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The Enzymatic Role of Lipid Metabolism in *Yarrowia lipolytica* Grown on Glycerol

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Abstract: More attention has been paid to the production of commercial fatty acids by microbial fermentation. The basic biochemistry of lipid accumulation in oleaginous microorganisms under nitrogen limitation has been extensively studied. As an oleaginous microorganism, *Yarrowia lipolytica* can produce fatty acid-derived biofuels and biochemicals. However, the response of the enzymes in *Y. lipolytica* to glycerol remains unclear. Therefore, we aimed to identify the rate-limiting enzymes associated with the mechanism of lipid accumulation of *Y. lipolytica* grown on glycerol. The variations in key enzyme activities of fatty acid accumulation in *Y. lipolytica* were investigated with glycerol as the single carbon source. The nitrogen source was exhausted at 10 h in the medium, leading to substantial changes in key enzyme activities associated with lipid accumulation. Adenosine monophosphate deaminase activity increased immediately by approximately twofold. NAD⁺-isocitrate dehydrogenase activity decreased by 65% after the nitrogen source was exhausted when compared to the initial maximum activity. ATP citrate lyase activity, which provides the substrate acetyl coenzyme A for lipid biosynthesis, increased by approximately fourfold when compared to the activity before nitrogen depletion. Malic Enzyme (ME) activity decreased considerably after nitrogen was exhausted so that the reducing power Nicotinamide Adenine Dinucleotide Phosphate (NADPH) required for lipid synthesis cannot be produced by ME. However, glucose-6-phosphate dehydrogenase activity increased from 550 nmol·min⁻¹·mg⁻¹ to 771 nmol·min⁻¹·mg⁻¹, suggesting that the main source of NADPH required for fatty acid accumulation may be provided by the pentose phosphate pathway when *Y. lipolytica* grew on the medium with glycerol as the only carbon source.

Keywords: *Yarrowia lipolytica*, Malic Enzyme, Lipid Accumulation, Glucose-6-Phosphate Dehydrogenase

Introduction

Some microbial organisms can accumulate fatty acids of more than 20% of their cell dry weight including yeasts, fungi, and algae, and are labeled oleaginous microorganisms (Thorpe and Ratledge, 1973). Microorganisms have several advantages, such as simple culture conditions, and high growth rates, and their growth is not easily affected by weather or climate, compared with traditional plant oils (Beopoulos *et al.*, 2011). Thus, fatty acid production by microorganisms is a sensible alternative to that by plants.

The basic mechanism of fatty acid overproduction is that the microorganism is grown on a culture medium in which the carbon source is excessive and other

important nutrients (particularly nitrogen) are insufficient (Ratledge and Wynn, 2002; Ratledge, 2004). Numerous studies have been carried out to elucidate the basic biochemistry of fatty acid synthesis in oleaginous microorganisms under nitrogen limitation and a clear understanding of microbial lipid production provides a theoretical basis for improving lipid production through genetic modifications. The deamination of Adenosine Monophosphate (AMP) to release ammonium and inosine monophosphate was catalyzed by AMP Deaminase (AMPD) at the beginning of lipid accumulation (Li *et al.*, 2022). The activity of NAD⁺-isocitrate dehydrogenase (NAD-ICDH) relies on AMP, therefore the loss of AMP causes a rapid decrease activity of NAD-ICDH (Wynn *et al.*, 2001; Papanikolaou and Aggelis, 2002). The attenuation

of NAD⁺-ICDH activity leads to the accumulation of isocitrate which rapidly equilibrates back to citrate, which is then transported from the mitochondria into the cytosol (Tang *et al.*, 2015). As the substrate of ATP Citrate Lyase (ACL), citrate was converted to acetyl Coenzyme A (acetyl-CoA), which can be catalyzed to fatty acid (Botham and Ratledge, 1979; Liu *et al.*, 2013). The ability of the accumulation of fatty acid in oleaginous microorganisms is closely related to the presence of ACL (Zhang *et al.*, 2014). In microorganisms with a low activity of ACL, the production of fatty acid is significantly low. However, many microorganisms with the activity of ACL still cannot synthesize plenty of fatty acids (Ratledge and Wynn, 2002), suggesting that lipid synthesis needs more enzyme activities.

Similarly, the conversion of acetyl-CoA into lipid needs the reducing power of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) (Zhang *et al.*, 2013). The enzyme Glucose-6-Phosphate Dehydrogenase (G6PD) and 6-Phosphogluconate Dehydrogenase (6PGD) of the Pentose Phosphate Pathway (PPP) can provide a good deal of NADPH (Zhang *et al.*, 2013). In yeasts and fungi, another enzyme that has also been considered to supply NADPH for fatty acid biosynthesis is Malic Enzyme (ME) (Ratledge and Wynn, 2002; Wynn *et al.*, 2001). Lipid overproduction will cease if the activity of ME is receded either by inhibition or mutation. Therefore, it is hypothesized that ME is the key enzyme to provide the additional NADPH to synthesize fatty acid.

