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Biodiesel production from oleaginous *Saccharomyces cerevisiae* by adaptive laboratory evolution method using glycerol as an alternative carbon source

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ABSTRACT: In this study, oleaginous *Saccharomyces cerevisiae* was subjected to adaptive laboratory evolution (ALE) to improve its growth on glycerol. After 25 sequential serial passages, the ALE-evolved strain *S. cerevisiae* TP-25 grown on glycerol exhibited a sevenfold increase in growth compared with the parental strain. In addition, C/N molar ratio was evaluated for lipid production, and the highest lipid content was observed at a C/N molar ratio of 75. At this ratio, the TP-25 strain produced 33.27 ± 0.55% of lipid content and 0.61 ± 0.01 g/l of lipid production. To assess the feasibility of these lipids for biodiesel production, the lipid properties were analyzed and compared with international biodiesel standards, including ASTM D6751 and EN 14214. The findings indicated that the lipids produced by oleaginous *S. cerevisiae* using glycerol as the carbon source met the necessary criteria for biodiesel production. This suggested that glycerol, a byproduct of many industrial processes, could serve as a valuable carbon source for sustainable biodiesel production in oleaginous *S. cerevisiae* strains.

KEYWORDS: biodiesel, oleaginous yeast, *Saccharomyces cerevisiae*, glycerol, adaptive laboratory evolution

INTRODUCTION

Nowadays, the issue of insufficient petroleum-based resources is a concern for all countries. Moreover, this problem is associated with increased greenhouse gas emissions. These significant challenges have prompted researchers to look into alternative energy sources to replace fossil fuels. One option that has received attention for a long time is biofuels, such as biogas, bioethanol, and biodiesel. These are considered renewable and sustainable sources because they are produced from biological materials. Biodiesel can be generated from various vegetable oils such as palm, soybean, corn, and rapeseed oils [1]. According to the Biodiesel Global Market Report 2023, the global biodiesel market is expected to reach 49.34 billion USD by 2027 and 72.29 billion USD by 2030. However, the massive demand for biodiesel has also raised various concerns, including the extensive space required for cultivating plants and their dependence on climatic conditions. Moreover, there are worries about its impact on global human food security because the leading source for producing biodiesel is edible plant oil [2]. Therefore, to overcome this obstacle, finding new sources of raw materials is an urgent necessity.

Microbial lipids, also known as single-cell oils, have garnered attention as an alternative source for biodiesel production. There are several advantages of using microbial lipids compared with vegetable oils. For instance, microbial lipids can be produced independently of seasonal and geographical conditions. Additionally, the microbes have a short life cycle during culture, and their management is not labor-intensive. Microbial lipids can be produced by various microorganisms, e.g., bacteria, algae, fungi, and yeasts [3]. These microorganisms are considered oleaginous, as they can accumulate intracellular lipids more than 20% of the cell dry weight. Oleaginous microalgae and yeasts are regarded as promising sources of microbial lipid production due to their enhanced lipid productivity. However, microalgal cultivation still has drawbacks, such as the requirement for sunlight, CO_2, and plenty of water [4], which can pose challenges when scaling up production.

Oleaginous yeasts are considered a promising candidate for the production of microbial lipids. Yeasts are unicellular organisms known for their rapid growth and high lipid content. In addition, yeast cultivation allows for easy scalability and does not require light for growth [5]. They can utilize various substrates for growth and lipid production. Considering all these properties, yeasts are the best candidates for developing industrial biotechnological approaches for lipid production. However, culturing yeast for lipid production on a commercial scale still poses challenges because it depends on the use of a glucose-composition medium, leading to high costs [6]. As a result, many studies have reported using different low-cost feedstocks for lipids production such as lignocellulosic biomass, acetate, wastewater, molasses and glycerol [7].

Glycerol is an attractive feedstock due to its abun-

dance and higher degree of reduction per carbon when compared with glucose [8]. Glycerol is a major by-product in the biodiesel industry of about 10% of biodiesel production. In addition, glycerol is generated from several other industries, including fat saponification, stearin production, and alcoholic beverage production [9]. Glycerol production was estimated to reach 680 kilotonnes in 2024 [10]. Therefore, glycerol could be a feedstock for microbial lipid production.

Many studies have reported successful bioconversion of glycerol into lipids by microbes. A majority of the oleaginous yeast using glycerol as a feedstock are classified within *Yarrowia lipolytica*, *Rhodotorula toruloides*, *Rhodotorula glutinis*, *Cryptococcus curvatus*, *Trichosporon oleaginosus*, and *Lipomyces starkeyi* [11]. However, few studies have reported lipid production by *S. cerevisiae* utilizing glycerol. One such study reported lipid production of 23.0 mg/l from glycerol, with a lipid content of 12% [12]. This is still considered a non-oleaginous yeast and unsuitable for large-scale lipid production. *S. cerevisiae* is an extensively studied model microorganism for commercial and industrial scaling up and has been successfully applied in the bioethanol industry. *S. cerevisiae* is classified as a GRAS (Generally Recognized as Safe) organism and a highly safe one. Moreover, *S. cerevisiae* has been extensively studied, with comprehensive whole-genome data available and significant genetic research conducted. Generally, *S. cerevisiae* is regarded as a non-oleaginous yeast. Nevertheless, some strains of *S. cerevisiae* were identified as oleaginous, and their primary carbon sources for lipid production were glucose and xylose [13, 14]. To our knowledge, there has been only one study on lipid production in oleaginous *S. cerevisiae* using glycerol reported by Berikten et al [15]. Although most existing studies focus on developing oleaginous *S. cerevisiae* strains capable of high lipid production from glucose [13, 14], it remains unclear whether these strains can be adapted to utilize alternative carbon sources, such as glycerol, for enhancing lipid production. No studies have yet explored the development of an oleaginous *S. cerevisiae* strain capable of high lipid production from glucose, nor whether such a strain could be adapted to utilize alternative substrates, such as glycerol, for enhancing lipid production.

