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Biosynthesis of omega 3, 6, and 9 polyunsaturated fatty acids by *Pichia kudriavzevii,* and *Yarrowia lipolytica*

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ABSTRACT

Polyunsaturated fatty acids offer a wide variety of health advantages. Fourteen yeast isolates were obtained from different dairy products. Preliminary lipid production screening revealed that five yeast isolates capable to accumulate lipids within its cells, as detected by Sudan black B staining. Quantitative analysis of lipid content after yeast growth on nitrogen-limited medium showed significant variations of lipid content among the yeast isolates. Notably, majdoula cheese isolate presented the highest lipid content 27.69%, followed by rayeb milk, labneh, mozzarella cheese, and ras cheese isolate recorded 22.73%, 22.37%, 21.79%, and 8.04%, respectively. Furthermore, Gas chromatography of fatty acids reveled that ras cheese yeast isolate exhibited oleic acid as the abundant unsaturated fatty acid, afterwards linoleic acid, linolenic acid, palmitoleic acid, homo-γ-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid sequentially, and it represent about 64.9 % of all fatty acids. Similarly, mozzarella cheese isolate and rayeb milk isolate showed a predominance of oleic acid, linoleic acid, and palmitoleic acid, representing over 62% and 56% of the total fatty acids, respectively. Three yeast isolates with the highest unsaturated fatty acids content were identified as *Pichia kudriavzevii PP527343, Yarrowia lipolytica PP527342 and Yarrowia lipolytica PP701998 by* 18s rRNA.

***Keywords*:** Polyunsaturated fatty acids, Gas chromatography, *Pichia, Yarrowia*

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1. INTRODUCTION

Owing to the high need for nutritional supplements, the production PUFAs has been important in new research. Consumption of foods containing a high percentage of PUFAs is important for human health. As mentioned in FAO/WHO reports; it appears that adequate PUFAs intake should be between 6% and 11% of the total calories in an adult human diet. PUFAs display a wide range of health benefits. Essential and non-essential fatty acids represent part of the components of phospholipids in the cell membrane, in addition to being necessary to maintain brain functions, it may also alleviate Alzheimer's, cancer and chronic intestinal disorder disease symptoms **(FAO, 2008; Finco *et al.*, 2017**). **Finco *et al.* (2017) and Wang and Tontonoz (2019)** declared that PUFAs has a role in regulating the fluidity of cellular membranes and modifying the activities of enzymes, transporters, and membrane receptors. It may also be important in preventing and managing many chronic diseases, such as problems resulting from autoimmune disorders, high blood pressure, diabetes, inflammatory, coronary heart disease, atopic eczema, depression, schizophrenia, and multiple sclerosis. Among the relevant ones, most are classified within the omega series, such as omega-3, 6 and 9 fatty acids. Converting linolenic acid to arachidonic acid involves adding another double bond to the fatty acid chain. Alpha-linolenic acid, which belongs to omega 3 group, is an analogue of linolenic acid and is gained by de novo synthesis. EPA and DHA are essential and have gained a lot of attention due to their great functional health potentialities and usefulness **(Spector and Kim 2015).** Oleaginous yeasts capable of accumulating lipids more than 20% of its biomass **(Patel *et al*., 2020),** some oleaginous yeasts can accumulate up to 90% of their dry biomass lipids as triglycerides linoleic and oleic acid represent more than 80% of them. The division Basidiomycota included the most studied species such as *Trichosporon oleaginosus, Cutaneotrichosporon curvatum,* and *Rhodotorula toruloides*) while *Yarrowia lipolytica and Lipomyces starkeyi* belong to ascomycota subdivision. Some *Candida* species e.g. *C. viswanathii, C. tropicalis,C. oleophila* also exhibit oleaginous phenotypes **(Ratledge and Wynn 2002).** Gas chromatography is the most widely used technique for analyzing fatty acids **(Seppänen-Laakso *et al*., 2002).** This study aims to isolate and identify oleaginous yeasts with great cellular lipid and characterize its fatty acids profile.

