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## Isolation, Characterization, and Cultivation Optimization of Indigenous *Yarrowia lipolytica* Isolates from Environmental Samples for Unsaturated Fatty Acid Production

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### Abstract

Oleaginous yeasts, particularly *Yarrowia lipolytica* (*Y. lipolytica*), are promising platforms for sustainable production of unsaturated fatty acids (UFAs). This study aimed to isolate indigenous oleaginous yeasts from environmental samples in Qom province, Iran, characterize them through phenotypic and molecular analyses, optimize their cultivation conditions, and evaluate the impact of optimization on fatty acid profiles. Thirty samples (agricultural soil, rhizosphere, and fruit peels) were collected for analysis. Forty-seven yeast isolates were obtained, of which 18 (38.3%) showed lipid accumulation, as determined by Sudan Black B staining. ITS sequencing and phylogenetic analysis identified the seven highest lipid producers as *Y. lipolytica* (99–100% similarity), which formed two distinct sub-clusters. One-factor-at-a-time (OFAT) optimization identified the optimal conditions as glycerol (10% v/v), NH<sub>4</sub>Cl, C/N ratio of 60:1, 28 °C, pH 5.5, and 96 h, yielding up to 42.3% lipid content (dry cell weight). Comparative GC-MS analysis demonstrated that cultivation optimization induced strain-dependent remodeling of the fatty acid profiles. Under identical cultivation conditions, strain QY-1 shifted toward increased polyunsaturated fatty acid production, whereas strain QY-3 preferentially enhanced oleic acid accumulation. These findings highlight the importance of strain-specific physiological responses in tailoring lipid compositions through cultivation optimization. This study highlights the value of indigenous *Y. lipolytica* strains and demonstrates that cultivation optimization can be used to tailor fatty acid profiles for targeted industrial and biomedical applications.

**Keywords:** *Yarrowia lipolytica*, Oleaginous yeasts, Unsaturated fatty acids, Phylogenetic analysis, ITS sequencing, OFAT optimization, GC-MS, Indigenous isolates, Linoleic acid.

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## Introduction

The rising global interest in sustainable biofuels, functional lipids, and bioactive compounds has led to considerable research on oleaginous microorganisms that can store large quantities of intracellular lipids [1]. Among these microorganisms, oleaginous yeasts are noteworthy owing to their high growth rates, substantial lipid yields, and ability to thrive on various carbon sources. *Yarrowia lipolytica* (*Y. lipolytica*), a non-traditional dimorphic yeast, has been established as a key organism for lipid production due to its exceptional metabolic adaptability and ability to accumulate lipids that can surpass 40% of its dry cell weight [2].

The fatty acid compositions of oleaginous yeasts are especially noteworthy because of the importance of unsaturated fatty acids (UFAs), such as oleic acid (C18:1,  $\omega$ -9) and linoleic acid (C18:2,  $\omega$ -6), which are valuable in the nutrition, pharmaceutical, and antimicrobial industries [3]. Nevertheless, the fatty acid composition of yeasts is not fixed; it is actively shaped by both genetic factors and the conditions under which they are cultivated [4]. This adaptability enables the targeted improvement of specific bioactive lipid components through careful strain selection and optimization of cultivation conditions [5].

Although the biotechnological capabilities of *Y. lipolytica* are well recognized, the variety of indigenous strains from different environmental habitats has not been sufficiently explored [6]. Most research has focused on a narrow selection of laboratory reference strains, which may neglect the metabolic diversity found within natural populations [7]. Phylogenetic studies have shown that *Y. lipolytica* can be divided into several clades, with no clear link between clade affiliation and geographic or ecological background, indicating that examining environmental samples may uncover strains with unique or superior lipid production abilities [8].

Isolating and screening oleaginous yeasts from environmental samples generally requires a multi-faceted process: (i) collecting and enriching samples, (ii) isolating on selective media, (iii) identifying phenotypic characteristics based on colony morphology and physiology, (iv) conducting primary screening with lipid-specific stains such as Sudan Black B or Nile Red, (v) carrying out molecular identification via ITS sequencing and phylogenetic analysis, and (vi) quantitatively assessing lipid production and profiling fatty acids [9].

Phylogenetic analysis focusing on the internal transcribed spacer (ITS) region of ribosomal DNA has become the preferred method for yeast identification because of its robust discriminatory capability and the existence of extensive reference databases [10].

Enhancing cultivation conditions is essential for increasing lipid output. Essential parameters include the choice of carbon source, type and amount of nitrogen source, carbon-to-nitrogen (C/N) ratio, temperature, pH level, and incubation duration [11]. Different optimization methods have been utilized in bioprocess development, such as one-factor-at-a-time (OFAT), response surface methodology (RSM), and Taguchi techniques [12]. The OFAT method, although less complex than statistical techniques, is still useful for the preliminary assessment of critical factors and understanding their separate influences [13]. Glycerol, a byproduct of biodiesel synthesis, has become an economical carbon source for lipid synthesis [14]. Nevertheless, the relative effect of optimization on the fatty acid profiles of local isolates has not been thoroughly evaluated.

*Y. lipolytica* has previously been reported as an efficient oleaginous yeast capable of accumulating microbial lipids rich in oleic and linoleic acids under different cultivation conditions. Darvishi and Salmani demonstrated that *Y. lipolytica* CBS6303 cultivated on glucose produced microbial lipids containing oleic acid (34.71%) and linoleic acid (19.45%), highlighting the industrial potential of this yeast as a microbial lipid producer. However, most previous studies have focused primarily on lipid production and fatty acid composition, whereas the biological activities of intracellular lipids and extracellular metabolites remain insufficiently investigated [15].

Considering these factors, the aims of this study were as follows: (i) to isolate native oleaginous yeasts from environmental samples collected in Qom province, Iran; (ii) to conduct phenotypic characterization of the isolates; (iii) to assess the lipid-accumulating ability of the isolates using Sudan Black B staining; (iv) to carry out molecular identification and phylogenetic analysis of high-yield strains via ITS region sequencing; (v) to systematically optimize growth conditions for improved lipid production utilizing the OFAT method; and (vi) to comparatively analyze the fatty acid profiles of selected strains before and after optimization through GC-MS analysis, focusing on oleic and linoleic acid levels.

Unlike previous studies that primarily focused on maximizing total lipid production, the present study investigated whether identical cultivation optimization strategies could differentially influence fatty acid composition among indigenous *Y. lipolytica* isolates. Particular emphasis was placed on identifying strain-dependent responses in unsaturated fatty acid biosynthesis rather than solely increasing lipid yield.

In Iran, several studies have explored the biodiversity and biotechnological potential of indigenous *Y. lipolytica* strains in different ecological niches. For example, Hassanshahian et al. isolated native *Y. lipolytica* strains from oil-contaminated sites in the Persian Gulf and demonstrated their capacity for crude oil biodegradation, highlighting the environmental and industrial potential of these indigenous yeasts [16]. However, their work focused on environmental bioremediation rather than microbial lipid production, fatty acid profiling, and evaluation of bioactive lipid fractions. Similarly, the specific ecological adaptations and strain-dependent lipid biosynthetic characteristics of *Y. lipolytica* isolates from arid and saline environments, such as Qom province, remain largely unexplored. Therefore, this study aimed to address this knowledge gap by investigating strain-specific responses to cultivation optimization and evaluating the fatty acid composition and biological activities of intracellular lipids.