This study reports on lipid production by oleaginous *S. cerevisiae* utilizing glycerol as a carbon source, for the first time using a strain that was previously reported to be unable to grow on glycerol [13], developed through the adaptive laboratory evolution (ALE) method. The C/N molar ratio was evaluated to optimize lipid production. Additionally, fatty acid composition concerning the biodiesel properties was analyzed by comparing them to the biodiesel standards, EN 14214 (Europe) and ASTM D6751 (USA). These studies ensured that the lipids obtained from oleaginous *S. cerevisiae* using glycerol would be suitable for biodiesel production.

MATERIALS AND METHODS

Strains and culture medium

The oleaginous *S. cerevisiae* strain CU-TPD4 was obtained from the Biofuels by Biocatalysts Research Unit (BBRU) [13]. The yeast strain was cultured in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) and YPG medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glycerol) in preparation for the ALE method.

ALE experiments of *S. cerevisiae* on glycerol

ALE was performed using single colony cultured in YPD medium at 30 °C under a rotary shaking at 200 rpm. Cells were collected, centrifuged, and washed twice with sterile distilled water to remove the YPD medium. The cells were reinoculated into YPG medium, and the initial OD600 of 0.1 was measured. After cell growth reaching a stationary phase, cells were reinoculated into fresh YPG medium and sequentially passaged 15 and 25 times to obtain the TP-15 and TP-25 strains, respectively.

Determination of C/N molar ratio

The TP-25 strain was pre-cultivated in YPG medium at 30 °C and under a rotary shaking at 200 rpm. It was then transferred into 100 ml of lipid production medium with an initial OD600 of 0.1 and re-incubated for five days. To evaluate the C/N molar ratio for lipid production, the nitrogen source was fixed at 5 g/l each for peptone and yeast extract. The C/N molar ratios were calculated based on the composition of peptone (14% w/w nitrogen, 8% w/w carbon) and yeast extract (7% w/w nitrogen, 12% w/w carbon) [13]. Initial glycerol concentrations ranging from 70 to 333 g/l were added to achieve the desired initial C/N molar ratios of 27, 50, 75, 100, and 125.

Determination of glycerol

Glycerol in the culture medium was determined using the Malaprade and Hantzsch reaction, as described by Kuhn et al [16]. Briefly, the culture medium was treated with periodate reagent, and then acetylacetone reagent was added to initiate a subsequent reaction, leading to the formation of a colored complex. The intensity of the complex solution was measured by the absorbance at 410 nm using a spectrophotometer.

Determination of lipid production

Lipids were extracted following the methodology described in a previous study [13]. Briefly, 25 ml of biomass culture was subjected to two rounds of washing with sterile distilled water and centrifugation. Then, 10 ml of a 1:1 (v/v) methanol-chloroform solution was added and incubated at 30 °C. Subsequently,

the lower phase of the solution, which contained the extracted lipids, was collected and put in a hot air oven until the solvent completely evaporated. The biomass was investigated by collecting a 1 ml aliquot of the culture and having it centrifuged, washed twice with sterile distilled water, and dried at 80 °C until a constant weight was obtained. The lipid production, lipid content, and biomass were determined by weight gravimetrically and calculated using the equations as follows:

$$\text{Lipid production (g/l)} = \frac{\text{Weight of lipid (g)}}{\text{Medium volume (l)}}$$

$$\text{Lipid content (\%)} = \frac{\text{Weight of lipid (g)}}{\text{Cell dry weight (g)}} \times 100$$

$$\text{Biomass (g/l)} = \frac{\text{Cell dry weight (g)}}{\text{Medium volume (l)}}$$

Fluorescence assay and qualitative analysis of lipids

Fluorescence assay was performed as previously described [13]. In brief, 20 µl of yeast cell culture was mixed with 25 µl of Nile red staining solution and 280 µl of 1:1 (v/v) DMSO:phosphate buffer and incubated for 10 min at 30 °C. Fluorescent microscopy was performed using emission and excitation wavelengths of 590 and 488 nm, respectively.

Determination of fatty acid composition

Fatty acid composition was determined from fatty acid methyl esters (FAMEs) by transesterification of lipids. Briefly, the lipids were converted to FAMEs by adding methanol and NaOH, and the solution was incubated at 60 °C for 30 min. Then, hexane was added, and the mixture was incubated at 30 °C for 60 min with shaking at 200 rpm. After incubation, two phases of the solution was obtained. The upper phase was collected and analyzed using gas chromatography with a flame ionization detector. The fatty acid composition was identified based on retention times. The proportions of different fatty acids were expressed in relative percentages calculated from the peak areas of individual FAMEs.