2. MATERIALS AND METHODS

2.1 Dairy samples

Thirty random samples of raw milk and some dairy products (raw milk, Rayeb milk, yoghourt and cheese as Karish, Cheddar, Talaga, Mozzarella, Ras, Syrian string (shalal), Syrian Halloumi, Syrian Majdoula and Syrian Labneh) were collected from different shops and dairy markets in Fayoum for yeast isolation.

2.2 Preparation of the tested samples

For raw milk, yoghourt and Labneh samples; 1 ml of the sample was inserted to 9 ml sterile saline. Each sample was thoroughly mixed to make a 10^{-1} dilution from which decimal serial dilutions were prepared. In case of cheeses, one gram was transferred

under aseptic conditions to a sterile test tube which 9 ml sterile sodium citrate solution (2%) was added and mixed on vortex apparatus till completely emulsified to make a dilution of 10^{-1} from which decimal dilution were prepared using sterile saline solution **(APHA, 1992).**

2.3 Isolation and purification of yeasts

The yeast was isolated on yeast and mold agar medium **(Bridson, 2006).** Separated colonies were selected and transferred to the slope agar, then the yeast isolates morphologically accomplished macroscopic and microscopic description. Yeast isolates were characterized based on their colony morphology (color, size and shape) and cell shape, size and arrangement under microscope.

2.4 Lipids detection using sudand black B staining technique

The purified yeast cultures were cultivated on Nitrogen –limited medium (NLM) for their cellular lipid accumulation screening (**Enshaeieh *et al.*, 2013).** After incubation for five days at 30 °C, the yeast isolates stained by Sudan black B method as described by **Jape *et al.* (2014)**.

2.5 Cultivation of yeast for lipids production

Standard inocula of selected yeast isolates were prepared by inserting 0.5 ml (10^6 CFU/ml) of the yeast culture into a conical flask (250 ml) that contain fifty ml of media (**Dai *et al.*, 2007)**. The inoculated flasks were placed on a rotary shaker (160 rpm) for two days at 30 ^{o}C. 5 ml of cultivated culture of the tested yeast isolates were utilized as a standard inoculum for 50 ml of the production medium, subsequently; flasks were incubated at 30 ^{o}C in a shaker incubator set at 160 rpm for 120 h. **(Pan *et al.*, 2009)**. The lipid content of collected yeast isolates was determined in accordance with **Bligh and Dyer (1959).**

2.6 Determination of yeast dry biomass

Yeast dry biomass was determined following the procedure outlined in **Granger *et al.* (1993)**. Fifteen ml of yeast culture was collected and centrifuged at 5,000 rpm for five minutes. Collected yeast cells were washed two times with five ml of distilled water then it was dried at 60^{o}C until a constant weight. Yeast dry biomass was assessed.

2.7 Total lipids extraction

Yeast lipids were extracted using the method described by **Bligh and Dyer (1959)**, with a modification provided by **Pan *et al*. (2009)**. Fifty milliliters of yeast culture were centrifuged at 5000 rpm for 10 minutes to separate the yeast cells. The precipitated cells were then washed twice with 50 milliliters of distilled water. After that, 10 milliliters of HCl (4M) were added, and the mixture was heated at 60°C for one to two hours. The acid-hydrolyzed mass was then mixed with a 20 ml combination of chloroform and methanol (1:1) at room temperature for two to three hours. Subsequently, centrifugation was performed at 5000 rpm for 10 minutes to separate the upper aqueous layer from the lower organic layer. The organic lower layer containing lipids was collected using a Pasteur pipette and evaporated at room temperature. The lipids were then weighed after drying. Lipid content was estimated in accordance with the equation mentioned by **Kraisintu *et al.* (2010)**:

Lipids content (%) = Lipids weight (g l^{-1}) / Cell dry weight (g l^{-1}) × 100

2.8 Fatty acids analysis of extracted lipids

2.8.1 Lipid sample preparation

Fatty acids methyl esters were generated through an alkali catalyzed reaction, with presence of KOH (2M), and then injected into hexane.