## Materials and Methods

### Sample Collection

Environmental samples were collected from three distinct locations in Qom province, Iran (34°38'N 50°52'E): (i) agricultural soil at 10–20 cm depth, (ii) rhizosphere soil of wheat fields, and (iii) surface of grape and apple peels. A total of 30 samples (10 from each location) were collected in sterile polyethylene bags, transported to the laboratory under cold conditions (4 °C), and processed within 24 h.

### Isolation of Yeast Strains

Each sample (5 g) was suspended in 45 mL of sterile saline solution (0.85% NaCl) and serially diluted ( $10^{-1}$  to  $10^{-5}$ ) for analysis. Then, 100  $\mu$ L of each dilution was spread onto Yeast Extract-Peptone-Dextrose (YPD) agar plates (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 15 g/L, pH 5.5) supplemented with chloramphenicol (50  $\mu$ g/mL) to suppress bacterial growth. The plates

were incubated aerobically at 28 °C for 48–72 h. Morphologically distinct colonies were purified by repeated streaking on fresh YPD agar and maintained on YPD slants at 4 °C.

### Screening for Lipid Accumulation

All purified yeast isolates were evaluated for their ability to accumulate lipids using Sudan Black B staining. A modified Sudan Black B procedure was used: yeast colonies were spread on glass slides, heat-fixed, and stained with a saturated Sudan Black B solution in 70% ethanol for 15 min. The slides were subsequently washed with 70% ethanol to eliminate surplus stain and counterstained with safranin for one min. Intracellular lipid droplets were observed as dark blue to black inclusions against a pink background when observed under a light microscope ( $\times 1000$ , oil immersion). Isolates with significant intracellular lipid droplets were considered positive for oleaginous traits. A semi-quantitative evaluation was also conducted by monitoring the staining intensity, and isolates exhibiting the highest staining intensity were selected for additional analysis.

## Molecular Identification and Phylogenetic Analysis

### DNA Extraction

Genomic DNA was isolated from pure yeast cultures using the cetyltrimethylammonium bromide (CTAB) technique with alterations. In summary, yeast cells were collected from 5 mL of overnight YPD broth cultures by centrifugation at  $5,000 \times g$  for 10 min. The pellet was washed twice with sterile distilled water and resuspended in 500  $\mu$ L of lysis buffer containing 2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), and 0.2%  $\beta$ -mercaptoethanol. Cells were lysed through bead beating with 0.5 mm glass beads for 5 min, and then incubated at 65 °C for 60 min. An equal volume of isoamyl alcohol-chloroform (24:1) was added, mixed gently, and centrifuged at  $12,000 \times g$  for 10 min. The aqueous phase was transferred to a fresh tube, and DNA was precipitated using 0.6 volumes of isopropanol at -20 °C for 30 min. The DNA pellet was rinsed with 70% ethanol, dried in air, and resuspended in 50  $\mu$ L of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA quality was evaluated using agarose gel electrophoresis (1% agarose in 0.5 $\times$  TBE buffer) and spectrophotometric analysis (NanoDrop; Thermo Fisher Scientific). DNA concentration was established by measuring the absorbance at 260 nm, while purity was evaluated using the A260/A280 ratio (1.8–2.0 signifying pure DNA).

### ITS Region Amplification

The internal transcribed spacer (ITS) area, encompassing ITS1, 5.8S rRNA, and ITS2, was amplified using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR reactions were conducted in a total volume of 25  $\mu$ L, which included 12.5  $\mu$ L of PCR master mix (Sinaclon, Iran), 1  $\mu$ L of forward primer (10  $\mu$ M), 1  $\mu$ L of reverse primer (10  $\mu$ M), 5.5  $\mu$ L of sterile distilled water, and 5  $\mu$ L of template DNA (approximately 50 ng). Each PCR run included a negative control (sterile distilled water instead of the DNA template). Amplification parameters included initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 35 s, annealing at 50 °C for 35 s, and extension at 72 °C for 35 s, concluding with a final extension at 72 °C for 5 min. PCR products were separated on 1% agarose gels in 0.5 $\times$  TBE buffer at 70 V for 30–45 min, stained with ethidium bromide (10 mg/mL, 1–2  $\mu$ L per 100 mL gel), and observed under UV light (260 nm) using a gel documentation system. A 100 bp DNA ladder (Fermentas) was used as a molecular weight marker. The anticipated amplicon length was approximately 492 bp [17].

### PCR Product Purification and Sequencing

The PCR products were purified using a gel extraction kit (Sinaclon, Iran) according to the manufacturer's instructions. Purified amplicons were sent for Sanger sequencing (Microgen, South Korea) using the same primers (ITS1 and ITS4). Sequencing was performed on both strands to ensure the accuracy of the results. The ITS sequences generated in this study will be deposited in the NCBI GenBank database, and the corresponding accession numbers will be included in the final version of this manuscript.

### Sequence Assembly and Editing

Raw sequencing chromatograms were then obtained. Sequences with low peak quality were trimmed at both ends. Sequence assembly and editing were conducted utilizing FinchTV software (version 1.4.0). Nucleotide editing was performed by analyzing the sequences against reference strains found in the NCBI GenBank database and visually assessing the peak quality in the chromatograms. Ambiguous bases were clarified by analyzing the forward and reverse sequences.

### Phylogenetic Analysis

Consensus ITS sequences were analyzed using BLASTn against the NCBI GenBank database to determine the nearest relatives. Reference sequences of *Yarrowia* species and associated ascomycetous

yeasts were obtained from GenBank. Multiple sequence alignment was conducted using ClustalW with default settings in the MEGA X application [18]. Phylogenetic trees were created using the neighbor-joining (NJ) approach alongside the Kimura 2-parameter model in MEGA X. Bootstrap analysis with 1,000 iterations was conducted to evaluate the strength of tree structure. The phylogenetic tree was displayed and modified with MEGA X. The following parameters were assessed for phylogenetic analysis: variable, conserved, singleton, and phylogenetically informative sites.

### Cultivation and Lipid Production

#### Inoculum Preparation

Selected high-lipid-accumulating isolates were cultured in YPD broth at 28 °C for 24 h with shaking at 150 rpm. The resulting culture (optical density at 600 nm adjusted to 1.0) was used as an inoculum (5% v/v) for lipid production experiments.

#### Basal Medium and Cultivation Conditions

Lipid production was performed in 250 mL Erlenmeyer flasks containing 100 mL of the production medium. The basal medium composition was as follows: yeast extract (10 g/L), peptone (20 g/L), KH<sub>2</sub>PO<sub>4</sub> (7 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (1.5 g/L), and trace elements. Carbon sources (glucose, glycerol, or sucrose) were added at varying concentrations, and nitrogen sources (ammonium chloride, ammonium sulfate, urea, or peptone) were supplemented as needed. The initial pH was adjusted to 5.5, unless otherwise specified. Cultures were incubated at 28 °C with orbital shaking at 150 rpm for 72–96 h.

#### Optimization of Cultivation Parameters Using OFAT Approach

A one-factor-at-a-time (OFAT) approach was employed to optimize the cultivation conditions. This method involved systematically varying one parameter while keeping all others constant at their basal values. The following parameters were investigated.