Analysis of lipid composition

FAMEs were collected and determined by a GC-2010 GC setup (Shimadzu, Japan) with a flame ionization detector and a 30 m × 0.53 mm i.d. × 0.25 mm DB-WAX column (Agilent, USA). One µl of sample was injected into the column with a split ratio of 1:10, and the inlet temperature was maintained at 250 °C. The column temperature program started at 50 °C, held for 2 min, followed by an increase of 25 °C/min to 125 °C, and then 10 °C/min to 220 °C, where it was held for 15.5 min. The detector temperature was set to 250 °C, with hydrogen flow at 40 ml/min, air zero at 400 ml/min, and helium makeup gas at 30 ml/min.

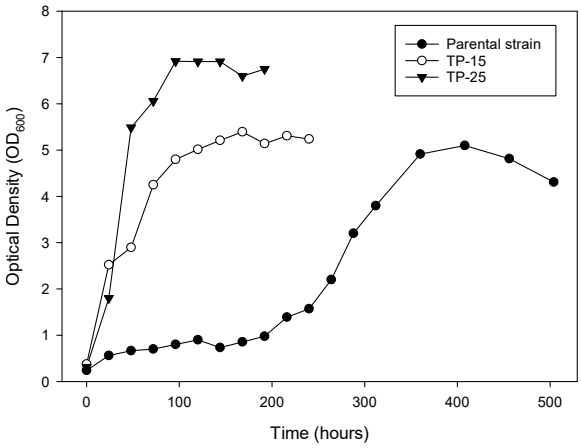

Fig. 1 Growth curves of adaptive laboratory evolution for *S. cerevisiae* on glycerol as a carbon source. Filled circles, *S. cerevisiae* CU-TPD4; open circles, the 15th passage; and filled triangles, the 25th passage.

The FAMEs were identified by comparing with standard reference solutions based on GLC-60 (Nu-Chek Prep, USA).

Qualitative analysis of the extracted lipids was performed using an LC-20 A series high-performance liquid chromatography (HPLC) (Shimadzu, Japan) with an ELSD detector. Lipids were mixed with hexane and injected into Shim-pack VX-SIL (250 mm, 4.6 A). The column was maintained at 40 °C, with a flow rate of 1.5 ml/min, and a 20 µl of sample was injected. A gradient mobile phase system was employed, where solvent A being hexane, and solvent B containing hexane, isopropanol, and ethyl acetate in a ratio of 42.5:10:5 (v/v). The gradient started with solvent B at 0%, increased to 25% over 18 min and held for 1 min, then raised to 99% over 25 min and held for 5 min. The gradient was then reduced to 1% solvent B over 2 min, and the column was washed for 13 min.

Investigation of physical properties of biodiesel

Physical properties of biodiesel, including the cetane number (CN), higher heating value (HHV), cloud point (CP), cold filter plugging point (CFPP), iodine value (IV), saponification value (SV), and density value of the FAMEs were analyzed using the Biodiesel Analyzer© Version 1.1, designed to the predict biodiesel properties [17].

RESULTS AND DISCUSSION

ALE of *S. cerevisiae* on glycerol as a carbon source

The present study employed the oleaginous *S. cerevisiae* strain CU-TPD4 to investigate lipid production using glycerol as a carbon source, as this strain possessed a high capacity for lipid accumulation. Nevertheless, the early endeavors to produce lipids from

glycerol in the *S. cerevisiae* CU-TPD4 strain were considerably restricted. The findings revealed that *S. cerevisiae* displayed either extremely slow growth or an inability to grow on glycerol (Fig. 1). The lag phase lasted for 168 h, then transitioned into the log phase at approximately 192 h, exhibiting a specific growth rate of 0.008 h^{-1}. Subsequently, the culture reached a stationary phase after 408 h, when growth gradually declined. Therefore, the ALE method, a strategy for improving phenotypes and characteristics in microorganisms under specific conditions, was used to overcome constraints. This method involves a process of serial transfers to enhance the characteristics of yeast. In this part, 15 and 25 sequential serial passages through fresh medium were performed until the yeast cells improved their ability to grow on glycerol. The experimental setup for lipid production from *S. cerevisiae* TP-25 using glycerol was given in Fig. S1.

The results showed that the TP-15 and TP-25 strains grew much better than the parental strain on glycerol (Fig. 1). The 15th passage exhibited a short lag phase, with a specific growth rate of 0.05 h^{-1}, sevenfold higher than the wild-type strain. Moreover, at the 25th passage, the specific growth rate of the TP-25 was 0.058 h^{-1}, and its OD600 of 6.9 was higher than the wild-type and the TP-15 strains, indicating an improvement in the abilities of TP-15 and TP-25 to grow on glycerol as a carbon source. This adaptation was consistent with numerous previous reports in which *S. cerevisiae* showed an improved growth on glycerol. For example, the specific growth rate of the *S. cerevisiae* MG16C strain increased threefold after several subcultures in a glycerol-containing medium [18], and the glycerol assimilation capacity of *S. cerevisiae* W303-1B improved by threefold [19].

