated fatty acids and produced a significant amount of unsaturated fatty acids ranged between 68 to 73 % of the total fatty acids make them a sustainable source for single-cell oil productionas well as pharmaceutical and nutritional industries.

KEYWORDS: Unsaturated fatty acids, Lipid production, Lipid screening, Oleaginous yeast, *Candida parapsilosis, Pichia kuriavzevii*, *Meyerozyma guilliermondii.*

1-INTRODUCTION

Essential fatty acids are vital components of structural lipids and contribute to the regulation of membrane properties like fluidity, flexibility, permeability, and modulation of membrane-bound proteins.[1] Fatty acids are valuable for human nutrition and health and show big compositional differences. Many studies have positively correlated essential fatty acids as a novel approaches to treat and prevent many diseases like cardiovascular morbidity and mortality, infant development, cancer prevention, optimal brain and vision functioning, hypertension, arthritis, diabetes mellitus, and neurological/neuropsychiatric disorders.[2]

Polyunsaturated fatty acids (PUFAs), particularly, omega-6 and omega-3 fatty acids are important bioactive nutrients that regulate several physiological parameters.[3] Although fish is the main source of Long Chain Polyunsaturated Fatty Acids (LC-PUFAs), there are several limitations in using fish oil as a source of essential fatty acids including: (1) the presence of teratogen, a carcinogen, and mutagen contaminants, (2) non-carcinogen contaminants such as methyl mercury, heavy metals, and antibiotics, fish oils possess undesirable odors, flavors, and tastes, (3) stability problems, (4) high cost and difficulty of purification, and (5) populations where pregnant and lactating women and young children have a higher risk for marine pollution.[4] Besides that, the decreasing fish stocks throughout the world have raised concerns about these resources. Therefore, different attempts have been carried out to supply LC-PUFA with alternative safe sources. Under appropriate cultivation conditions, oleaginous microorganisms can accumulate high amounts of lipids. Therefore, their potential for application as lipid producing sources has attracted much attention.[4, 5]

Microbial oil has the potential to substitute the plant oil in the market. Microorganisms that produce lipid more than 20% of their biomass are called oleaginous.[6] Oleaginous yeasts attract much attention because of their high growth rate and their ability to use different carbon sources. Unlike other oil-producing microorganisms, such as microalgae, oleaginous yeast does not require a long fermentation period and their resulting lipid profiles could be simply manipulated by varying the fermentation conditions.[7] They can also utilize low-cost substrates as waste materials such sugarcane molasses, crude glycerol, agricultural and industrial products and can store lipids in the form of triacylglycerol which considers the main objectives for improving the biotechnological products.[8, 9] Therefore, the aim of this study was the isolation and screening of oleaginous yeasts with high potential for lipid production from marine environments, identifying the most promising isolates, and evaluating fatty acids content by each strain that can be used for several biotechnological applications.

2. MATERIALS AND METHODS

2.1. Samples collection

Eight seawater and sediment samples were collected from the Mediterranean Sea in Alexandria (Abu Qir Bay) and the Red Sea in the Suez Canal (Suez Bay) in Egypt. The collected samples were stored at 4 °C in sterile plastic bags and transported to the laboratory for yeast isolation.

2.2. Isolation and cultivation conditions

Basal liquid medium was used for isolation and cultivation of oleaginous marine yeast. This medium prepared by natural seawater and consisted of (g/L); glucose 20, yeast extract 10.0, and peptone 20.0. The medium pH was adjusted to 5.5 before sterilization. After 5 days of incubation, cell mass was harvested for fatty acid analysis.[10] The potential oleaginous yeast colonies for lipid accumulation were grown in Glycerol & YPD (1:1) media which contained (g/L): glucose 20, peptone 20, and yeast extract 10 at 4 °C and continuously transferred once every two months. Stock cultures were incubated for two days and then stored in a refrigerator before use.

2.3. Screening techniques for polyunsaturated fatty acid production

The isolated yeast colonies grown on a basal liquid medium to test their abilities to produce polyunsaturated fatty acids and the screening was conducted by using two different techniques. The first was conducted by fluorescence methods using Nile Red staining according to Kimura *et al.,* (2004).[11] The yeast isolates were examined using a fluorescence microscope (Olympus BX 40) and the isolates which give positive (the greatest intensity of light) for lipid production capacity were selected as single cell oil producers. The second technique by the colorimetric method using Triphenyltetrazolium chloride (TTC) an oxidant that could be reduced from colorless compound to red color compound triphenylformazan [TF]**.** In brief, solution of 0.6% TTC was prepared bydissolve 0.6 g of TTC powder 100 ml of 0.5 mol^{-1} phosphate buffer (pH 7.8) at room temperature then sterilized through 0.22 μm filter and stored at −20°C until use.[12] Fresh samples of yeast cells were harvested using suction filtration and the staining procedures were done according to Vadivelan & Venkateswaran (2014) method with some modification using methanol instead of ethyl acetate. The staining level was quantified by measuring the absorbance of TF in the methanol solvent at wavelength 485nm.[13] The yeast isolates with positive results were picked up for further characterization and identification.

2.4. Identification of the most promising yeast isolates

2.4.1. Phenotypic characteristics

Morphological properties of the most promising yeast isolates including surface appearance, color, margin, and elevation were investigated on YPD agar plates after incubation at 25 °C for 48 hr. Purity and cell shape were inspected under an oil immersion lens (100X) using fixed smears stained with methylene blue (0.1%, *w/v*).

Physiological and biochemical properties including the ability to utilize and grow aerobically on different carbon sources (D-glucose, lactose, xylitol, sucrose, soluble starch, D-xylose, glycerol, citrate, and arabinose) and nitrogen sources (nitrate, urea, and ammonium sulfate) as a sole source of energy was estimated and compared with a negative control without carbon and nitrogen. Assimilation tests were achieved by replica plate method after 24–48 h of incubation. The capability of yeast isolates to grow at wide range of temperature (4.0°C–40.0°C) was also determined.[14]

2.4.2. Genotypic characteristics

Genomic DNA of the most promising strains was extracted using the CTAB method, followed by amplification of the internal transcribed spacer (ITS) using primers ITS5 (5'- GACTCCTTGGTCCGTGTT -3' as forward) and ITS4 (5'-ATTACCGCGGCTGCTGGCACC-3' as reverse).[15] PCR amplification was conducted in a total volume of 25 µl. The composition of each PCR reaction was 5 µl PCR buffer, 1.5 µl of dNTP, 1 µl ITS5 primer (10 µM), 1 µl ITS4 primer (10 µM) and 0.3 µl TaqDNA polymerase, DNA template 0.3 µl and Nuclease free water 15.2 µl. The content of the tubes was mixed properly by a brief spin of micro-centrifuge. The tubes were placed in a PCR machine (Thermo-scientific, USA) and the reaction parameters were as follows: The PCR amplification started with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 2 minutes, annealing at 54 °C for 90 seconds, and extension at 72°C for 2 minutes. The final extension was done at 72°C for 10 min. The PCR products were electrophoresed on 1% agarose gel containing 0.1 µg/mL ethidium bromide in 1X TBE buffer; electrophoresis was carried out at 125 volts till the dye was reached 3/4th of the gel. The gel was removed and examined on a UV

trans-illuminator and confirmed by gel documentation and photographed. The amplified product was purified by using EZ-10 Spin Column PCR Products Purification Kit BS664 (Bio-basic, USA). The sequencing was carried out by Sanger Sequencing Technology on Applied Bio-systems automated DNA sequencer (model; ABI 3730XL DNA Analyzer-Applied Bio-systems, USA; service provided by Macrogen Inc., South Korea). The sequence analyses and alignments were performed by NCBI-BLAST programs and the phylogenetic relationship of these yeast strains was displayed in a distance-based Neighbor-Joining tree.