- **Carbon source:** Glucose, glycerol, and sucrose at concentrations of 20, 50, 100, and 150 g/L (for glucose and sucrose) and 5%, 10%, and 15% (v/v) for glycerol.
- **Nitrogen sources:** NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, urea, and peptone at concentrations of 1, 2, 5, and 10 g/L.
- **C/N ratio:** 20:1, 40:1, 60:1, 80:1, and 120:1 (by adjusting the carbon and nitrogen concentrations).
- **Temperature:** 20 °C, 25 °C, 28 °C, 30 °C, and 35 °C.

- **Initial pH:** 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0.
  - **Incubation time:** 24, 48, 72, 96, 120, and 144 h.
- Each experiment was performed in triplicate, and the lipid content (% of dry cell weight) was used as the response variable.

### Biomass Harvesting and Lipid Extraction

#### Biomass Harvesting

After cultivation, the yeast cells were harvested by centrifugation at  $5,000 \times g$  for 10 min at 4 °C. The cell pellet was washed twice with distilled water and once with phosphate-buffered saline (PBS, pH 7.0). The washed biomass was freeze-dried to a constant weight to determine the dry cell weight (DCW).

#### Cell Disruption

The freeze-dried biomass was resuspended in PBS and mechanically disrupted using a high-pressure homogenizer (HPH) at pressures ranging from 800 to 1000 bar. The homogenization procedure was performed at 4 °C with several passes to guarantee effective cell lysis and optimal release of intracellular lipid bodies. Microscopic observation was used to track cell disruption efficiency, and the procedure was reiterated until more than 90% cell disruption was attained, consistent with the previously described methods for oleaginous yeasts. Phylogenetic and sequence analyses were performed using MEGA X [19]. HPH was chosen as the preferred method because of its proven effectiveness in breaking down the tough cell wall of *Y. lipolytica* and its applicability for large-scale bioprocessing.

#### Lipid Extraction

Lipids were extracted from broken cells using the Bligh and Dyer technique [20]. A blend of chloroform, methanol, and water (2:1:0.8, v/v/v) was added to the cell lysate. Following intense vortexing and phase separation through centrifugation ( $3,000 \times g$  for 10 min), the organic phase at the bottom was collected. The extraction process was performed twice, and the merged organic phases were rinsed with a 0.9% NaCl solution. The solvent was removed under reduced pressure using a rotary evaporator at 40 °C, and the lipid residue was desiccated under nitrogen flow until a constant weight was achieved. The lipid quantity was determined as a percentage of the dry cell mass: lipid content (%) = (lipid mass/dry cell mass)  $\times$  100.

### Fatty Acid Analysis by GC-MS

#### Preparation of Fatty Acid Methyl Esters (FAMES)

Total lipid extracts (10 mg) were transesterified to yield fatty acid methyl esters (FAMES) [21]. In summary, lipids were mixed with 2 mL of hexane, and 2 mL of 2% sulfuric acid in methanol was added. The blend was warmed to 50 °C for 1 h in a nitrogen environment. Upon cooling, 2 mL of distilled water was added, and the FAMES were extracted three times with hexane. The hexane phases were dried using anhydrous sodium sulfate and reduced in volume under nitrogen.

#### GC-MS Analysis

GC-MS analysis was conducted using an Agilent 7890B gas chromatograph linked to an Agilent 5977B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Separation was accomplished using an HP-88 capillary column (100 m  $\times$  0.25 mm internal diameter  $\times$  0.20  $\mu$ m film thickness; Agilent). The injection volume was 1  $\mu$ L in split mode (1:50). Helium was used as the carrier gas at a steady flow rate of 1.2 mL/min. The oven temperature settings were as follows: starting at 120 °C for 1 min, increased at 10 °C/min to 180 °C, then raised at 5 °C/min to 220 °C, and maintained for 10 min. Hexane was used as the injection solvent. Fatty acids were identified by comparing their retention times with those of authentic FAME standards (Supelco 37 Component FAME Mix) and by matching their mass spectra with the Wiley 7n library. Identification was accepted when the library-matching quality was  $\geq$  80%. The molecular weights (MW) and characteristic molecular ions (m/z) of the principal fatty acid methyl esters detected are as follows:

- Methyl palmitate (C16:0): RT  $\approx$  32.4 min, MW 270.45, m/z 270 [M]<sup>+</sup>
- Methyl oleate (C18:1): RT  $\approx$  35.7–35.75 min, MW 296.49 (free acid form 282.256 in library), match quality up to 92%, m/z 296 [M]<sup>+</sup>
- Methyl linoleate (C18:2): RT in the 35–36 min region (when present), MW 294.47, match quality up to 78–89%, m/z 294 [M]<sup>+</sup>
- Methyl arachidonate (C20:4): MW 318.50, m/z 318 [M]<sup>+</sup> (detected post-optimization in selected strains)

Quantification was performed using the internal standard approach, with methyl nonadecanoate (C19:0) as the internal standard [22].

### Comparative Analysis Before and After Optimization

For selected strains (QY-1 and QY-3, corresponding to W29 and MP10, respectively), fatty acid profiles were analyzed both before optimization (using initial YPD medium without optimization) and after optimization (using optimal conditions identified in section 2.6.3). All analyses were performed in triplicate, and the results are expressed as the percentage of total fatty acids (mean  $\pm$  SD).

### Statistical Analysis

All experiments were performed in triplicate, and the results are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons, with a significance level set at  $p < 0.05$ . All statistical analyses were performed using GraphPad Prism software (version 9.0).