Effects of C/N molar ratio on lipid production

As TP-25 strain showed the highest cell density and growth rate, it was chosen for lipid production. The C/N molar ratio is a critical factor for lipid production in yeasts. Yeast cells accumulate lipids when cultured in a limited amount of nitrogen or under high C/N molar ratio conditions. Therefore, an optimal C/N molar ratio is crucial for lipid production. The C/N molar ratio of glycerol was optimized for the highest lipid production to evaluate the effects of different C/N molar ratios on lipid accumulation. The TP-25 strain was cultured with five C/N molar ratios, including 27, 50, 75, 100, and 125. The results indicated that the total lipid production and the lipid content increased proportionally with the C/N molar ratios (Fig. 2). At the ratios of 27, 50, and 75, the total lipid productions were 0.48 ± 0.07, 0.57 ± 0.03, and 0.61 ± 0.01 g/l; while the lipid content were $15.18 \pm 2.03\%$, $21.95 \pm 1.12\%$, and $33.27 \pm 0.55\%$, respectively. These results were similar to previous research findings in *L. starkeyi* [20]. However, when the C/N molar ratio was 100, it negatively affected lipid production and lipid content to the values of 0.32 ± 0.03 g/l and $31.48\% \pm 1.70\%$, respectively. Moreover, the C/N molar ratio of 125 inhibited growth, which could result from the adverse influence of high osmotic pressure caused by the extreme glycerol concentration, contributing to stress and subsequently affecting cell growth [21]. Thus, the C/N molar ratio of 75 was considered an optimal value for lipid production in TP-25.

Table 1 Effects of C/N molar ratios on lipid yield and glycerol consumption in oleaginous *S. cerevisiae* TP-25.

C/N molar ratio	Initial glycerol (g/l)	Lipid yield (g/g)	Glycerol consumption (g/l)
27	70	0.015 ± 0.002	31.39 ± 3.87
50	132	0.016 ± 0.002	30.66 ± 3.44
75	199	0.017 ± 0.006	30.56 ± 10.70
100	266	0.026 ± 0.008	19.08 ± 5.54

The results demonstrated that varying the C/N molar ratios significantly impacted the growth and lipid production in oleaginous *S. cerevisiae*. A lower C/N molar ratio supported higher biomass production, which could be attributed to increased nitrogen availability and cell growth. However, lipid accumulation was relatively low at this ratio, likely because nitrogen-rich conditions favored growth over lipid biosynthesis. As the C/N molar ratio increased, both lipid production and lipid content improved. This could indicate that high C/N molar ratio triggered lipid accumulation, consistent with the known metabolic shift toward lipid storage under nutrient-deprived conditions. Moreover, glycerol consumption remained relatively stable across different C/N molar ratios, suggesting that the capacity of *S. cerevisiae* to metabolize glycerol might be inherently limited, as shown in Table 1.

S. cerevisiae TP-25 exhibited the highest lipid content when cultured at a C/N molar ratio of 75. This yeast strain is of particular interest due to its ability to accumulate substantial amounts of lipids, even when utilizing glycerol as a carbon source. In this study, *S. cerevisiae* TP-25 demonstrated the ability to adapt and efficiently synthesize lipids using glycerol, highlighting the potential of TP-25 strain for lipid production and its significant applications in industrial biotechnology, particularly in the production of biofuels. However, despite its capacity for high lipid accumulation, represented by high lipid content (%), the lipid yield (g/g) and productivity of the TP-25 strain was low compared with other yeast strains (Table 2).

Several oleaginous yeasts, such as *Y. lipolytica* and *Rhodotorula* species, have already been shown to be highly efficient in utilizing glycerol for both growth and lipid production. Nonetheless, it has been well-established that *S. cerevisiae* generally demonstrates poor growth when glycerol is used as the sole carbon source. This presents a challenge for improving lipid

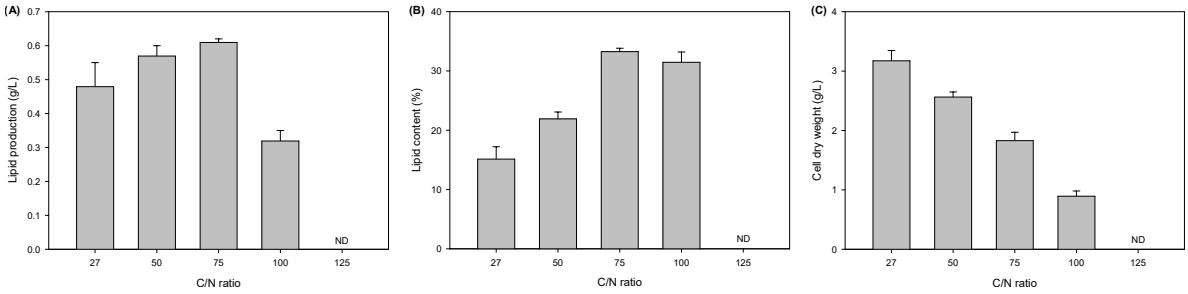

Fig. 2 (A) Lipid production; (B) lipid content; and (C) biomass of *S. cerevisiae* TP-25 strain grown on different C/N molar ratios of glycerol as a carbon source. Data are shown as mean ± standard deviation of triplicates. ND indicates no detection.

Table 2 Lipid production from glycerol in various yeast strains.