2.5. Production conditions

2.5.1. Inoculum preparation

The most potent strains were inoculated in YPD broth medium and incubated at 25 °C for 24-48 h.The optical density (OD) of the cultures was adjusted at 1 nm. Two milliliters of cultured cells were centrifuged at 8000 *xg* for 5 min, washed with normal saline (0.9 % NaCl), and centrifuged again. The pellets were re-suspended in 1 ml normal saline and used as inoculum.[16]

2.5.2. Lipid accumulation and biomass determination

Lipid accumulation by the most potent oleaginous yeast isolates (E2, W6, D1, and G2) was conducted by cultivating 1 ml of culture cells on nitrogen-limited medium containing (g/L): 50 glucose, 3 yeast extract, and 5 peptone dissolved in one liter of seawater and kept in an orbital shaker at 25 °C ±2 °C at 150 rpm at initial pH 5.8. Then the cells were collected and washed twice with sterile saline solution after centrifugation at 5,000 *xg* and 15 °C. Cell dry weight (CDW, g L^{-1}) of the biomass was measured after the cell pellets were kept at 55 °C until their dry weight became constant.[17]

2.6. Lipid extraction and purification

Lipid content in yeast cells was extracted, dried, and weighed, based on the method described by Bligh and Dyer (1959).[18] Briefly, 50 ml of sample was centrifuged at 5000 *xg* for 5 min, and the cells were rinsed twice with 50 ml of distilled water then added into 10 ml of 4 M HCl, and incubated at 60°C for two hours to breakdown the yeast cell wall. The acid-hydrolyzed mass was stirred with 20 ml of chloroform/methanol mixture (2:1) at room temperature for 2 to 3 h, followed by centrifugation at 2000 *xg* for 5 min at room temperature to separate the aqueous upper phase and organic lower phases. The aqueous upper layer containing methanol, water, and non-lipid compounds was discarded and the lower phase containing chloroform with lipid layer was filtered through filter paper and collected in glass vials. Finally the lower phase containing lipids became recovered with a Pasteur pipette and dried by oven at 55 °C until reach constant weight and the dry lipids were weighed and expressed as gram lipid per liter of fermentation broth and percentage of gram lipid dry biomass.

2.7. Fatty acid analysis

2.7.1. Fatty Acid Methyl Esters (FAMEs) Formation

After extraction procedures, the fatty acids are converted into methyl esters by adding 2 ml of methanolic sulfuric acid (6% H_2SO_4) into crude lipid extract and mixed for 30 sec in close tightly screw tubes and incubated at 70°C for 1-2 h. The obtained fatty acid methyl esters (FAMEs) was mixed with 2 ml petroleum ether and 1 ml distilled water for 30 sec, the upper petroleum ether layer was transferred to a 2 ml vial and evaporated at 38°C under nitrogen atmosphere until dryness. Residual FAMEs were re-dissolved in 200 ml hexane to be analyzed by gas chromatography-mass spectroscopy.[19]

2.7.2. Gas Chromatography/Mass Spectroscopy Analysis

The collected fatty acid methyl ester was analyzed by Agilent 6890N Gas Chromatography connected by Agilent 55973 Mass Spectrometer with an HP-5ms capillary column (30 m0.25 mm Inner Diameter, 0.25 mm film thickness; J&W Scientific, USA). The carrier gas (helium) was maintained at a flow rate of 1.0 ml/min. The inlet temperature was maintained at 300 °C and the oven was programed for 2 min. at 150 °C, then increased to 300 °C at 4 °C /min and maintained for 20 min at 300 °C. Peaks of FAMEs were identified by comparison of the retention time and mass spectra to the spectral reference data obtained from the Wiley and NIST.[20]

3. RESULTS

3.1. Isolation and screening of lipid producers

Ten isolates with the morphology of typical yeast were obtained from marine water and sediment samples. Each isolate was analyzed to detect the presence of lipid droplets in different proportions, whose size was visually estimated concerning to the cell area, based on the yellow gold fluorescence emitted by neutral lipids after treatment with Nile red dye. Amongst all isolates, only four strains encoded as E2, D1, G2, and W6 showed a significant amount of lipid accumulation by exhibiting positive Nile red staining (Fig. 1) with lipid accumulation between 25 to more than 35% of the cell area, therefore these yeast isolates were selected for further screening and investigations.

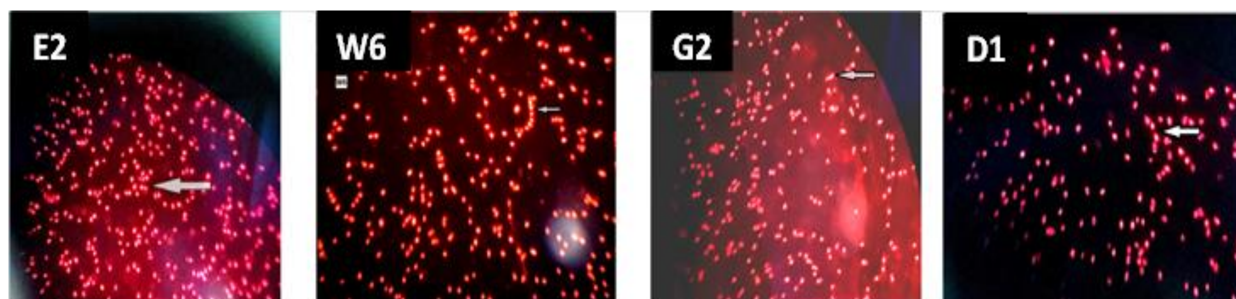

Fig 1: Fluorescence microscopy of the Nile Red stained isolates E2, W6, G2 and D1.

To confirm the selection of the most promising isolates, the isolates were secondary screened by TTC stain based on lipid content and the degree of staining. The four yeast isolates formed various degrees of staining red color with TTC dye at 485 nm which considered being a positive result (Fig. 2; Table 1). The highest degree of color was shown by isolates E2, and W6 followed by strains G2, and D1.

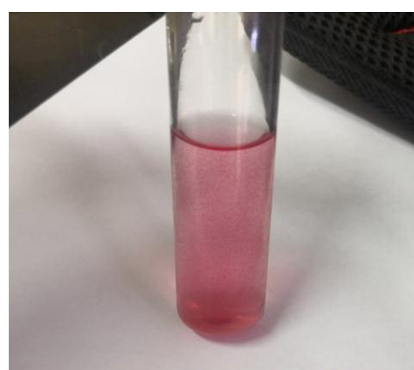

Fig 2: Positive (Red) and negative (colorless) sample in the TTC staining method.