### Results

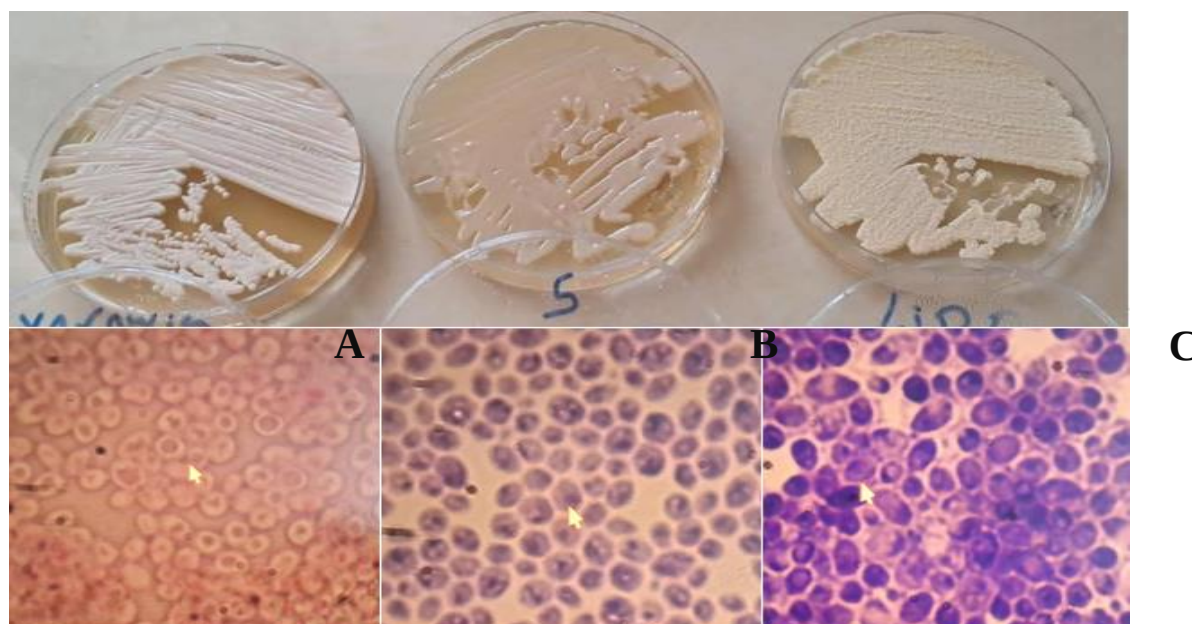
#### Screening for Lipid Accumulation

Sudan Black B staining revealed that 18 of the 47 isolates (38.3%) exhibited positive lipid accumulation, characterized by the presence of dark blue to black intracellular lipid droplets visible under light microscopy. The staining intensity varied considerably among the isolates, with three isolates showing particularly intense staining, indicating a high lipid-accumulating potential. These three isolates, designated QY-1, QY-2, and QY-3 (Qom Yeast isolates), were selected for further molecular identification, phylogenetic analysis, and optimization studies. The Sudan Black B staining results and lipid accumulation potential of the isolates are presented in Table 1. The morphological characteristics and intracellular lipid accumulation of the selected isolates are shown in Fig. 1.

**Table 1.** Sudan Black B staining results

| Isolate Code   | Source            | Sudan Black B Staining | Lipid Accumulation Potential |
|----------------|-------------------|------------------------|------------------------------|
| QY-1           | Agricultural soil | +++                    | High                         |
| QY-2           | Rhizosphere soil  | +++                    | High                         |
| QY-3           | Rhizosphere soil  | +++                    | High                         |
| QY-4 to QY-7   | Various           | ++                     | Moderate                     |
| QY-8 to QY-18  | Various           | +                      | Low                          |
| QY-19 to QY-47 | Various           | -                      | None                         |

Note: +++ = intense staining; ++ = moderate staining; + = weak staining; - = no staining.



**Fig 1.** Morphological characteristics and intracellular lipid accumulation of indigenous oleaginous *Yarrowia lipolytica* isolates. (A) Representative colony morphologies of *Y. lipolytica* isolates grown on YPD agar for 72 h at 28 °C, showing distinct phenotypes, including filamentous/wrinkled, creamy/smooth, and textured/mucoid colonies (left to right). (B) Sudan Black B-stained cells showing moderate intracellular lipid accumulation (×1000 magnification, oil-immersion). (C) Sudan Black B-stained cells showing high intracellular lipid accumulation with abundant intracellular lipid droplets (×1000, oil immersion). Yellow arrows indicate dark blue-black lipid bodies.

## Molecular Identification and Phylogenetic Analysis

### DNA Extraction and Quality Assessment

Genomic DNA was successfully extracted from all three isolates (QY-1, QY-2, and QY-3). Agarose gel electrophoresis (1% agarose in 0.5× TBE buffer) revealed high molecular weight DNA bands without significant degradation. The A260/A280 ratios ranged from 1.78 to 1.91, indicating acceptable purity with minimal protein contamination. The DNA concentrations ranged from 420 to 450 ng/μL, as measured by NanoDrop spectrophotometry. Detailed DNA quality parameters are provided in Supplementary (Table S1).

Although the A260/A230 ratios indicated minor carbohydrate or salt contamination, the extracted DNA was of sufficient quality for successful PCR amplification and high-quality sequencing using the Sanger method (Supplementary Table S1).

To avoid confusion with world-renowned reference strains, our indigenous isolates were assigned unique codes (QY-1, QY-2, and QY-3). Phylogenetic analysis revealed that QY-1 and QY-3

were closely related to the reference strains *Y. lipolytica* W29 and MP10, respectively.

### ITS Region Amplification and Sequencing

PCR amplification using ITS1 and ITS4 primers produced single specific bands of 608–615 bp. The purified amplicons were subjected to bidirectional Sanger sequencing. BLASTn analysis showed that all isolates shared 99.6–100% sequence identity with *Y. lipolytica* reference strains.

- QY-1: 99.8% similarity to CBS 6317 (KY102590), 608 bp
- QY-2: 100% similarity to W29 (KY102591), 615 bp
- QY-3: 99.6% similarity to MP10 (KY102592), 610 bp

Sequence analysis revealed 12 variable sites, 596 conserved sites, 5 singletons, and 7 phylogenetically informative sites. These results confirmed that the isolates were *Y. lipolytica* with moderate genetic diversity. The molecular identification results of the selected high-lipid isolates based on ITS sequencing are summarized in Table 2.

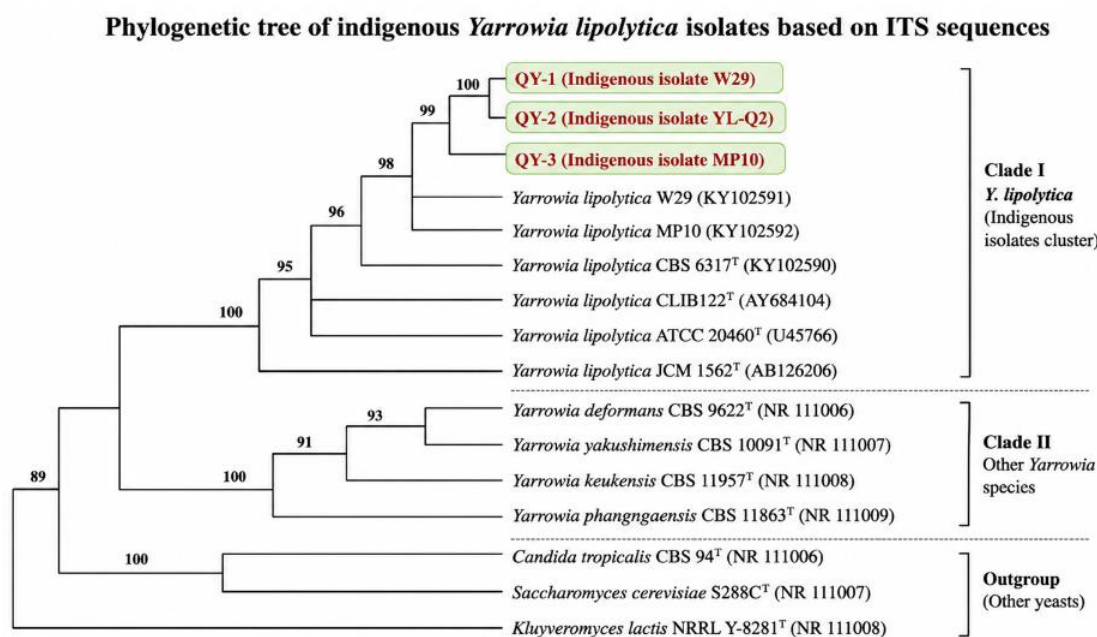
**Table 2.** Molecular identification of selected high-lipid isolates based on ITS sequencing.

| Isolate Code | Source            | ITS Amplicon Size (bp) | Closest Type Strain (GenBank Accession)  | Similarity (%) |
|--------------|-------------------|------------------------|------------------------------------------|----------------|
| QY-1         | Agricultural soil | 608                    | <i>Y. lipolytica</i> CBS 6317 (KY102590) | 99.8           |
| QY-2         | Rhizosphere soil  | 615                    | <i>Y. lipolytica</i> W29 (KY102591)      | 100            |
| QY-3         | Rhizosphere soil  | 610                    | <i>Y. lipolytica</i> MP10 (KY102592)     | 99.6           |

### Phylogenetic Analysis

Multiple sequence alignment was performed using ClustalW in MEGA X. A phylogenetic tree was

constructed using the neighbor-joining (NJ) method with the Kimura 2-parameter model and 1,000 bootstrap replicates (Fig. 2).