Yeast strain	Biomass (g/l)	Lipid production (g/l)	Time (h)	Productivity (g/l/h)	Reference
Y. lipolytica QU21	4.9 ± 0.68	1.48 ± 0.27	96	0.015	[28]
Y. lipolytica ACA-DC 5010	6.71 ± 0.52	1.37	50	0.027	[29]
C. wangnamkhiaoensis	3.63 ± 0.08	0.88 ± 0.05	120	0.007	[30]
R. toruloides NRRL Y-6984	16	3.23	215	0.015	[31]
C. curvatus NRRL Y-1511	16.6	0.95	211	0.005	[31]
R. glutinis TISTR 5159	7.20 ± 0.19	0.87 ± 0.03	144	0.006	[32]
S. cerevisiae TP-25	1.83 ± 0.13	0.61 ± 0.01	120	0.005	This study

yield and lipid productivity in oleaginous *S. cerevisiae*. To address these challenges, strategies to improve lipid yield include metabolic engineering targeting lipid biosynthesis pathways, such as overexpressing genes involved in fatty acid synthesis or redirecting metabolic fluxes toward lipid accumulation, offering an additional approach to further develop this strain [22]. Additionally, optimizing bioprocess conditions, such as employing alternative cultivation modes or two-stage processes, which involve maintaining two different culture conditions during yeast growth to maximize lipid production, would be highly interesting to explore further. Previous studies demonstrated that the implementation of two-stage processes could significantly enhance lipid productivity [23]. Thus, optimizing bioprocess conditions in oleaginous *S. cerevisiae* should be further investigated.

Fluorescence assay and qualitative analysis of lipid droplets

Fluorescence microscopy was employed to determine the existence of lipid droplets within TP-25 cells when glycerol was used as a carbon source. Fig. 3 shows the golden color lipid droplets in TP-25 cells. At a C/N molar ratio of 27, the amount of lipid droplets observed were relatively small, and the number of droplets per cell was limited. In contrast, at C/N molar ratio of 75, a multitude of lipid droplets were clearly exhibited and evenly distributed inside the cell in a consistent size. These results proved that the TP-25 strain could synthesize and store lipids inside the cells. Additionally, the qualitative analysis of lipids using HPLC showed that the lipid components consisted of triacylglycerols, free fatty acids, and a small amount of 1,3- diacylglycerol (Fig. 4).

Analysis of fatty acid composition

To investigate the fatty acid composition of TP-25 strain, the total lipids were converted into FAMEs by base transesterification. The findings were presented in Table 3. The dominant fatty acids had chain lengths ranging from 16 to 18 carbons with the highest being palmitoleic acid (C16:1; 38.7%), followed by oleic (C18:1; 30.9%), palmitic (C16:0; 18.1%), stearic (C18:0; 11.7%), myristic acids (C14:0; 0.5%), and lauric acids (C12:0; 0.1%). The highest levels of saturated fatty acids were palmitic acid (C16:0) and stearic acid (C18:0), while the highest levels of unsaturated fatty acids were palmitoleic (C16:0) and oleic acids (C18:0), as shown in Fig. S2.

Table 3 shows the fatty acid composition of various vegetable oils and oleaginous yeasts using glycerol as a carbon source. The predominant fatty acids found in the yeast species were mainly long-chain fatty acids with 16 and 18 carbon atoms, which included palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic acids (C18:3). Additionally, the four primary fatty acids were oleic (39.4%–48.9%), palmitic (17.8%–31.0%), stearic (2.8%–12.9%), and linoleic (0.5%–2.2%) acids; and the results corresponded to ranges of saturated, monounsaturated, and polyunsaturated fatty acids of 29.2%–48.2%, 42.5%–51.8%, and 6.7%–24.4%, respectively. Interestingly, the highest percentage of lipids from the yeast strains including *S. cerevisiae* TP-25 was primary unsaturated fatty acids, similar to