Table 1: Staining degree, biomass production and lipid content of the most potent yeast isolates after 5 days of cultivation on nitrogen limiting medium.

Yeast isolate	Staining degree(A_{485} nm)	Dry biomass (g/L)	Total lipid content (%)
E2	1.43±0.01	16.4±0.54	29.3±0.51
W6	1.30±0.02	15.5±0.22	26.2±0.43
G2	1.20±0.03	14.3±0.29	25.6±0.41
D1	1.12±0.02	13.8±0.33	23.4±0.33

3.2. Identification of oleaginous yeast strains

3.2.1. Phenotypic characteristics

Morphological and microscopical properties of selected isolates showed that the isolate G2 is grown in a butyrous manner, smooth, glistening, and brown to white color with bipolar budding, its cell shape is ovoid to elongate, occur singly or pairs or in short chains. Isolates E2 and D1 appear flat, dull to shiny, cream white color, and butyrous like manner with bipolar budding and oval to spherical shape. The isolate W6 is seems smooth, flat with an elevated center, white, and has an entire edge with multilateral budding and spherical to ovoidal shape, short elongates, and form pseudohyphae. All yeast strains utilized glycerol, D-glucose, and sucrose, as a sole carbon sources but not able to utilized lactose and they utilized ammonium sulfate as the best nitrogen source. Biochemical characterizations of these isolates including the assimilation of different carbon and nitrogen sources as well as the growth at varied temperature ranges are illustrated in Table 2.

3.2.2. Genotypic characteristics and phylogenetic tree construction

The agarose gel electrophoresis of the genomic DNA profile of four isolates displayed that only one band represents the genomic DNA appear and compared with a ladder (100 bp kb to 3 kb size). The DNA concentration obtained from E2, G2, D1, and W6 isolates was found to be 345.6, 422.5, 337.4, and 340.9 ng μL^{-1}, respectively and the extracted DNA was amplified by PCR and a band of DNA was observed in the agarose gel. Phylogenetic analysis revealed that many available sequences exhibit evolutionary closeness with the isolates obtained in this study (Table 3). Based on the evolutionary distance between the yeast strains, the isolates E2 and D1 are closely related to *Candida*

parapsilosis strain CBS 604 with similarity 96.94%, 96.90%, respectively (Fig. 3). While the isolate G2 was revealed to be closely related to *Meyerozyma guilliermondii* strain CBS2030 with similarity of 98% (Fig. 4). Eventually, strain W6 was found to be closely related to *Pichia kuriavzevii* with similarity of 97.73% (Fig. 5).

Table 2: Biochemical characteristics of the most promising yeast strains E2, G2, D1, and W6.

Test	Most potent isolates			
	E2	G2	D1	W6
Glycerol	+ve	weak	+ve	+ve
Starch	+ve	-ve	+ve	+ve
D-Glucose	+ve	+ve	+ve	+ve
Lactose	-ve	-ve	-ve	-ve
Xylitol	+ve	+ve	+ve	-ve
Sucrose	+ve	+ve	+ve	+ve
L-Arabinose	+ve	weak	+ve	-ve
D-Xylose	+ve	+ve	+ve	-ve
Citrate	+ve	+ve	+ve	-ve
Urea	-ve	ND	-ve	+ve
Nitrate	+ve	-ve	+ve	-ve
Ammonium Sulfate	+ve	+ve	+ve	+ve
Growth at 4°C	-ve	-ve	-ve	-ve
Growth at 25°C	+ve	+ve	+ve	+ve
Growth at 37°C	+ve	+ve	+ve	+ve
Growth at 40°C	+ve	+ve	+ve	+ve
+ve, Positive; -ve, Negatve; ND, Not detected.				

Table 3: Taxonomic identification of yeast strains based on the genetic distance of the query sequence of 18S rRNA showed the similarity to related genera, using BLAST in Gen Bank.

Strain	Species	Gen-bank access number	Similarity (%)	Cover (%)
D1	*Candida parapsilosis*	MH545914.1	96.90	99
E2	*Candida parapsilosis*	MH545914.1	96.94	98
D1	*Meyerozyma guilliermondii*	MH545918.1	98.00	94
W6	*Pichia kudriavzevii*	AB536782.1	97.73	98

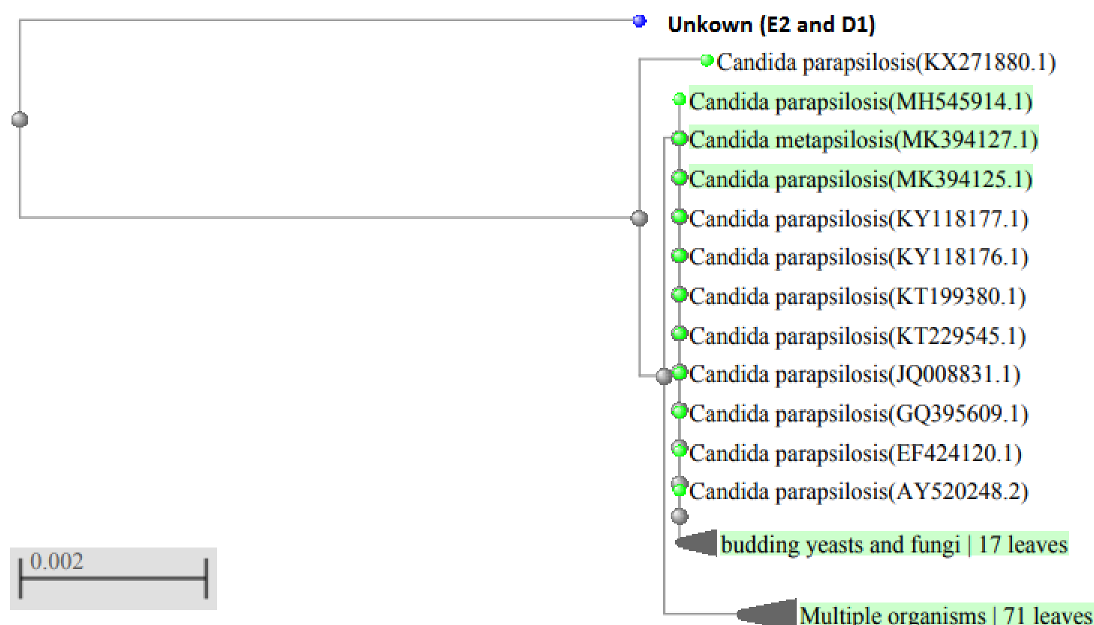

Fig. 3: The 18S rRNA Phylogenetic tree for isolates (D1 and E2) based on their similarity to closely related strains.