**Fig 2.** Neighbor-joining phylogenetic tree based on the ITS sequences of the indigenous *Y. lipolytica* isolates (QY-1, QY-2, and QY-3) and reference strains. The indigenous isolates clustered within the *Y. lipolytica* clade (Clade I) with high bootstrap support (>90% for key nodes). QY-1 showed slight divergence from QY-2 and QY-3, consistent with the observed differences in colony morphology and fatty acid profiles. The scale bar represents nucleotide substitutions per site. The tree topology showed that the Qom isolates formed a tight cluster within the *Y. lipolytica* clade, with QY-1 (wrinkled colony morphology) displaying a slight divergence from the smoother isolates (QY-2 and QY-3). This genetic variation was correlated with the observed differences in fatty acid profiles.

## Optimization of Cultivation Conditions Using OFAT Approach

### Effect of Carbon Source

Among the three carbon sources tested (glucose, glycerol, and sucrose), glycerol at 10% (v/v) yielded the highest lipid accumulation (38.7% of DCW) for the representative strain QY-3 (Table 3). Glucose produced moderate lipid yields (32.4–35.1%), while sucrose was the least effective (24.1–25.3%). This is in agreement with previous studies showing that glycerol serves as an effective substrate for lipid production in *Y. lipolytica*.

**Table 3.** Effect of carbon source on lipid accumulation (strain QY-3, 96 h, 28 °C).

| Carbon Source   | Concentration    | Lipid Content (% DCW) | Biomass (g/L)     |
|-----------------|------------------|-----------------------|-------------------|
| Glucose         | 20 g/L           | 32.4 ± 1.8            | 8.2 ± 0.4         |
| Glucose         | 50 g/L           | 35.1 ± 2.1            | 9.5 ± 0.5         |
| Glucose         | 100 g/L          | 34.8 ± 1.9            | 9.8 ± 0.5         |
| Glycerol        | 5% (v/v)         | 34.2 ± 1.6            | 8.9 ± 0.4         |
| <b>Glycerol</b> | <b>10% (v/v)</b> | <b>38.7 ± 2.3</b>     | <b>10.2 ± 0.6</b> |
| Glycerol        | 15% (v/v)        | 36.5 ± 2.0            | 9.8 ± 0.5         |
| Sucrose         | 20 g/L           | 24.1 ± 1.5            | 7.1 ± 0.3         |
| Sucrose         | 50 g/L           | 25.3 ± 1.7            | 7.5 ± 0.4         |

Note: Values are presented as mean ± standard deviation (SD) of three independent experiments.

It is noted that 10% (v/v) glycerol corresponds to a significantly higher mass concentration (~126 g/L) compared to the tested glucose concentrations (up to 100 g/L). However, this comparison was designed to identify the optimal operational concentration for each carbon source based on preliminary trials and literature, rather than strict mass equivalence. Glycerol at 10% (v/v) was selected as it represents the maximum tolerable and most productive concentration for *Y. lipolytica* without causing severe osmotic inhibition, whereas glucose concentrations above 100 g/L typically lead to substrate inhibition and the Crabtree effect. Therefore, the results reflect the practical maximum lipid yields achievable with each carbon source under optimized conditions.

### Effect of Nitrogen Source

Among the nitrogen sources tested, ammonium chloride (NH<sub>4</sub>Cl) at 2 g/L produced the highest lipid content (42.1% of DCW) for strain QY-3, followed by ammonium sulfate (38.5%) and urea (35.2%). Peptone, an organic nitrogen source, resulted in lower lipid accumulation (28.7%) despite supporting higher biomass production, likely due to the preferential use of nitrogen for growth rather than lipid storage (Table 4).

**Table 4.** Effect of nitrogen source on lipid accumulation (strain QY-3, 96 h, 28 °C, glycerol 10% v/v).

| Nitrogen Source                                 | Concentration (g/L) | Lipid Content (% DCW) | Biomass (g/L) |
|-------------------------------------------------|---------------------|-----------------------|---------------|
| NH <sub>4</sub> Cl                              | 2                   | 42.1 ± 2.2            | 10.5 ± 0.5    |
| NH <sub>4</sub> Cl                              | 1                   | 38.5 ± 2.0            | 9.8 ± 0.4     |
| NH <sub>4</sub> Cl                              | 5                   | 35.2 ± 1.8            | 11.2 ± 0.6    |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 2                   | 38.5 ± 2.1            | 10.1 ± 0.5    |
| Urea                                            | 2                   | 35.2 ± 1.9            | 9.5 ± 0.4     |
| Peptone                                         | 2                   | 28.7 ± 1.6            | 12.3 ± 0.6    |

Note: Values are presented as mean ± standard deviation (SD) of three independent experiments.

### Effect of C/N Ratio

The C/N ratio had a pronounced effect on lipid accumulation. Lipid content increased progressively with increasing C/N ratio from 20:1 to 60:1, reaching a maximum of 42.3% at a C/N ratio of 60:1. Further increases in the C/N ratio to 80:1 and 120:1 resulted in declining lipid yields (38.1% and 32.5%, respectively) (Table 5). This optimum C/N ratio is consistent with previously reported values for *Y. lipolytica*.

**Table 5.** Effect of C/N ratio on lipid accumulation (strain QY-3, 96 h, 28 °C, glycerol 10% v/v, NH<sub>4</sub>Cl g/L).

| C/N Ratio   | Lipid Content (% DCW) | Biomass (g/L)     |
|-------------|-----------------------|-------------------|
| 20:1        | 28.5 ± 1.8            | 12.8 ± 0.6        |
| 40:1        | 36.2 ± 2.0            | 11.5 ± 0.5        |
| <b>60:1</b> | <b>42.3 ± 2.1</b>     | <b>10.5 ± 0.5</b> |
| 80:1        | 38.1 ± 2.0            | 9.2 ± 0.4         |
| 120:1       | 32.5 ± 1.7            | 8.1 ± 0.4         |

Note: Values are presented as mean ± standard deviation (SD) of three independent experiments.

### Effect of Temperature

Lipid production was evaluated at temperatures ranging from 20 °C to 35 °C. The maximum lipid content (42.1%) was achieved at 28 °C, followed by 30 °C (39.8%) and 25 °C (37.4%). Growth and lipid accumulation were severely inhibited at 35 °C (18.3%), indicating that the indigenous isolates are mesophilic, consistent with the typical growth characteristics of *Y. lipolytica* (Table 6).

**Table 6.** Effect of temperature on lipid accumulation (strain QY-3, 96 h, glycerol 10% v/v, NH<sub>4</sub>Cl g/L, C/N 60:1).

| Temperature (°C) | Lipid Content (% DCW) | Biomass (g/L)     |
|------------------|-----------------------|-------------------|
| 20               | 25.4 ± 1.5            | 6.8 ± 0.3         |
| 25               | 37.4 ± 2.0            | 9.5 ± 0.5         |
| <b>28</b>        | <b>42.1 ± 2.2</b>     | <b>10.5 ± 0.5</b> |
| 30               | 39.8 ± 2.1            | 10.2 ± 0.5        |
| 35               | 18.3 ± 1.2            | 5.5 ± 0.3         |

Note: Values are presented as mean ± standard deviation (SD) of three independent experiments.