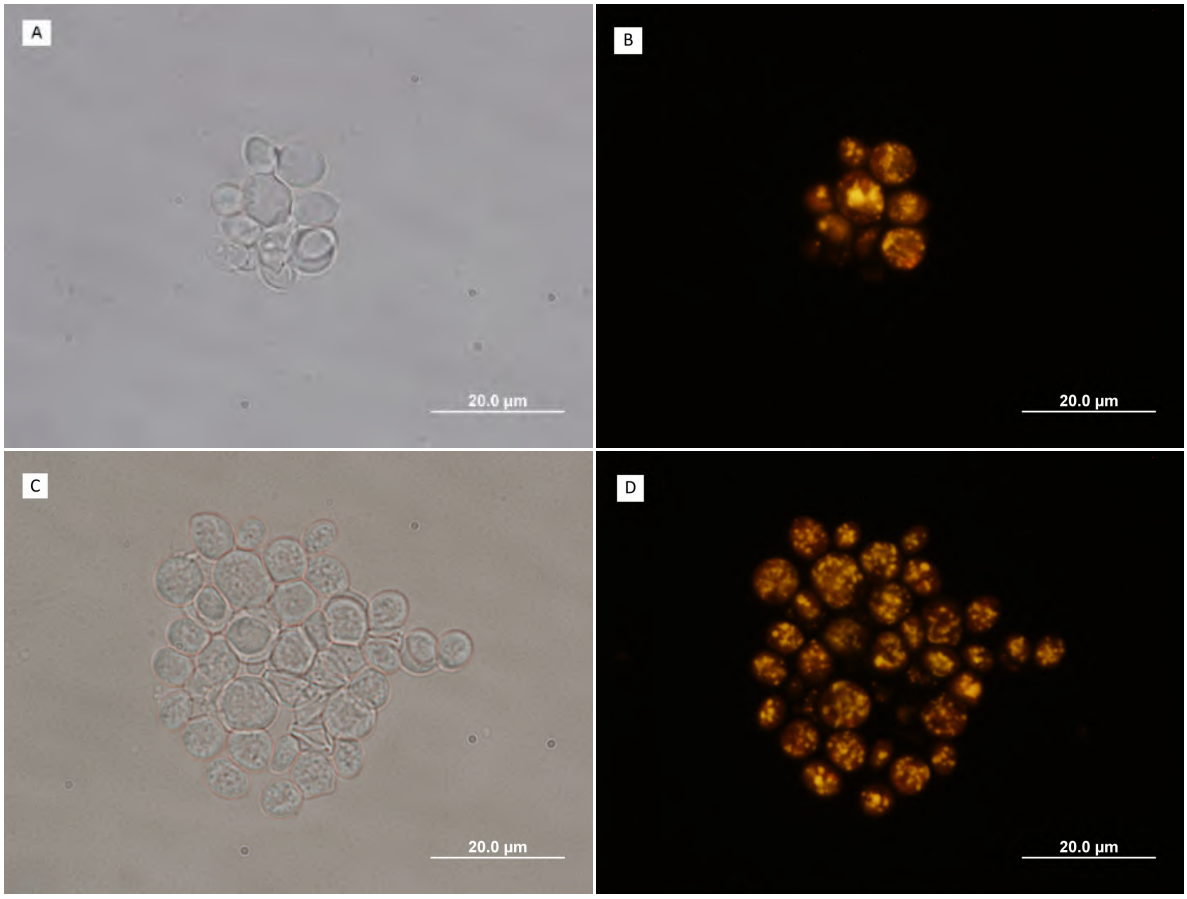

Fig. 3 Lipid droplets in TP-25 cells under brightfield and fluorescent microscopy. (A) and (B) at C/N molar ratio of 27; (C) and (D) at C/N molar ratio of 75.

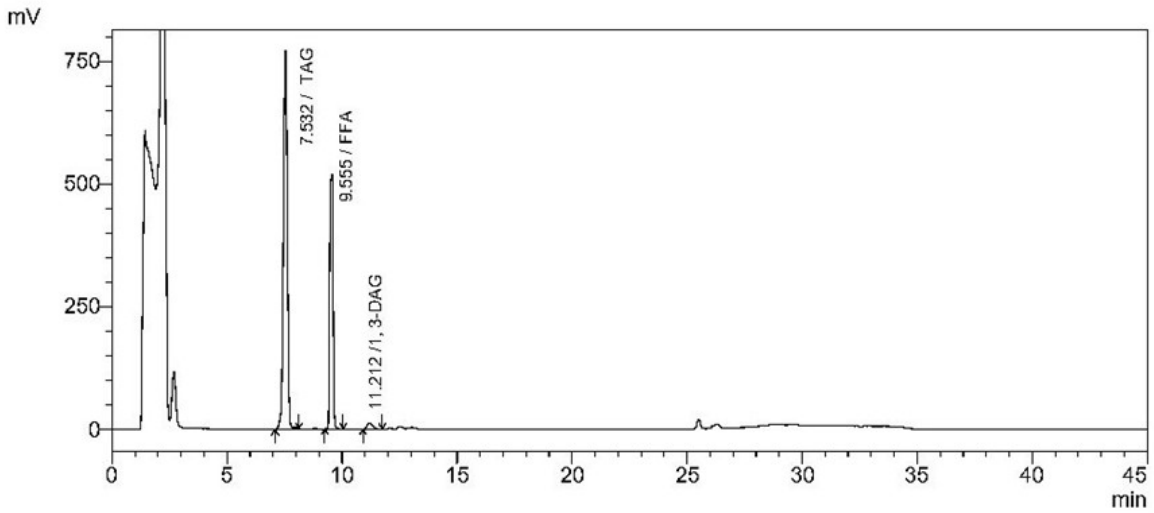

Fig. 4 Qualitative analysis of lipids from TP-25 strain. TAG, triacylglycerol; FFA, free fatty acids; and DAG, diacylglycerol.

Table 3 Fatty acid composition of various vegetable oils and oleaginous yeasts using glycerol as a carbon source.

Source	Fatty acid composition (%)											Reference
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	SFA	MUFA	PUFA	
Palm oil	—	1.1	42.5	0.2	4.2	41.3	9.5	0.3	47.8	41.5	9.8	[33]
Sunflower oil	0.1	0.1	6.4	0.1	3.6	21.7	66.3	1.5	10.2	21.8	67.8	[33]
Rapeseed oil	0.1	—	4.2	0.2	1.6	59.5	21.5	8.4	5.9	59.7	29.9	[33]
R. glutinis	—	1.5	22.2	2.9	2.8	48.9	15.4	2.2	30.6	51.8	17.6	[34]
C. curvatus	—	—	21.6	—	6.1	42.5	19.4	5.0	29.2	42.5	24.4	[35]
L. starkeyi	—	3.1	31.0	4.3	12.9	39.4	7.6	0.5	48.2	43.7	8.1	[35]
Y. lipolytica	—	4.6	17.8	—	12.0	44.1	6.7	—	34.4	44.1	6.7	[36]
S. cerevisiae	0.1	0.5	18.1	38.7	11.7	30.9	—	—	30.4	69.6	—	This study

Table 4 Comparison of predicted characteristics of biodiesels derived from vegetable oil, yeast oil, and the biodiesel standards.