2.8.2 Gas chromatography (GC-FID)

Using the gas chromatography model 7890B by Agilent Technology was fitted with a flame ionization detector (GC-FID). The separation was accomplished utilizing a Zebron ZB-FAME column with dimensions of 60 m in length, 0.25 ml in internal diameter, and a film thickness of 0.25 micrometers. The analyses were conducted with hydrogen carrier gas, flowing at a rate of flow 1.8 ml/min in a split-1:50 mode, with an injection volume of 1 µl. The temperature program was as follows: starting at 100 °C for 3 minutes, then increasing at a rate of 2.5 °C/min until reaching 240 °C, where it was maintained for 10 minutes. The injector was maintained at 250 °C, while the detector (FID) was set to 285 °C.

2.9 Molecular identification of yeast isolates

The yeast isolates were grown as described by **Pitt and Hocking (2009**). The growing cultures were transferred to the Molecular Biology Research Unit at Assiut University to extract DNA. Extraction of DNA process utilized the Patho-gene-spin DNA/RNA extraction kit, which was supplied by Intron Biotechnology Company based in Korea. Following that, the yeast DNA was dispatched to SolGent Company located in Daejeon, South Korea, for PCR and sequencing of the rRNA gene. PCR was conducted utilizing ITS1 (forward) and ITS4 (reverse) primers that were included in the reaction mixture. The primers possess the following sequences: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). The PCR purified product was sequenced using the same primers, with the addition of dideoxynucleotide triphosphates (ddNTPs) in the reaction mixture, as described by **White *et al*. (1990)**. The sequences which obtained were analyzed by the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website, and phylogenetic analysis of the sequences was conducted using MegAlign (DNA Star) software version 5.05.

3. RESULTS AND DISCUSSION

3.1 Isolation, characterization and screening of oleaginous yeasts

Fourteen yeast isolates were obtained from various dairy products, including three isolates from raw milk, two isolates from Rayeb milk, one isolate from Karish cheese, one isolate from Talaga cheese, two isolates from Mozzarella cheese, two isolates from Ras cheese, one isolate from Labneh, one isolate from Halomi cheese, and one isolate from Majdoula cheese. Preliminary screening of lipid accumulation was conducted for all fourteen yeast isolates using Sudan black B staining. The results revealed that five yeast isolates demonstrated the ability to accumulate lipids, as indicated by the presence of black or greenish particles within the cells.

3.2 Quantitative determination of lipid content

The quantitative analysis of lipid content was conducted on the selected five yeast isolates by extracting and estimating lipids after their growth on

NLM. The results revealed significant variations in lipid content among the examined isolates. Maj.c exhibited the highest lipid content percentage (27.69%), followed by Ray.m, Lab, M.c, and R.C, which showed percentages of (22.73%), (22.37%), (21.79%), and (8.04%), respectively, as depicted in Table 1. These findings align with **Enshaeieh *et al*. (2013)**, which indicated that oleaginous microorganisms are capable of accumulating lipids exceeding 20% of their dry weight. During nitrogen starvation, both oleaginous and non-oleaginous yeasts continue to metabolize carbon sources. However, only oleaginous yeasts metabolize it, resulting in an increased ATP/AMP ratio in their cells **(Hammoud and Glatz, 1988).**

Table 1. The lipids produced by the selected yeast isolates

Isolate code	Isolate source	Biomass dry weight (g l^{-1})	Lipid dry weight (g l^{-1})	Lipid content (%)
Lab	Labneh	15.2	3.4	22.37
M.C	Mozzarella cheese	15.6	3.4	21.79
Maj. C	Majdouli cheese	13.0	3.6	27.69
R.C	Ras cheese	39.8	3.2	8.04
Ray	Rayeb milk	13.2	3.0	22.73