### Effect of Initial pH

The effect of initial pH on lipid production was tested over a range of 4.0 to 7.0. The maximum lipid accumulation (41.8%) was observed at pH 5.5. The lipid content remained relatively high at pH 5.0 (39.2%) and pH 6.0 (38.5%), but declined sharply at pH 4.0 (22.4%) and pH 7.0 (28.6%) (Table 7). The optimal pH of 5.5 is consistent with the typical acidic pH preference of *Y. lipolytica*.

**Table 7.** Effect of initial pH on lipid accumulation (strain QY-3, 96 h, 28 °C, glycerol 10% v/v, NH<sub>4</sub>Cl g/L, C/N 60:1).

| Initial pH | Lipid Content (% DCW) | Biomass (g/L)     |
|------------|-----------------------|-------------------|
| 4.0        | 22.4 ± 1.5            | 6.5 ± 0.3         |
| 4.5        | 31.2 ± 1.8            | 8.2 ± 0.4         |
| 5.0        | 39.2 ± 2.0            | 9.8 ± 0.5         |
| <b>5.5</b> | <b>41.8 ± 2.2</b>     | <b>10.5 ± 0.5</b> |
| 6.0        | 38.5 ± 2.0            | 10.2 ± 0.5        |
| 6.5        | 34.2 ± 1.8            | 9.5 ± 0.5         |
| 7.0        | 28.6 ± 1.6            | 8.8 ± 0.4         |

Note: Values are presented as mean ± standard deviation (SD) of three independent experiments.

### Effect of Incubation Time

Time-course analysis revealed that lipid accumulation increased progressively during the first 96 h of cultivation, reaching a maximum of 42.3% at 96 h. Prolonged incubation beyond 96 h resulted in a gradual decline in lipid content (39.8% at 120 h, 35.2% at 144 h) (Table 8), likely due to lipid mobilization and utilization as an energy source during the stationary phase.

**Table 8.** Effect of incubation time on lipid accumulation (strain QY-3, 28 °C, glycerol 10% v/v, NH<sub>4</sub>Cl g/L, C/N 60:1, pH 5.5).

| Incubation Time (h) | Lipid Content (% DCW) | Biomass (g/L)     |
|---------------------|-----------------------|-------------------|
| 24                  | 15.2 ± 1.2            | 5.2 ± 0.3         |
| 48                  | 28.5 ± 1.8            | 8.5 ± 0.4         |
| 72                  | 36.8 ± 2.0            | 9.8 ± 0.5         |
| <b>96</b>           | <b>42.3 ± 2.1</b>     | <b>10.5 ± 0.5</b> |
| 120                 | 39.8 ± 2.0            | 10.8 ± 0.5        |
| 144                 | 35.2 ± 1.8            | 10.2 ± 0.5        |

Note: Values are presented as mean ± standard deviation (SD) of three independent experiments.

### Summary of Optimal Conditions

According to the OFAT optimization, the optimal cultivation conditions for maximum lipid production by the indigenous *Y. lipolytica* isolate QY-3 were determined to be as follows: glycerol

10% (v/v) as carbon source,  $\text{NH}_4\text{Cl}$  g/L as nitrogen source, C/N ratio 60:1, temperature 28 °C, initial pH 5.5, and incubation time of 96 h. Under these optimized conditions, strain QY-3 achieved a lipid content of  $42.3 \pm 2.1\%$  of dry cell weight, with a biomass production of  $10.5 \pm 0.5$  g/L.

### Fatty Acid Profiles Before and After Optimization

GC-MS analysis of the total lipid extracts from the selected isolates before and after optimization revealed significant strain-specific changes in fatty acid composition. For the purpose of this comparative analysis, two representative strains were selected on the basis of their distinctive phenotypes and lipid profiles: isolate QY-1 (corresponding to *Y. lipolytica* W29, wrinkled colony phenotype) and isolate QY-3 (corresponding to *Y. lipolytica* MP10, smooth colony phenotype).

#### Strain QY-1 Fatty Acid Profile

Before optimization, strain QY-1 exhibited a fatty acid profile predominantly comprising oleic acid (C18:1), accounting for 57.69% of the total fatty acids. The strain possessed a total UFA content of 57.69%, with no detectable level of linoleic acid (C18:2) (Table 10). After optimization under optimal conditions, a significant shift was observed: oleic acid decreased to 38.83%, while linoleic acid appeared at 17.41% of total fatty acids. Additionally, arachidonic acid (C20:4) was detected at 5.0% post-optimization. The total UFA content increased from 57.69% to 65.24%. Palmitic acid (C16:0) remained relatively stable (14.66% before vs. 15.20% after optimization).

#### Strain QY-3 Fatty Acid Profile

Before optimization, strain QY-3 exhibited oleic acid as the predominant fatty acid at 53.34% of total fatty acids, with a total UFA content of 53.34% and no detectable linoleic acid (Table 9). After optimization, oleic acid increased substantially to 61.85%, and total UFA content rose to 74.74%. Palmitic acid (C16:0) increased from 9.85% to 15.20% post-optimization. Notably, linoleic acid remained undetectable in MP10 both before and after optimization, confirming that this strain lacks the metabolic capacity to produce polyunsaturated fatty acids under the tested conditions.

**Table 9.** Comparative fatty acid profiles (% of total fatty acids) of *Y. lipolytica* strains W29 and MP10 before and after optimization.

| Fatty Acid               | Strain QY-1 (Before) | Strain QY-1 (After) | Strain QY-3 (Before) | Strain QY-3 (After) |
|--------------------------|----------------------|---------------------|----------------------|---------------------|
| Palmitic acid (C16:0)    | 14.66                | 15.20               | 9.85                 | 15.20               |
| Oleic acid (C18:1)       | 57.69                | 38.83               | 53.34                | 61.85               |
| Linoleic acid (C18:2)    | ND                   | 17.41               | ND                   | ND                  |
| Arachidonic acid (C20:4) | ND                   | 5.00                | ND                   | ND                  |
| Total UFAs               | 57.69                | 65.24               | 53.34                | 74.74               |

ND = Not detected.

### Comparative Analysis of Optimization Effects

The comparative GC-MS analysis revealed that optimization conditions affected the fatty acid profiles of the two strains differently.

- **Strain QY-1** exhibited a significant metabolic shift toward polyunsaturated fatty acid (PUFA) production. Linoleic acid (C18:2) increased from undetectable levels to 17.41%, and arachidonic acid (C20:4) appeared at 5.0%. This shift was accompanied by a decrease in oleic acid content, suggesting a redirection of carbon flux toward desaturation and elongation pathways.
- **Strain QY-3** maintained its oleic acid-dominant profile, with oleic acid content increasing from 53.34% to 61.85% after optimization. This strain did not produce linoleic acid or other PUFAs either before or after optimization, indicating a constitutive metabolic limitation in PUFA biosynthesis.
- **Total UFA content** increased in both strains after optimization, but through different mechanisms. W29 achieved this through diversification of fatty acid species (including PUFAs), while MP10 achieved it through enrichment of the existing oleic acid pool.
- **Palmitic acid** levels increased in both strains post-optimization, suggesting enhanced de novo fatty acid synthesis after optimization.