Source	Biodiesel properties								Reference
	CN	HHV (MJ/kg)	CP (°C)	CFPP (°C)	IV (g I_2/100 g)	LCSF (% wt)	SV (mg KOH/g)	Density (g/cm^3)	
Sunflower oil	50.5	43.9	4.57	−3.7	—	—	—	0.84	[37]
Rapeseed oil	53.7	41.1	−3	−12	116	—	—	0.88	[33]
Palm oil	61.9	40.6	14	9	54	—	—	0.87	[33]
R. glutinis	54.8	39.5	—	−5.1	81	3.62	205	0.88	[34]
Y. lipolytica	53.3	—	—	−9.3		—	—	0.86	[38]
C. viswanathii	47	—	—	−0.5	120	—	198	0.86	[39]
S. cerevisiae TP-25	57.3	39.3	4.53	7.6	66	7.66	211	0.86	This study
ASTM D6751 Standard	⩾47	38.1–40.8	NS	NS	NS	NS	NS	NS	[40]
EN 14214 Standard	⩾51	NS	NS	NS	<120	NS	NS	0.86–0.90	[40]

CN, cetane number; HHV, higher heating value; CP, cloud point; CFPP, cold filter plugging point; IV, iodine value; LCSF, Long chain saturated factor; SV, saponification value. NS = Not specified.

rapeseed and sunflower oils. Biodiesel, which contains a high concentration of monounsaturated fatty acids, demonstrates good characteristics in terms of ignition quality, nitrogen oxide emissions, and fuel stability [24].

Properties of biodiesel derived from yeast lipids

Properties of biodiesel are influenced by the composition of fatty acids in the lipid feedstock. However, direct determination of the properties of biodiesel involves several factors and typically requires a large sample quantity. Therefore, predicting biodiesel properties using model equations based on fatty acid composition is a feasible and appropriate approach. In this study, biodiesel properties were estimated using established empirical equations. The theoretical properties of the obtained biodiesel were predicted based on the fatty acid composition of glycerol. To ascertain the suitability of the FAMEs derived from the TP-25 strain, various physical properties, including the CN, HHV, CP, CFPP, IV, SV, and density value, were assessed. These qualities were compared with those of vegetable oil-based biodiesel, various yeast species using glycerol, and international standards. As shown in Table 4, the characteristics of TP-25 biodiesel conformed to the established biodiesel standards and were within the ranges specified by the ASTM D6751 and EN 14214 standards. The CN is a measure of ignition characteristics of diesel fuel, and a high CN value contributes to a reduced ignition delay time and lesser emissions [25]. TP-25 biodiesel exhibited a CN value of 57.3, which was higher than the standard requirements, and the biodiesels derived from sunflower oil, rapeseed oil, *R. glutinis*, *Y. lipolytica*, and *C. viswanathii*. Similarly, HHV is one of the most critical properties of biodiesel and refers to the amount of heat released when one gram of fuel undergoes combustion, producing CO_2 and H_2O [25]. ASTM D6751 establishes a standard HHV range of 38.1–40.8 MJ/kg, and TP-25 biodiesel fell within this range with an HHV of 39.3.

CP and CFPP are significant parameters for determining the low-temperature applicability of fuels. While CP is the highest temperature at which wax becomes visible during the cooling of the fuel, CFPP refers to the minimum temperature at which biodiesel can readily pass through a standardized filter [25]. The CP and CFPP of TP-25 biodiesel were 4.53 and 7.6, respectively, similar to those of palm- and sunflower-derived biodiesels. With regard to IV, it refers to the addition of double bonds in fatty acids and their degree of unsaturation [26]. According to the EN 14214 standard, the requirement for IV is <120 gI_2/100 g; and TP-25 biodiesel exhibited an IV of 66 gI_2/100 g, complying with the established requirement. Furthermore, the density value, an essential parameter relating to fuel injection characteristics and engine performance [27], of the TP-25 biodiesel was 0.86 g/cm^3; and the value complied with the EN14214 requirement (Table 4).

Based on these results, lipids from oleaginous TP-25 could be considered suitable for biodiesel production, contributing to the United Nations goal on affordable and clean energy (SDG7) and promoting sustainable and modern energy in the future.

CONCLUSION

The present study reported, for the first time, a detailed investigation of enhancing lipid production in oleaginous *S. cerevisiae* grown on glycerol using the ALE method and its potential for biodiesel production. The ALE method was employed to obtain the oleaginous *S. cerevisiae* TP-25 strain. The results indicated that *S. cerevisiae* could grow in a glycerol medium and produce a maximum lipid content of 33.27%. Moreover, when these lipids were transesterified to FAMEs, their biodiesel-related properties. i.e., CN, HHV, CP, CFPP, IV, SV, and density, met the requirements of international standards. These results demonstrated the potential of oleaginous *S. cerevisiae* for using glycerol as a carbon source to produce lipids that can be a substitute for biodiesel conforming to international standards.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.041.