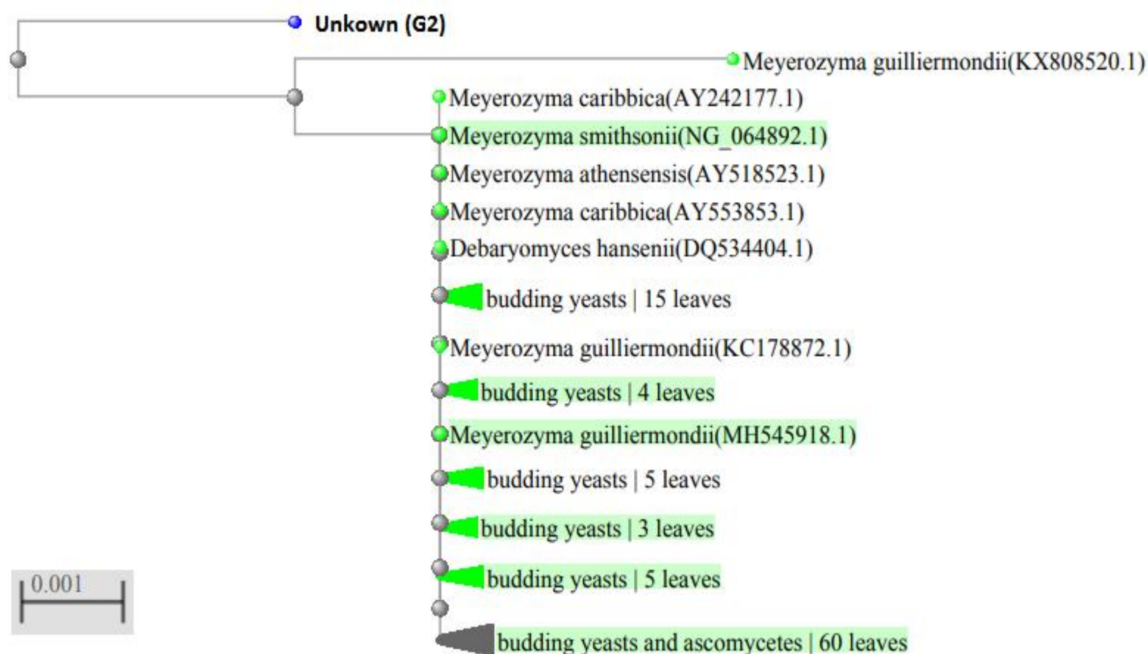

Fig. 4: The 18S rRNA Phylogenetic tree for isolate G2 based on its similarity to closely related strains.

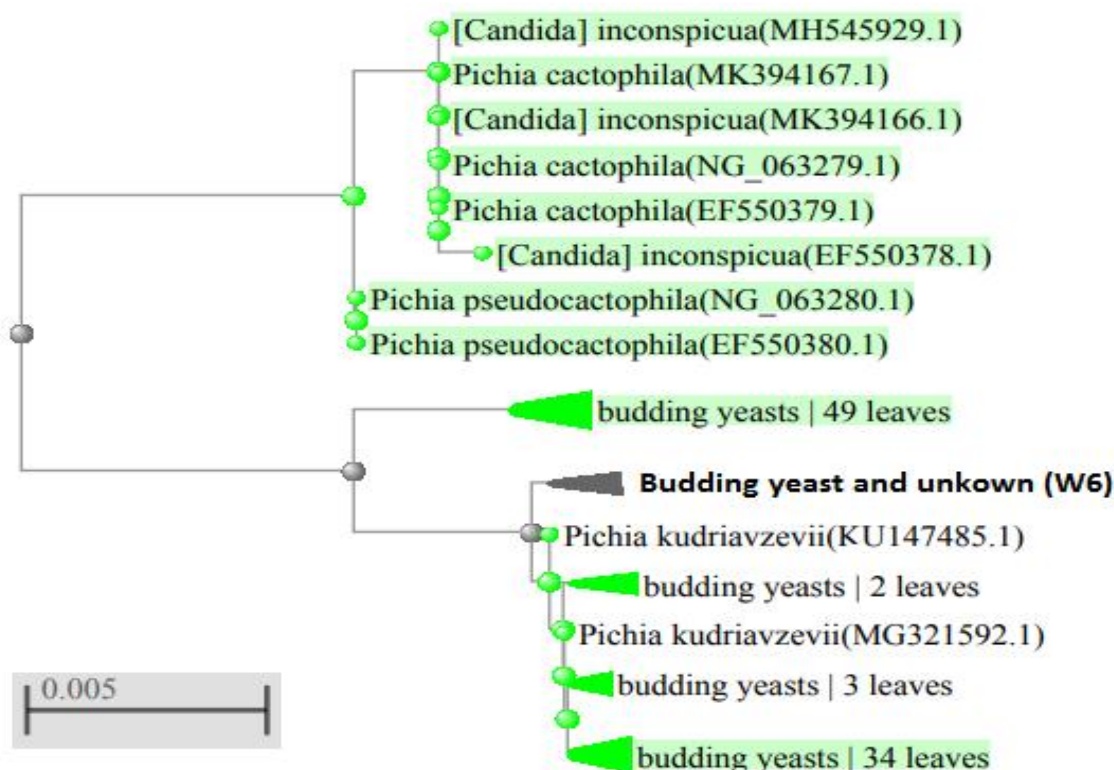

Fig. 5: The 18S rRNA Phylogenetic tree for isolate W6 based on its similarity to closely related strains.

3.3. Lipid production and biomass determination

Table 1 shows the lipid production concerning dry mass for the most potent isolates when cultivated at 25°C on YPD medium containing glucose as a carbon source at initial pH 5.8 in all cultivations. The highest lipid producer strain among the four isolates was observed by strain E2 with 29.3% lipid per dry biomass (16.4 dry weight /L) followed by the strains W6, and G2 that yielded 26.2%, and 25.6% per dry mass at 15.5 g/L, and 14.3 g/L, respectively. While the lowest potential ability for lipid production was displayed by strain D1 with 23.4 %, and 13.8 g/L of cell dry weight.

3.4. Fatty acid profiles analysis

Table 4 showed the fatty acid profiles of lipid production for the most promising strains including total lipid content, total saturated, and unsaturated fatty acid compared with the authentic standard by GC/MS. Based

on the GC analysis the composition of fatty acid of all studied strains is similar to each other. The major fatty acids produced by these strains were palmitic acid (C16:0), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) which represent over 90 % of the total fatty acids. While the lesser amount of fatty acids were capric acid (C10), undecanoic acid (C11), lauric acid (C12), and tridecanoic acid (C13). The content of total unsaturated fatty acid (USFA) was the highest in *Meyerozyma guilliermondii* strain G2 (72.23%), *Candida parapsilosis* strain E2 (72.20%) followed by *Candida parapsilosis* strain D1 (71.66%), and *Pichia kuriavzevii* strain W6 (68.07%). On the other hand, the content of total saturated fatty acids (SFAs) showed the highest content in strains W6 (31.93%) followed by D1 (28.34%), E2 (27.80%), and G2 (27.77%). The main fatty acids mostly produced by strains G2, E2, W6, and D1 were oleic acid (C18:1) with content (46.57%, 49.69%, 27.16%, and 46.44%), respectively, and relatively high yields of palmitic acid (C16:0) ranging from 12.89%, 17.01%, 19.43% to 11.16 % were obtained in the isolates G2, E2, W6, and D1, respectively (Figs. 6, 7, 8, and 9) and Alpha-linolenic acid (ALA) was present in G2, E2, W6 and D1 strains (~7.40, 7.65, 8.68, and 7.10 % respectively. Other fatty acids are produced by these isolates belonging to n-3, n-6, and n-9 families such as Arachidonic acid (ARA), Dihomo-gamma-linolenic acid (DGLA), Eicosapentaenoic acid, Erucic acid, Eicosatrienoic acid, Nervonic acid, and Eicosenoic acid.