Lab: yeast isolate from labneh M.C: yeast isolate from mozzarella cheese
Maj.C: yeast isolate from majdouli cheese R.C: yeast isolate from ras cheese
Ray: yeast isolate from Rayeb milk

3.3 Fatty acids profile analysis

Numerous studies have highlighted the potential of oleaginous yeasts to produce microbial lipids similar to vegetable or plant oils, specifically those with carbon chain lengths of 16 and 18 **(Kosa *et al*., 2018; Maza *et al*., 2020)**. Analyzing the fatty acid profiles of lipids synthesized by yeasts is crucial as their composition has implications for various biotechnological applications, including dietary supplements and disease treatments **(Maza *et al*., 2020)**. Figs. 1, 2, 3, 4, and 5 present data on the composition of yeast lipids, including saturated, monounsaturated, and polyunsaturated content accumulated by Ray, M.c, R.c Maj, and Lab isolates. The highest proportion of unsaturated fatty acids was observed in the R.c isolate, accounting for 64.98%, followed by the M.c and Ray isolates with 62.91% and 56.45%, respectively. These findings align with those reported by **Barghoth *et al*. (2018)**, where oleic acid (ω9) constituted the highest proportion of unsaturated fatty acids, followed by linoleic acid (ω6) and linolenic acid (ω3). The data obtained in this study indicates that the tested cultures synthesize fatty acids similar to those found in vegetable oils, such as triacylglycerides, linolenic acid, linoleic acid, and oleic acid. The main unsaturated fatty acids synthesized by the M.c isolate were oleic acid, followed by linoleic acid, palmitoleic acid, docosahexaenoic acid (DHA), linolenic acid, eicosapentaenoic acid (EPA) and homo-γ-linolenic acid, accounting for 38.68%, 14.01%, 9.09%, 0.49%, 0.27%, 0.2%, and 0.17%, respectively. These fatty acids collectively represented over 62% of the total fatty acids, as illustrated in Fig. 1.

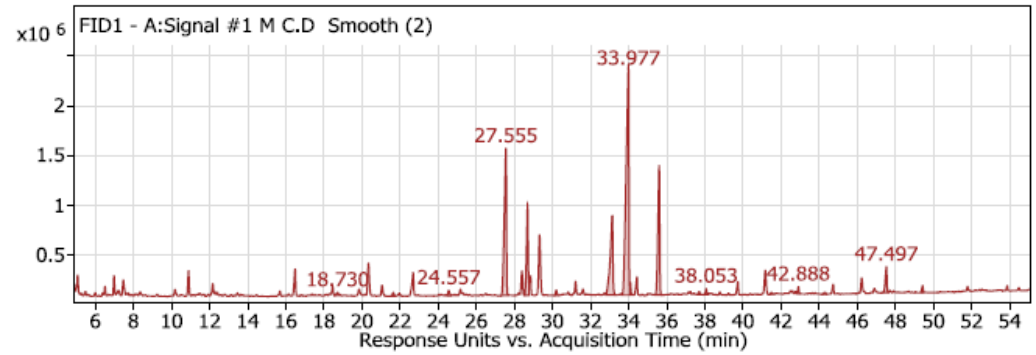

Fig 1. The major fatty acids synthesized by Mc. yeast isolate as illustrated from the GCMS-FID chromatogram.

In contrast, the data presented in Fig. 2 reveal the predominant unsaturated fatty acids produced by the R.c isolate. The primary unsaturated fatty acids synthesized were oleic acid, followed by linoleic acid, linolenic acid, and palmitoleic acid. Additionally, homo-γ-linolenic acid, EPA, and DHA were also detected, comprising 28.6%, 20.26%, 10.53%, 4.14%, 0.3%, 0.13%, and 0.81% of the total fatty acid content, respectively. Altogether, these fatty acids accounted for over 64% of the entire fatty acid composition.