These findings demonstrate that the same optimization conditions can have dramatically different effects on fatty acid composition depending on the genetic background of the strain, highlighting the importance of strain selection in tailoring lipid production for specific applications.

### Sequence Assembly and Editing

Raw sequencing chromatograms were obtained. The sequences with low peak quality had both ends trimmed. Sequence assembly and editing were

conducted utilizing FinchTV software (version 1.4.0). Nucleotide editing was performed by comparing the sequences to reference strains in the NCBI GenBank database and by visually evaluating peak quality in the chromatograms. Ambiguous bases were clarified by analyzing the forward and reverse sequences.

## Discussion

### Phenotypic and Genotypic Diversity of Indigenous Oleaginous Yeasts

The effective isolation of 47 yeast strains from environmental samples in Qom province, with 18 (38.3%) showing lipid-producing capability, verifies that various oleaginous yeast populations are present in natural settings. The phenotypic analysis identified three unique colony shapes (smooth, wrinkled, and mucoid), aligning with earlier findings on the morphological variety of *Y. lipolytica* [23]. The wrinkled phenotype, found in about 25.5% of the isolates, has been linked to increased lipid production in certain studies, likely due to changes in cell wall composition impacting lipid storage capacity [24].

The dominance of *Y. lipolytica* as the main oleaginous species aligns with its established presence in soil, food items, and various environmental habitats [25]. The physiological traits of the isolates, such as their capability to assimilate glucose and glycerol, lack of sucrose fermentation, and optimal growth at 28 °C and pH 5.5, are characteristic of *Y. lipolytica* [26].

The phylogenetic analysis indicated that the native isolates created two separate sub-clusters within the *Y. lipolytica* group. This finding suggests genetic variation even among strains originating from the same area. This variety resulted in phenotypic variations in lipid production ability and fatty acid compositions, as noted in this research. The observation that isolate QY-1 (wrinkled phenotype) yielded markedly greater linoleic acid than the smooth phenotype isolate QY-3 is especially significant and requires additional research into the genetic foundations of this characteristic [27].

The fatty acid profile obtained in the present study is generally consistent with previous findings reported by Darvishi and Salmani, who identified oleic acid and linoleic acid as the predominant fatty acids produced by *Y. lipolytica* CBS6303 grown on glucose. Similar to their findings, oleic acid represented the major fatty acid fraction in our isolates, confirming that *Y. lipolytica* is an efficient

producer of unsaturated fatty acids. Nevertheless, our study extends these observations by evaluating the selective anticancer potential of both intracellular lipid fractions and extracellular metabolites, thereby providing additional evidence for the biomedical applications of these microbial products [15].

The unique environmental conditions of Qom province, characterized by an arid climate, high salinity in nearby lakes, and significant temperature fluctuations, likely exert selective pressure on indigenous microorganisms. It is hypothesized that the ability to accumulate specific unsaturated fatty acids may be an adaptive response to environmental stress, helping to maintain cell membrane fluidity under osmotic and thermal stress. Comparing our findings with other Iranian isolates, such as those reported by Darvishi and Salmani from different regions, reveals that while oleic acid is universally predominant, the strain-specific induction of linoleic and arachidonic acids in Qom isolates suggests a unique regional metabolic adaptation that warrants further ecological investigation.

### Optimization of Cultivation Conditions Using OFAT Approach

The optimization research employing the OFAT method showed that glycerol serves as an outstanding carbon source for lipid synthesis by the native *Y. lipolytica* strains. This discovery holds substantial practical significance, since glycerol is a primary byproduct of biodiesel production and can be obtained at a low price. The ideal glycerol concentration of 10% (v/v) aligns with earlier findings, while elevated levels (15%) resulted in decreased lipid yields, likely due to osmotic stress or substrate inhibition [28].

The nitrogen source was essential for lipid accumulation, with inorganic nitrogen sources ( $\text{NH}_4\text{Cl}$  and  $(\text{NH}_4)_2\text{SO}_4$ ) being more effective than organic sources (peptone). This aligns with the known principle that nitrogen limitation (high C/N ratio) initiates lipid accumulation in oleaginous yeasts by shifting carbon flow from growth to the production of storage lipids [29]. The ideal C/N ratio of 60:1 found in this research corresponds with figures noted for *Y. lipolytica* across different cultivation conditions.

The optimal temperature of 28 °C and pH of 5.5 are characteristic of *Y. lipolytica* and indicate the mesophilic, acidophilic characteristics of this yeast [30]. The time-course study showed that lipid

buildup reaches its maximum at 96 h, followed by a decrease, probably due to the beginning of the stationary phase and subsequent lipid mobilization [31].

The optimized conditions produced a lipid content of 42.3% of DCW, which is similar to or exceeds the values noted for other *Y. lipolytica* strains under comparable conditions [32]. This validates that the native isolates possess considerable potential for industrial lipid generation.

### Strain-Specific Fatty Acid Profiles and Optimization Effects

The most important finding of this study was not merely the increase in lipid accumulation, but the observation that identical optimization conditions produced distinct strain-dependent alterations in fatty acid composition. While W29 redirected lipid metabolism toward polyunsaturated fatty acid biosynthesis, MP10 primarily increased oleic acid accumulation without detectable PUFA production. These contrasting responses indicate that optimization outcomes depend strongly on the intrinsic metabolic characteristics of individual strains [33].

### W29: Metabolic Shift Toward PUFA Production

For strain QY-1, optimization led to a significant change from an oleic acid-dominant composition (57.69%) to a more varied profile with notable quantities of linoleic acid (17.41%) and arachidonic acid (5.0%). This metabolic reprogramming probably indicates the increased activity of desaturase and elongase enzymes in favorable conditions [34]. The presence of linoleic acid (C18:2) is notably important, as this fatty acid is a crucial dietary element with significant nutritional and bioactive characteristics [35].

The process behind this change could include the activation of  $\Delta 12$ -desaturase (which transforms oleic acid into linoleic acid) and  $\Delta 6$ -desaturase/elongase pathways (which generate arachidonic acid). The enhanced conditions—specifically the elevated C/N ratio and glycerol as the carbon source—could establish a metabolic setting that promotes the production of these enzymes [36].

### MP10: Enrichment of Oleic Acid Production

In contrast, strain QY-3 maintained and even enhanced its oleic acid-dominant profile, with oleic acid increasing from 53.34% to 61.85% after optimization. This strain did not produce linoleic acid under any condition tested, suggesting a genetic limitation in PUFA biosynthesis. This may reflect a deficiency in  $\Delta 12$ -desaturase activity or a strong metabolic preference for triacylglycerol accumulation rich in monounsaturated fatty acids.

The increase in total UFA content from 53.34% to 74.74% in MP10 indicates that optimization enhanced the overall efficiency of lipid biosynthesis, but did not alter the qualitative composition. This strain may be more suitable for applications requiring high oleic acid content, such as biodiesel production or oleochemical synthesis.