Table 4: Fatty acid methyl ester profiles of the most potent yeast strains.

Name of fatty acid	Type of fatty acid	Percentage of each fatty acid			
		G2	E2	W6	D1
Caproic acid (C6)	**Saturated**	3.01	0.30	0.68	2.88
Caprylic acid (C8:0)	**Saturated**	1.66	1.22	1.64	1.97
Capric acid (C10)	**Saturated**	0.21	0.15	0.20	0.25
Undecanoic acid (C11)	**Saturated**	0.17	0.12	0.17	0.20
Lauric acid (C12)	**Saturated**	0.16	0.11	0.15	0.18
Tridecanoic acid (C13)	**Saturated**	0.18	0.16	0.25	0.25
Myristoleic acid (C14)	**Unsaturated**	0.50	0.36	0.50	0.60
Myristic acid (C14)	**Saturated**	0.30	0.22	0.31	0.37
cis-10-Pentadecenoic acid (C15)	**Unsaturated**	0.57	0.42	0.56	0.67
Pentadecanoiccid (C15)	**Saturated**	0.25	0.19	0.45	0.32
Palmitoleic acid (C16)	**Unsaturated**	1.36	0.95	12.14	1.07
Palmitic acid (C16)	**Saturated**	**12.89**	**17.01**	**19.43**	**11.16**
cis-10-Heptadecenoic acid (C17)	**Unsaturated**	1.49	1.96	1.24	0.84
Heptadecanoic acid (C17)	**Saturated**	1.33	1.13	0.66	1.57
gama-Linolenic acid (C18)	**Unsaturated**	3.00	2.74	4.08	3.06
Alpha-Linoleic acid (C18)	**Unsaturated**	**7.40**	**7.65**	**8.68**	**7.10**
Oleic acid (C18)	**Unsaturated**	**46.57**	**49.69**	**27.16**	**46.44**
Elaidic acid (C18)	**Unsaturated**	2.03	1.96	4.15	1.97
Stearic acid (C18)	**Saturated**	2.77	2.71	2.17	1.87
Arachidonic (C20)	**Unsaturated**	1.24	0.12	1.21	0.37
cis-5,8,11,14,17-Eicosapentaenoic acid (C20)	**Unsaturated**	1.09	0.84	1.22	1.32
cis-8,11,14-Eicosatrienoic (C20)	**Unsaturated**	1.22	0.90	1.19	1.45
cis-11,14-Eicosadienoic acid (C20)	**Unsaturated**	1.12	0.82	1.10	1.35
cis-11-Eicosenoic acid (C20)	**Unsaturated**	0.87	0.62	0.00	1.10
cis-11,14,17-Eicosatrienoic acid (C20)	**Unsaturated**	1.14	0.83	1.12	1.35
Arachidic acid (C20)	**Saturated**	0.61	0.45	0.61	0.74
Heneicosanoic acid (C21)	**Saturated**	0.86	0.66	0.85	1.06
cis-4,7,10,13,16,19-Docosahexaenoic acid (C22)	**Unsaturated**	1.40	0.89	1.24	1.45
cis-13,16-Docosadienoic acid (C22)	**Unsaturated**	0.00	0.00	1.24	0.00
Behenoic acid (C22)	**Saturated**	1.49	1.09	1.47	1.77
Tricosanoic acid (C23)	**Saturated**	0.00	0.78	1.01	1.26
Nervonic acid (C24)	**Unsaturated**	1.24	0.91	1.22	1.48
Lignoceric acid (C24)	**Saturated**	2.07	1.64	1.90	2.49
Total fatty acid		**100.00**	**100.00**	**100.00**	**100.00**
Total Saturated fatty acids		**27.77**	**27.80**	**31.93**	**28.34**
Total unsaturated fatty acids		**72.23**	**72.20**	**68.07**	**71.66**

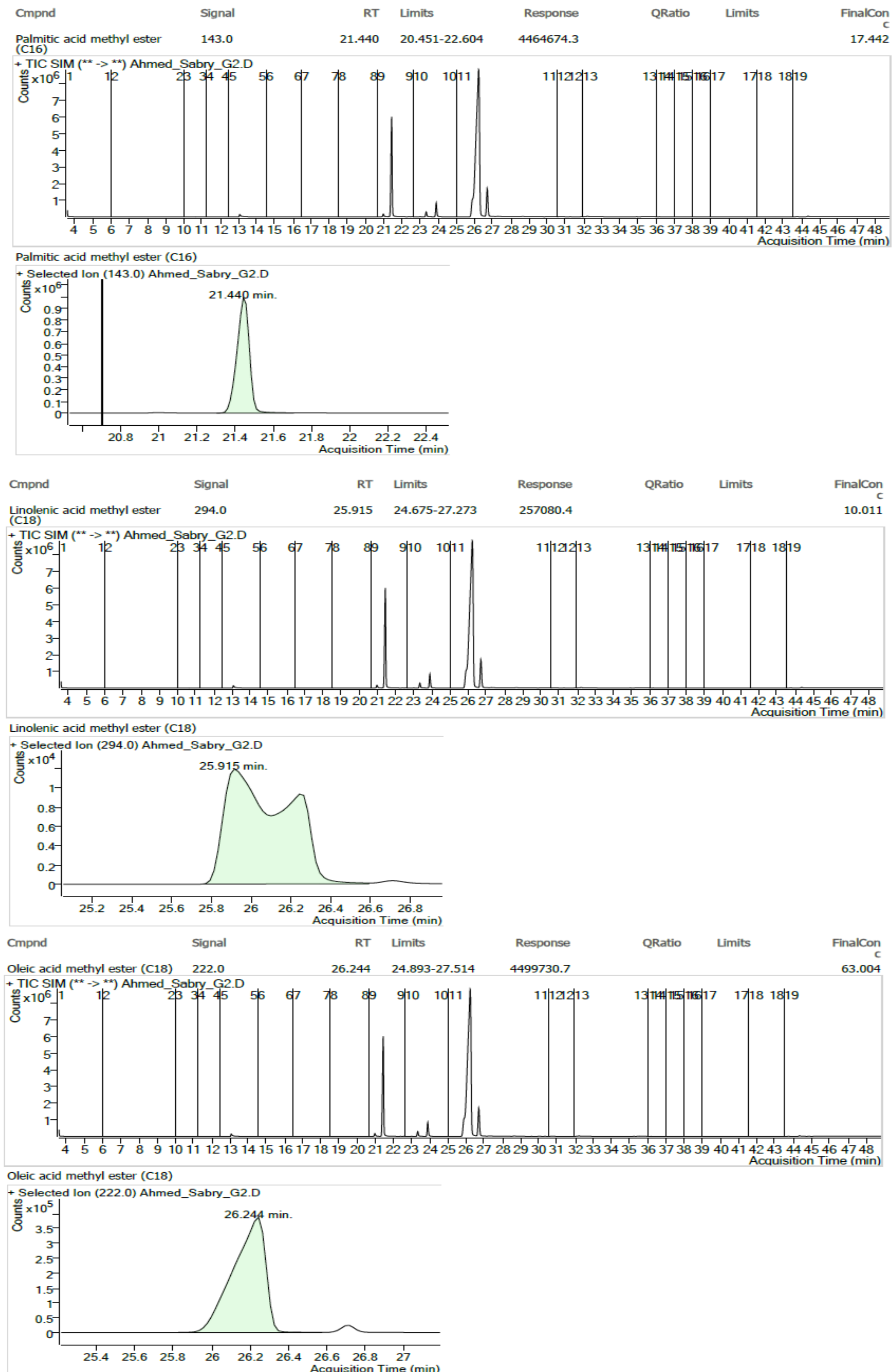

Fig. 6: The GC-MS chromatogram showed the main fatty acid accumulated by yeast isolate G2 when cultivated on glucose as a sole carbon source.