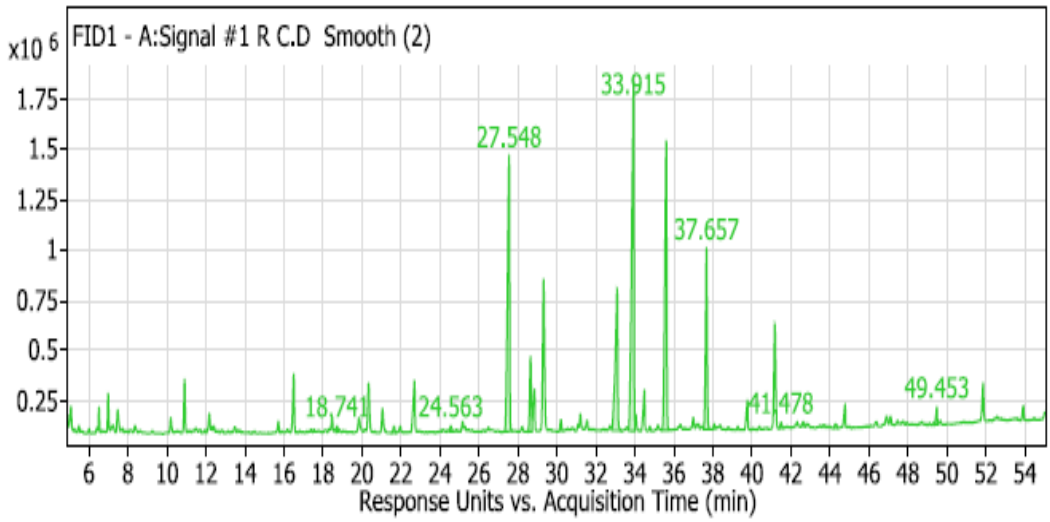

Fig. 2. Main fatty acids produced by Rc. isolate as illustrated from the GCMS-FID chromatogram.

In **Fig. 3** the majority of unsaturated fatty acids for Ray isolate were oleic (C18:1n9) followed by Linoleic acid, then Palmitoleic acid, Homo-γ-linolenic acid), DHA, and Linolenic with 33.56%, 12.92%, 7.48%, 0.91%, 0.83%, and 0.75% respectively, representing over 56% of all fatty acids content.

Data in **Fig. 4** illustrated that the main unsaturated fatty acids created by Lab. isolate were oleic followed by Linoleic acid, then Cis-13,16-Docosadienoic acid, DHA, Cis-11-Eicosenoic acid, Nervonic acid, and

Linolenic acid with 33.34%, 13.63%, 1.05%, 0.73%, 0.4%, 0.26% and 0.19% respectively, representing over 43% of fatty acids content.

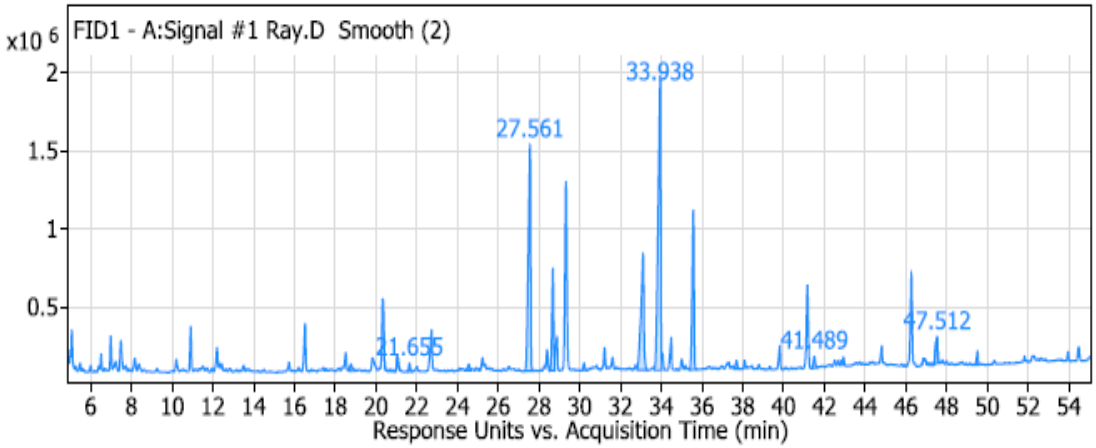

Fig. 3. Main fatty acids produced by Ray. isolate as illustrated from the GCMS-FID chromatogram