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Appendix A. Supplementary data

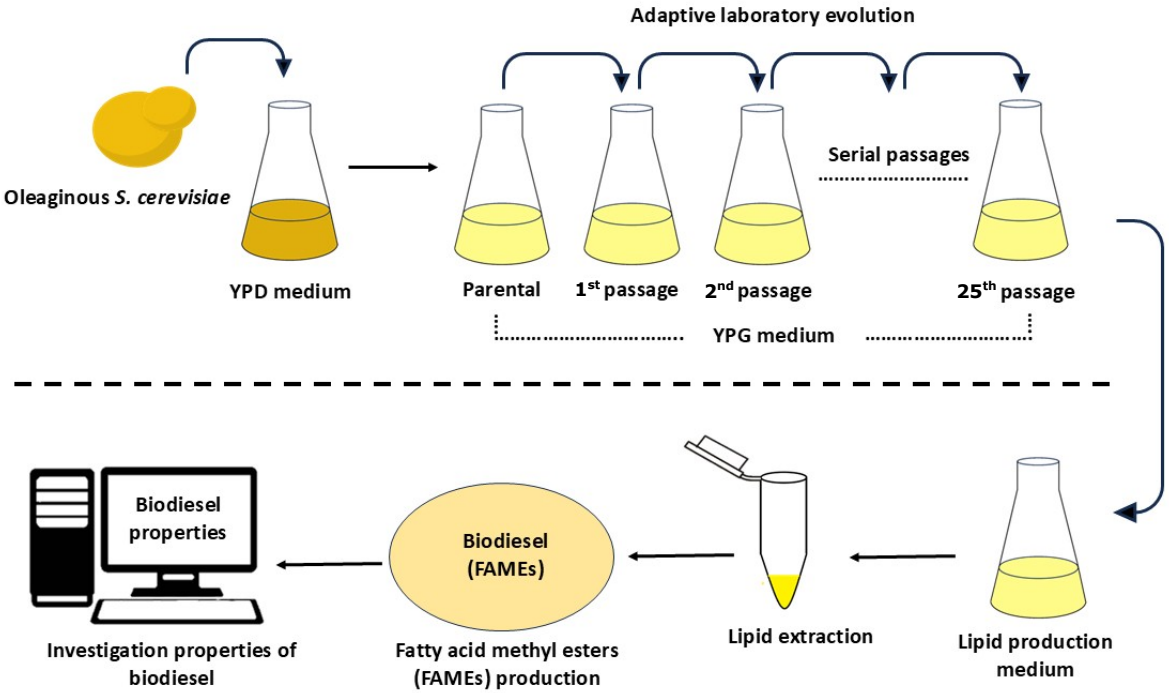

Fig. S1 Experimental setup for lipid production from *S. cerevisiae* TP-25 using glycerol.

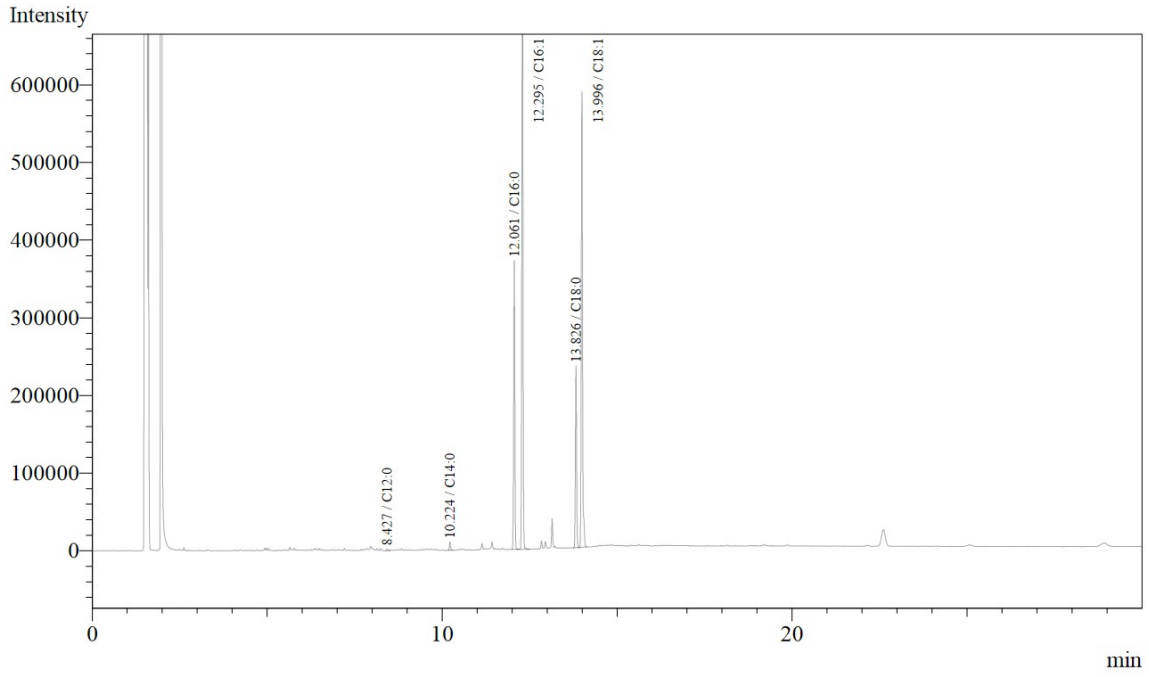

Fig. S2 Fatty acid composition analyzed by GC-FID.