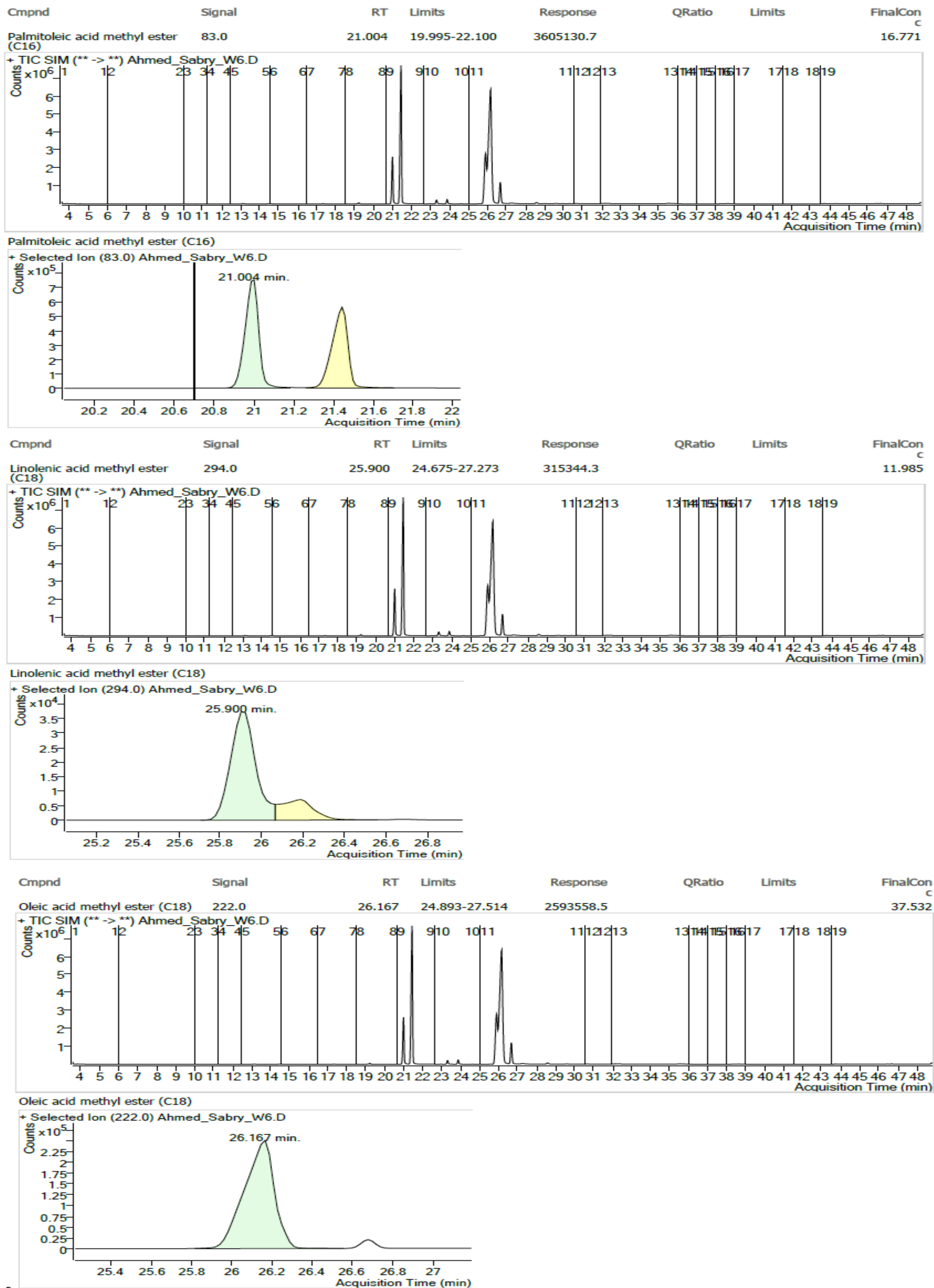

Fig. 7: The GC-MS chromatogram showed the main fatty acid accumulated by yeast isolate W6 when cultivated on glucose as a sole carbon source.