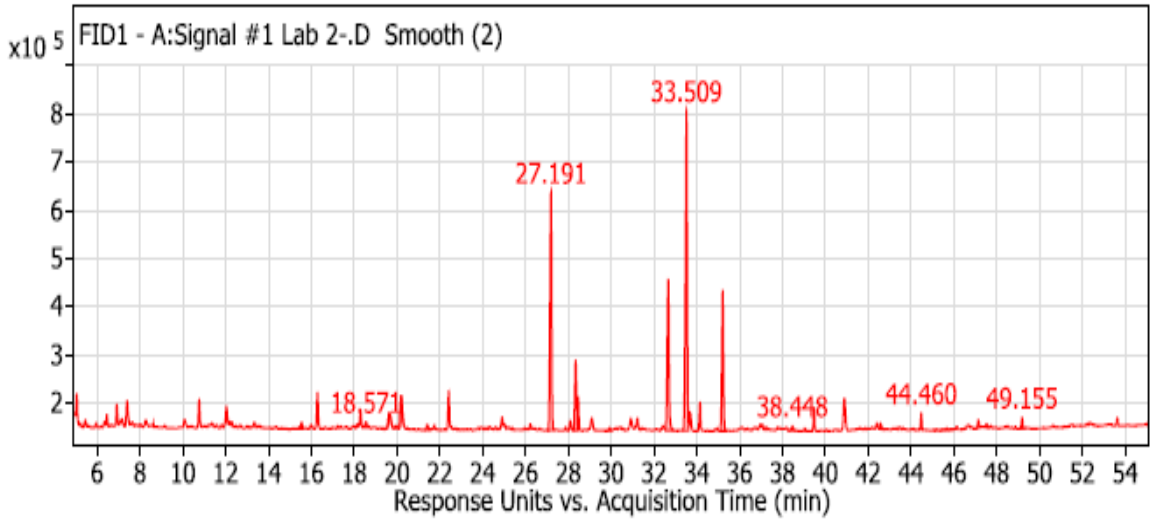

Fig. 4. Main fatty acids synthesized by Lab. yeast isolate as illustrated from the GCMS-FID chromatogram.

The main unsaturated fatty acids formed by Maj isolate were oleic acid C18:1n9 followed by Linoleic acid, then Cis-13,16-Docosadienoic acid, DHA, Linolenic acid, Nervonic acid, and Cis-11-Eicosenoic acid with 49.67%, 1.23%, 0.92%, 0.4%, 0.28%, 0.17% and 0.14% respectively, these fatty acids representing over 37% of all fatty acids content as shown in **Fig. 5**.