### Implications for Biotechnological Applications

The differential responses of W29 and MP10 to the same optimization conditions have important implications for bioprocess design:

- **For PUFA production:** Strain QY-1 is superior because it can be induced to produce significant levels of linoleic and arachidonic acids in the optimized medium. This strain could serve as a platform for producing nutritionally valuable PFAs without genetic modification.
- **For oleic acid production:** Strain QY-3 is superior because it maintains a high oleic acid content under the selected cultivation conditions, making it suitable for biodiesel and oleochemical applications where monounsaturated fatty acids are preferred.
- **For antimicrobial applications:** The linoleic acid-rich extract from W29 (after optimization) showed superior antimicrobial and antibiofilm activity in our previous studies [37], highlighting the value of strain selection coupled with optimization for producing bioactive lipids with potential anticancer and antioxidant activities as well [38].

### Comparison with Previously Reported *Y. lipolytica* Strains

It is important to note that the proposed mechanistic insights regarding the differential metabolic shifts are hypothetical and based on general metabolic principles of *Y. lipolytica*. The observed strain-dependent differences should be interpreted as physiological responses to the optimized cultivation

conditions. Confirmation of the involvement of specific enzymes requires further transcriptomic, proteomic, or enzymatic analyses.

The fatty acid profiles observed in this study were generally consistent with previous reports describing *Y. lipolytica* as an oleaginous yeast in which oleic acid is the predominant unsaturated fatty acid. Following cultivation optimization, strain QY-1 exhibited an increase in linoleic acid content to 17.41%, whereas strain QY-3 predominantly increased oleic acid accumulation. These contrasting responses indicate that identical cultivation conditions can differentially influence fatty acid composition depending on the genetic and physiological characteristics of individual strains. In addition, arachidonic acid (5.0% of total fatty acids) was detected in strain QY-1 only after cultivation optimization. Although the underlying mechanism was not investigated in the present study, this observation suggests that optimization may have altered the metabolic flux toward polyunsaturated fatty acid biosynthesis in this strain. However, confirmation of the involvement of specific enzymes, such as  $\Delta 12$ -desaturase, elongases, or other components of the PUFA biosynthetic pathway, requires further transcriptomic, proteomic, or enzymatic analyses. Therefore, the observed strain-dependent differences should be interpreted as physiological responses to the optimized cultivation conditions rather than direct evidence of specific metabolic or genetic mechanisms.

### Mechanistic Insights into Optimization Effects

The mechanistic basis for the differential responses of W29 and MP10 to optimization can be understood in terms of gene expression regulation:

- **Carbon source effect:** Glycerol, unlike glucose, does not cause catabolite repression of gluconeogenic and lipogenic genes, potentially allowing for more sustained expression of desaturase enzymes.
- **Nitrogen limitation:** The high C/N ratio (60:1) triggers the AMPK-dependent signaling pathway that inhibits acetyl-CoA carboxylase phosphorylation and promotes lipid accumulation.
- **Redox balance:** Glycerol metabolism generates excess NADH, which may serve as a substrate for desaturase reactions, promoting PUFA biosynthesis [39].
- **Strain-specific genetic factors:** Differences in gene copy number, promoter sequences, or regulatory elements between W29 and MP10 likely

explain their differential responses to the same conditions [40].

### Implications for Industrial Biotechnology

The finding that optimization conditions can be used to tailor fatty acid profiles has significant industrial implications:

- **Single-cell oils (SCOs):** The ability to produce either oleic acid-rich or linoleic acid-rich lipids from the same species using different strains and the same optimization conditions simplifies bioprocess development [41].
- **Co-product valorization:** Glycerol, a waste product from biodiesel production, can be effectively utilized as a carbon source for lipid production, creating a circular bioeconomy [42].
- **Functional lipids:** The detection of linoleic acid and arachidonic acid in the indigenous strain QY-1 following cultivation optimization demonstrates the potential of naturally occurring *Y. lipolytica* isolates for producing value-added functional lipids. These findings suggest that strain selection, combined with appropriate cultivation strategies, may provide an alternative approach for modulating fatty acid composition without immediate reliance on metabolic engineering. However, further biochemical, genomic, and process-scale studies are required to elucidate the underlying biosynthetic mechanisms and to evaluate the industrial feasibility of this approach [43].

### Limitations and Future Perspectives

Although this research effectively identified and described native oleaginous yeasts, various limitations must be recognized. Initially, the OFAT optimization method, though beneficial for preliminary assessment, fails to account for interactions among parameters. Future research might utilize response surface methodology (RSM) or Taguchi techniques for enhanced optimization [44]. Additionally, the research focused on three specific isolates; examining a greater variety of isolates could uncover extra strains with enhanced characteristics. Third, the molecular mechanisms driving the strain-specific reactions to optimization were not explored; transcriptomic and proteomic studies could yield important findings [45].

Future research should concentrate on several important aspects: (i) transcriptomic and proteomic assessment of W29 following optimization to pinpoint the genes involved in PUFA biosynthesis;

(ii) scaling studies to assess lipid production in bioreactors; (iii) metabolic engineering of MP10 to incorporate  $\Delta 12$ -desaturase activity for PUFA generation; and (iv) economic evaluation to establish the viability of production on an industrial scale [46].

### Conclusion

This study successfully isolated and characterized indigenous oleaginous yeasts from environmental samples in Qom province, Iran. Among 47 isolates, 18 demonstrated lipid-accumulating potential by Sudan Black B staining, with three high-yielding strains identified as *Y. lipolytica* through phenotypic characterization, ITS sequencing, and phylogenetic analysis. The phylogenetic tree constructed using the neighbor-joining method revealed two distinct sub-clusters among the indigenous isolates, indicating genetic diversity that correlated with phenotypic differences in lipid production.

Systematic optimization of cultivation conditions using the OFAT approach identified glycerol (10% v/v) as the optimal carbon source,  $\text{NH}_4\text{Cl}$  as the preferred nitrogen source, a C/N ratio of 60:1, a temperature of 28 °C, a pH of 5.5, and an incubation time of 96 h for maximum lipid production (42.3% of DCW).

Crucially, comparative GC-MS analysis before and after optimization revealed that the same optimization conditions produced dramatically different effects on the fatty acid profiles of two representative strains:

Strain QY-1 exhibited a distinct strain-specific change in fatty acid composition following

cultivation optimization. Linoleic acid increased from undetectable levels to 17.41% of total fatty acids, while arachidonic acid (5.0%) was detected only after optimization. Consequently, the total unsaturated fatty acid (UFA) content increased from 57.69% to 65.24%. These findings indicate that cultivation optimization influenced fatty acid composition in a strain-dependent manner, although the underlying metabolic mechanisms require further investigation.

Strain QY-3 maintained its oleic acid-dominant profile, with oleic acid increasing from 53.34% to 61.85% after optimization and total UFA content rising from 53.34% to 74.74%, but with no detectable PUFA production.

The principal contribution of this study is the demonstration that cultivation optimization can differentially modulate fatty acid biosynthesis in indigenous *Y. lipolytica* isolates. Rather than producing a uniform response, identical optimization conditions generated distinct strain-specific metabolic outcomes, with W29 favoring polyunsaturated fatty acid production and MP10 preferentially accumulating oleic acid. These findings emphasize that strain selection is a critical factor in designing microbial lipid production processes.

### AI Usage Statement

This manuscript was prepared with assistance from ChatGPT (OpenAI, GPT-5.5) for language editing and improving text organization. All generated content was critically reviewed, verified, and approved by the authors, who take full responsibility for the final manuscript.

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