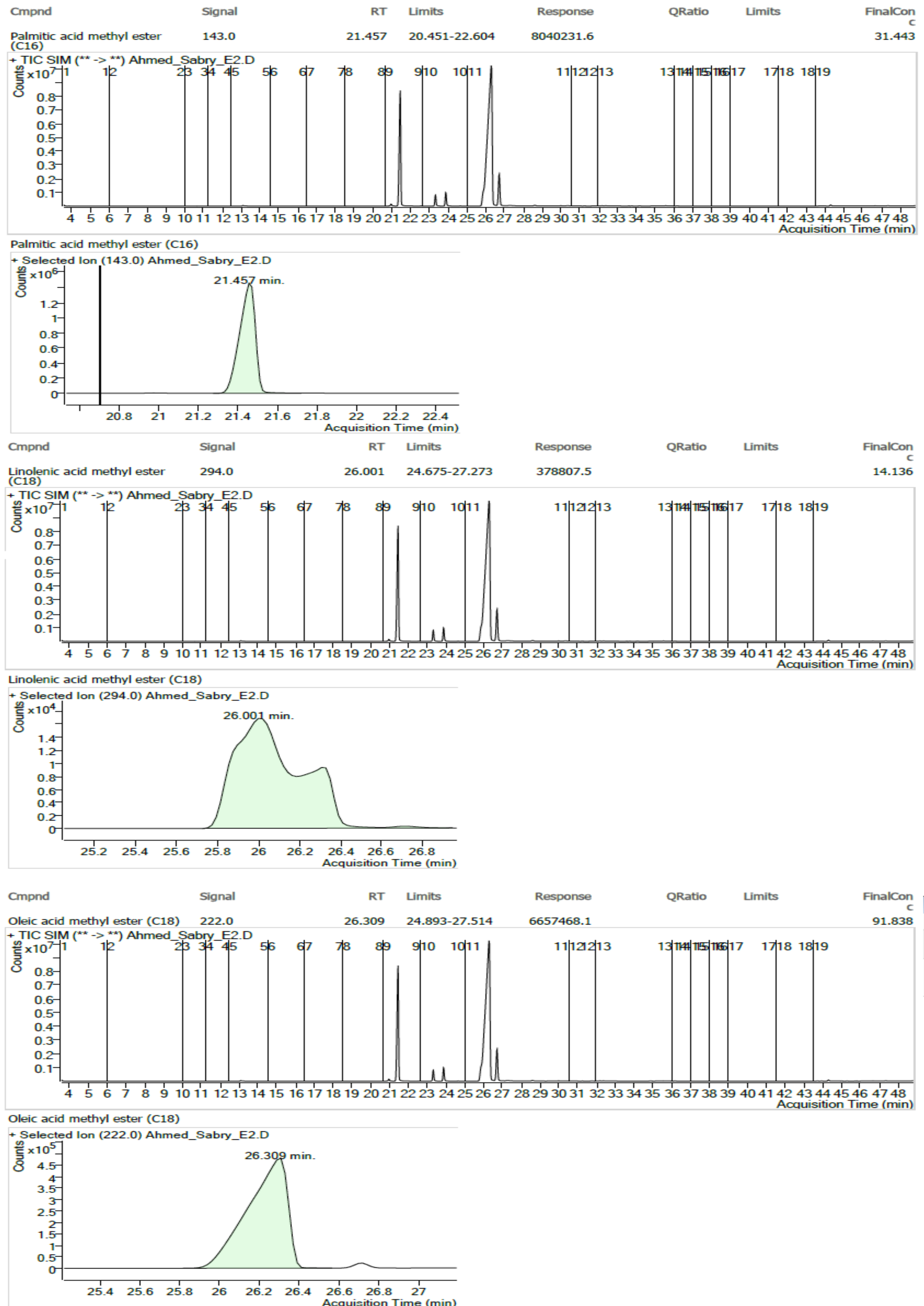

Fig. 8: The GC-MS chromatogram showed the main fatty acid accumulated by yeast isolate E2 when cultivated on glucose as a sole carbon source.

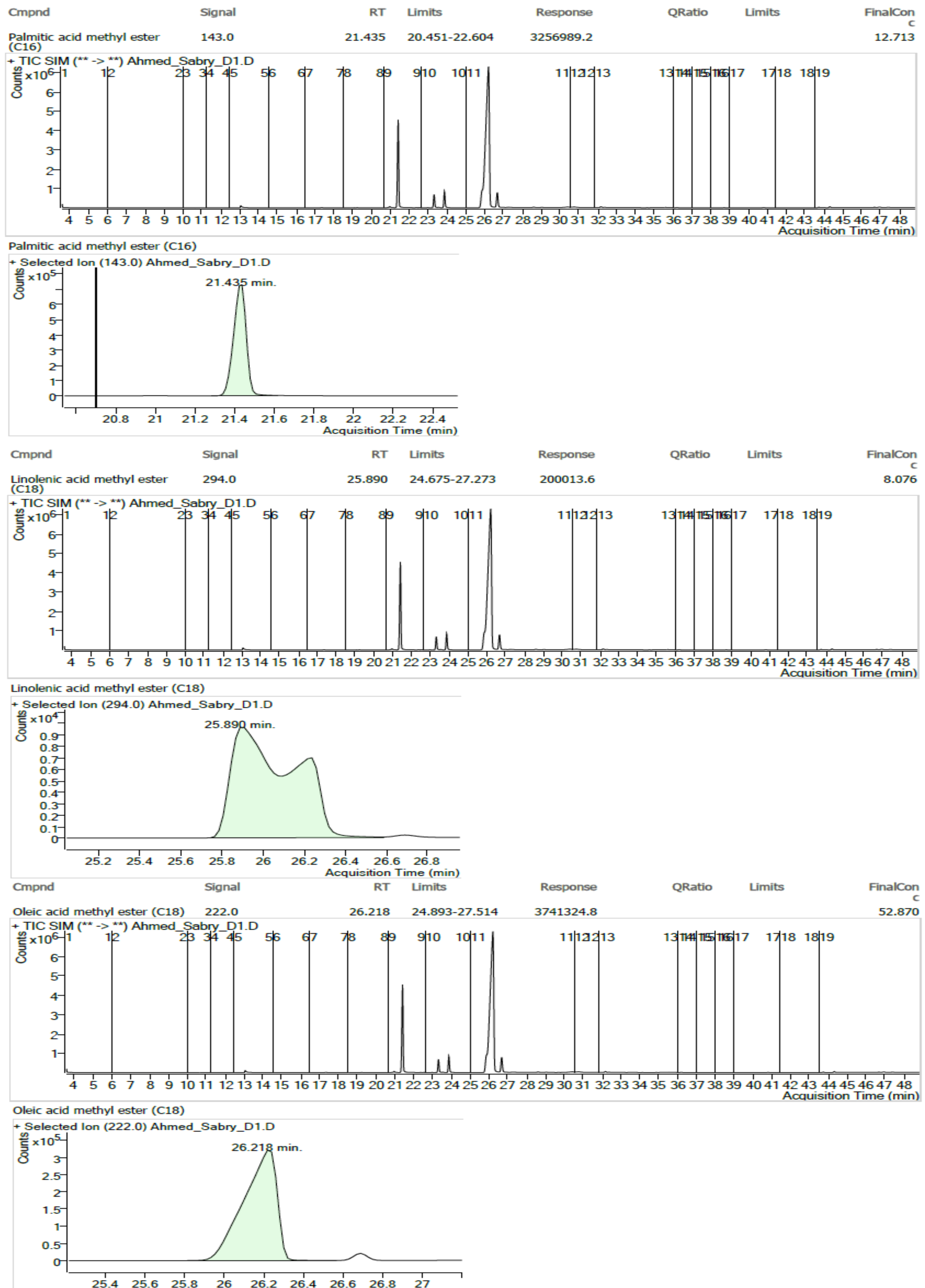

Fig. 9: The GC-MS chromatogram showed the main fatty acid accumulated by yeast isolate D1 when cultivated on glucose as a sole carbon source.

4. DISCUSSION

Microbial lipid production is studied as an alternative tool that can be used in many industrial raw materials and human food additives besides biofuel production, so yeast especially oleaginous ones is recommended for more investigation.[21] Marine environments are considering one of the most promising sources to isolate oleaginous yeasts, therefore, in this study eight seawater and sediment samples were collected from the Mediterranean Sea in Alexandria and the Red Sea in the Suez Canal in Egypt. The primary screening was performed by Nile red staining as was preferred by many investigators for the quickly selection and separation of oil producing yeast strains [22] through demonstrating and determining the intracellular lipid content. Nile red staining was able to recognize lipid producing and non-producing organisms because it reacts only with a hydrophobic compound like lipids and emits strongly positive red fluorescence signals which can be detected by fluorescence microscopy.[23] The fluorescence intensity of stained cells under UV light depends on lipid concentration in which the microorganisms that not able to accumulate lipid are produced light fluorescence.[24] The treatment with Nile Red not only proved efficient for a preliminary large scale screening of oleaginous yeasts, but the whole screening process consumed 96 hours for yeast cultivation in addition to 20 to 30 minutes for Nile Red smear preparation and fluorescence detection. Hence the process would greatly benefit from a reduction in the cultivation time, especially if a high throughput methodology is established.[25]

These isolates were subjected for a second round of screening by TTC stain. Reduction of TTC is mostly used as a biochemical test for the viability of living cells where the fatty acids producing strain reduced TTC stain using their dehydrogenases activity from colorless to red and the degree of staining depends on the fatty acids content accumulated by these strains as reported by.[26] There are many dehydrogenases secretes by fungi, however specific dehydrogenases only worked such as fatty acyl desaturases, a kind of dehydrogenases regulated the formation of the double bond in fatty acids.[27] The ability for accumulation of lipid with high percentage inside yeast cell has reported by many investigators.[28- 31] Our study revealed that only four isolates designated as D1, E2, G2, and W6 are capable to produce fluorescence through Nile Red dye and reduced TTC stain from colorless to red with the highest degree of staining showed by isolate E2 also they observed the highest potential to lipid accumulation among all isolates (Table 1; Figs. 1 and 2).