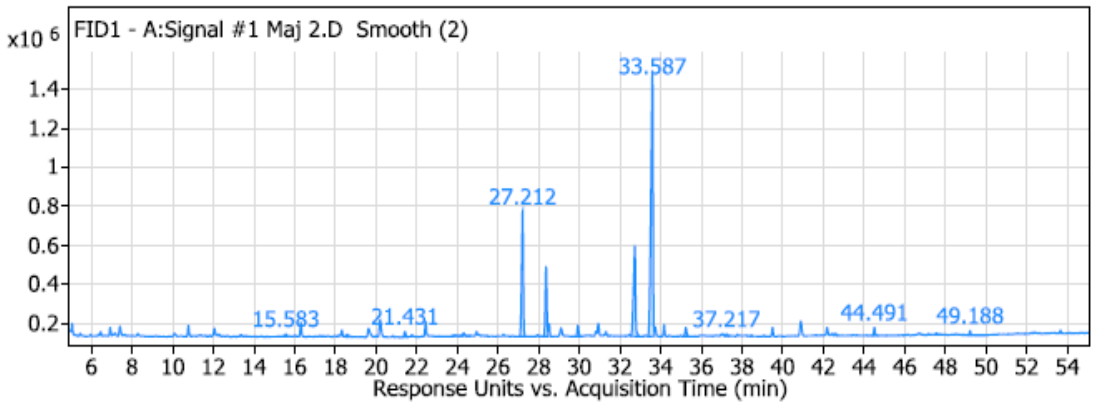

Fig. 5. The major fatty acids generated by Maj. yeast isolate as illustrated from the GCMS-FID chromatogram.

The highest concentration of total unsaturated fatty acids was observed in the R.c isolate, accounting for 64.98%. It was closely followed by the M.c isolate with 62.91% and the Ray isolate with 56.45%. In contrast, the primary content of total saturated fatty acids (SFAs) was found in the (Ray) isolate, representing 43.57%. This was followed by the (M.c) isolate with 37.07% and the R.c isolate with 35.03%. These findings align closely with the results reported by **Dalia *et al.* (2015)**, who found that the *Pichia kudriavzevii* D5 strains produced 27.55% of all saturated fatty acids and 71.43% of all unsaturated fatty acids.

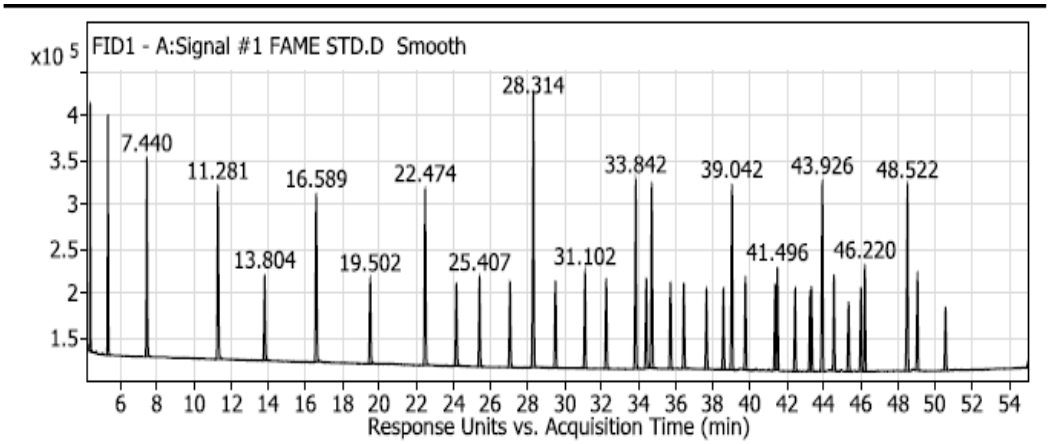

Fig. 6. FAME standard chromatogram

3.4 Molecular characterization and phylogenetic analysis of the yeast's rDNA sequences

18S rRNA gene exhibits a high degree of conservation within species. So, it is commonly utilized as a molecular marker in biodiversity research, with similarities approaching nearly 100%. The Outputs of the phylogenetic analysis indicate that the isolates belong to two separate genera, Pichia and Yarrowia. The amplified PCR product compared with the sequences in GenBank (http:// www. ncbi.nlm.nih.gov), yeast isolates were recognized, and it was found that they are a closely resemble known strains of *P. kudriavizvii* PP527343, *Y. lipolytica*

PP527342 and *Y. lipolytica* PP701998 as illustrated in Table 2. The similarity index utilized in the cluster analysis assure validates the resemblance between the isolates and known strains.

Table 2. Yeast isolates identification depending on basis of 18s rDNA gene sequences similarity

Isolates	Accession number	strains
R.C	PP527343	*Pichia kudriavizvii*
M.C	PP527342	*Yarrowia lipolytica*
Ray	PP701998	*Yarrowia lipolytica*

R.C: yeast isolate from ras cheese M.C: yeast isolate from mozzarella cheese

Ray: yeast isolate from Rayeb milk

Yeast strains were identified and recorded in gene bank with accession number *Pichia kudriavizvii* PP527343, *Yarrowia lipolytica* PP527342 and *Yarrowia lipolytica* PP701998 as mentioned in **Table 2**.

The phylogenetic tree indicates the division of the isolates into 2 clusters, each of them corresponds to a genus, exhibiting a noticeable similarity to known strains, as shown in **Fig. 7**. The Outputs of the cluster analysis indicate a significant level of similarity between the yeast isolates and yeast species previously identified.

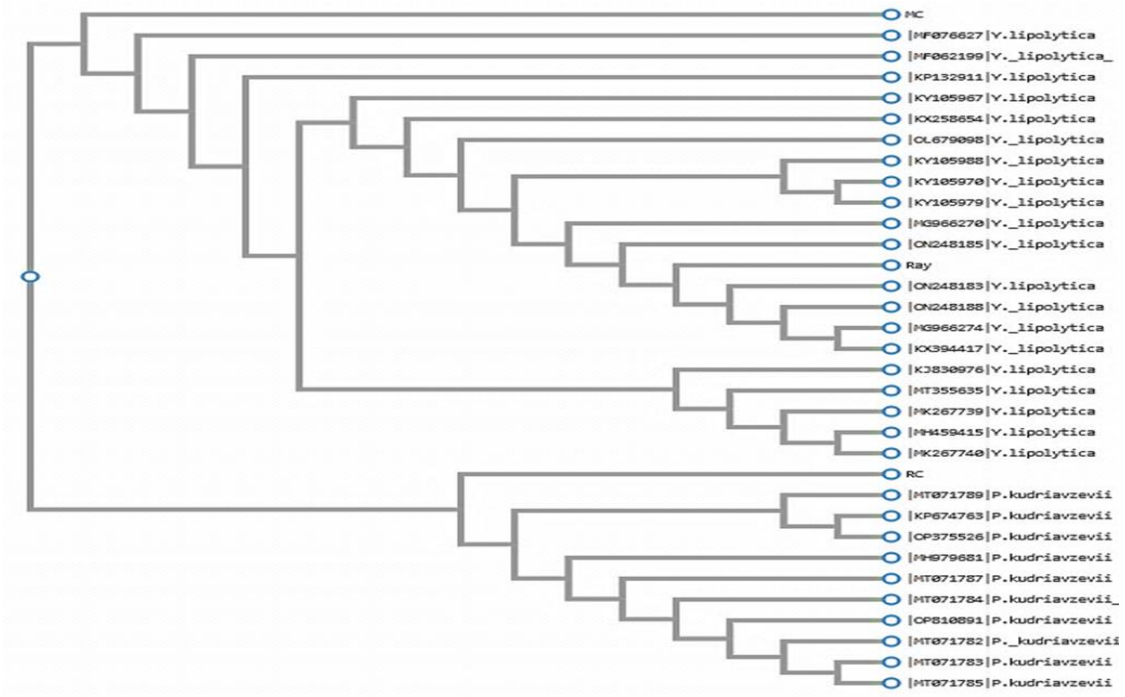

Fig. 7. Phylogenetic tree based on ITS region of yeast geneomic which shows the relationship between the selected isolates and their homologues strains in Genbank, analyzing were conducted in MEGA X. Rooted phylogentic tree (UPGMA)

4. CONCLUSION

In the recent years, polyunsaturated fatty acids (PUFAs) have significant attention of human health. Therefore, this research aimed to confirm the capability of yeast isolates biosynthesis and accumulation of lipids, especially PUFAs. For this reason, five isolates were chosen from fourteen different yeast isolates. Using Gas Chromatography (GCME-FID) analysis illustrated the varying capacities of the chosen isolates in producing PUFAs. The study extended to identify the three promising isolates. However, they belong to the genera of Pichia and Yarrowia. They could be utilized in producing essential omega 3, 6, and 9 fatty acids.

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