Ageitos et al., (2011) indicated that oleaginous yeasts can accumulate lipids up to 20% of their biomass.[6] The four strains which isolated and selected in this study are typically oleaginous yeasts because of internal lipid accumulating capacities exhibited by E2 and W6 strains were 29.3%, 26.2%, respectively and 25.6 %, 23.4% for strains G2 and D1, respectively. Only 3–10% of known yeast species are oleaginous including genera of *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon*, and *Lipomyces*.[7]

The assimilation of different carbon and nitrogen sources still has a role in primary screening and aiding the other methods in identification.[32] The identification of the most potent yeast strains was done using phenotypic and molecular taxonomic characteristics. It was difficult to differentiate among *Pichia* spp. and *Candida* spp. based on biochemical tests only because of the conflicting of their biochemical characteristics and variations between two strains.[33] So that, yeast identification using the DNA sequencing approach is the most preferred method.[34] Based on the 18S rRNA analysis, isolates E2 and D1 were revealed to be a good match with *Candida parapsilosis* and hence they were identified as *Candida parapsilosis* E2 and *Candida parapsilosis* D1 (Fig. 3). Strain W6 exhibited high similarity with the *Pichia* genera corresponding to *Pichia kuriavzevii* with similarity of 97.73% and hence it was identify as *Pichia kuriavzevii* W6 (Table 3) (Fig.5), while the isolate G2 exhibited the highest similarity with *Meyerozyma* genera with similarity 98% to *Meyerozyma guilliermondii* strain and hence it was identified as *Meyerozyma guilliermondii* G2 (Fig.4).

Lipid accumulation and biomass production of oleaginous yeast is affected by the availability of carbon and nitrogen sources during fermentation. To produce the maximum amount of cell biomass and lipid content in oleaginous yeasts, the growth medium should contain an excess of carbon source and a limited amount of nitrogen sources.[35]

Therefore, in this study, glucose was selected as a carbon substrate source; however, many other carbon sources are studied, such as lactose or sucrose.[36] Vijayakumar *et al*., (2010) estimated the lipid content from different carbon substrates by *Rhodotorulla glutinis*, between these substrates; glucose yielded 2.43 g/L and 23.78 % of lipid content.[37] Our recorded data yielded lipid percent higher than reported by Vijayakumar *et al*., (2010) and relatively near to those evaluated by Dai *et al*., (2007)and Easterling*et al*. (2009) using glucose as a start material as they reported that *Rhodoturula glutinis* and *Rhodosporidium toruloides* produced 25% and 36.6% lipid, respectively.[38, 39]

In this work, the production of polyunsaturated fatty acids was achieved under low nitrogen condition because nitrogen limiting media has a significant effect upon the microbial fatty acids composition. Several studies pointed out a close relationship between nitrogen limitation, and lipid storage contents.[31, 40, 21] Li *et al.* (2015) reported that the limitation of nitrogen sources stimulated the production of lipid in yeast. Under nitrogen limiting conditions, the yeast cells continue to utilize glucose and regulate the fermentation process to

produce big amounts of storage fatty acids in the form of lipid droplets.[40]

The efficiencies of oleaginous yeast accumulate oil are mainly related to the difference in their biochemistry and genetic constitution of their cells as reported by Jiru*et al*., (2016).[41] This diversity of oil storage can be exhibited not only among different oleaginous species but among strains of the same species as clearly shown between isolates E2 and D1 (*Candida parapslosis*), although they have belonged to the same species they differ in the lipid profile (Table 4).

Lipids produced by yeasts are mainly composed of saturated and unsaturated fatty acids.[42] Fatty acid profiles observed during this study confirmed that the highest value of unsaturated fatty acids ranging from 68.0 % up to 73 % of total fatty acids are present and this suggested that lipids from yeast isolates could be an alternative to fish and vegetable oil in different industrial fields. The major fatty acids constructed by the most potent strains were oleic acid 46.57%, 49.69%, 27.16%, and 46.44% for G2, E2, W6, and D1, respectively then palmitic acid with 12.89%, 17.01%, 19.43%, and 11.16 % for G2, E2, W6, and D1, respectively and finally linoleic acid with percent of 7.40% in G2, 7.65% in E2, 8.68 in W6 % and 7.10 % in D1. On the other hand, the minor fatty acids created were stearic acid (18:0) with percent ranging from 2.71% to 1.87 %, and myristic acid (14:0) with percent around 0.30% (Table 4; Figs 6-9). These results correspond with those reported by Zhu *et al.* (2008) and Li *et al*. (2010) as they reported that, the fatty acids formed by *Rhodotorula mucilaginosa* TJY15a and *Trichosporon fermentans* were mainly composed of stearic acid (C18:0), oleic acid (C18:1), linolenic acid (C18:2), palmitic acid (C16:0),and palmitoleic acid (C16:1).[43, 44] Also the results are consistent with the range of lipids generally found in other oleaginous yeast species noted by some authors.[45, 46] Based on results previously reported by Knothe (2005)[47], our findings indicated that there an appropriate ratio betweenthe content of saturated and unsaturated fatty acids produced by our selected strains makes these strains of the most important tools to obtain biodiesel with appropriate quality properties.

CONCLUSION

This study showed that marine environments are viable sources for isolation of PUFAs producing yeasts. Ten yeast isolates were isolated and screened for lipid production. Of these isolates, four yeast isolates encoded E2, W6, G2, and D1 were selected as the most promising strains for PUFAs. The nucleotide sequence analysis of these isolates indicated that these strains are belonged to the genera Ascomycota and identified as *Candida parapsilosis* E2, *Candida parapsilosis* D1, *Pichia kuriavzevii* W6, and *Meyerozyma guilliermondii* G2. These strains accumulated a valuable amount of lipid more than 20 % of total cell dry weight. The fatty acid profile produced by these strains consisted of a significant amount of unsaturated fatty acids accounting for 90% of total fatty acids including oleic acid (18:1), palmitic acid (C16:0), and linoleic acid (18:2) as the predominant fatty acid similar to present in vegetable oil. These strains are potential suitable candidate for oil production for pharmaceutical and nutritional industries due to their high growth, easy cultivation, and high fatty acid content when compared to other reported strains. Further studies are recommended to enhance and optimize the lipid production yield through the fermentation technologies or metabolic engineering methods.

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