Yarrowia lipolytica is a type of oleaginous yeast that has the potential to produce various fatty acids (Yuzbasheva *et al.*, 2019). *Y. lipolytica* is safe to produce food grade metabolites as its capacity of gathering high fatty acid content has been confirmed by the food and drug administration (Groenewald *et al.*, 2014; Wang *et al.*, 2022). *Y. lipolytica* can use various carbon sources, such as alkanes, lipids, ethanol, fructose, glucose, and glycerol (Zhang *et al.*, 2014). As a by-product of many soap industries, glycerol is a cheap carbon source for a yeast culture to reduce costs (Hapeta *et al.*, 2020). *Y. lipolytica* is a good option as a model microorganism to explore biochemical events involved in lipid synthesis in oleaginous microorganisms grown on a medium with glycerol as a carbon source (Papanikolaou and Aggelis, 2002; Souza *et al.*, 2014). To increase the accumulation of lipids when *Y. lipolytica* was cultivated on glycerol, an in-depth understanding of the regulation of fatty acid biosynthesis of oleaginous microorganisms is required. All enzymes thought to be crucial for the accumulation of substantial amounts of storage lipids are found in both filamentous fungi and oleaginous yeasts. However, the response of the enzymes in *Y. lipolytica* to glycerol remains unclear. Therefore, we aimed to make sure the rate-limiting enzymes related to the onset of fatty acid

accumulation in *Y. lipolytica* to glycerol. The findings could have implications for increasing single cell oil production in *Y. lipolytica* with genetic modification. Our results show that when *Y. lipolytica* is grown on glycerol, the substrate acetyl-CoA for the synthesis of lipids is provided by ACL cleavage of citric acid, and the reducing power NADPH is provided by G6PD and 6PGD of PPP.

Materials and Methods

Preparation of Yeast Strains and Culture Conditions

Y. lipolytica DSMZ1345 was used for the experiments. The yeast was cultivated in a nitrogen limited medium containing 160 g/L glycerol, 1.5 g/L yeast extract, 2 g/L ammonium tartrate, 1.5 g/L MgSO₄•7H₂O, 7 g/L KH₂PO₄, 0.1 g/L CaCl₂•2H₂O, 0.1 mg/L CuSO₄•5H₂O, 8 mg/L FeCl₃•6H₂O, 0.1 mg/L Co(NO₃)₂•6H₂O, 1 mg/L ZnSO₄•7H₂O and 0.1 mg/L MnSO₄•5H₂O. *Y. lipolytica* cultures were initially cultivated in 1 L bottles containing 200 mL of Yeast extract Peptone Dextrose (YPD) medium. The cultures were incubated for 24 h at 28°C and then inoculated into 5 L fermenters (10% v/v of the inoculum) containing the nitrogen deficiency medium. Fermenters were incubated at 28°C and stirred at 400 rpm with an aeration rate of 1.0 vvm. 10.0 m NaOH was added automatically to maintain the pH at 6.2. Culture samples were termly removed from fermenters for analysis.

Determination of Culture Dry Weights

A 4 mL sample of the culture was centrifuged at 3000 ×g for 5 min and washed twice with cold distilled water, then shifted into a 5 mL dried and pre-weighed centrifuge tube. The supernatant was retained and analyzed for glycerol and ammonium concentrations. The sediment was frozen at -80°C refrigerator and then to freeze dry. The dry weight of biomass was determined gravimetrically.

Analysis of Fatty Acid Content and Composition in Cells

Freeze-dried cells (10-20 mg) were weighed in special lipid extraction tubes and used for fatty acid content and composition analysis. The cells were acidified with 4 m HCl. A chloroform/methanol mixture (2:1, v/v) was added to the freeze-dried cells to extract cell lipids, and used pentadecanoic acid (C15:0) as an internal standard. Fatty acids were methylated using methanol and analyzed using a gas chromatograph (GC-2010; Shimadzu Corp., Kyoto, Japan) equipped with a DB-Waxetr column (30 m × 0.32 mm internal diameter; film thickness, 0.25 μm; J&W Scientific Inc., Folsom, CA, USA). The temperature program was set up as follows: 120°C for 3 min, ramped to 190°C at 5°C/min, then to 220°C at 4°C/min, and held for 20 min. Each experiment was performed in triplicate.

Analysis of the Culture Supernatant

The residual glycerol concentration in the culture medium was determined using a glycerol test kit (Rongsheng Biotech Co., Ltd., Shanghai, China), according to the manufacturer's instructions. Residual NH_4^+ concentration in the culture medium was determined as described by Chaney and Marbach (1962).

Determination of Enzyme Activities

Harvested cells by centrifugation were washed twice with cold distilled water and resuspended in Buffer A containing 100 mm $\text{KH}_2\text{PO}_4/\text{KOH}$ (pH 7.5), 20% glycerol, 1 mm dithiothreitol, and 1 mm benzamidine hydrochloride. The cells were lysed six times at 25 kpsi using a constant cell disruption system and centrifuged at $10,000 \times g$ for 10 min at 4°C .

ME activity was assayed as described by Hsu and Lardy (1969) using 80 mm $\text{KH}_2\text{PO}_4/\text{KOH}$ (pH 7.5), 0.6 mm NADP^+ , 3 mm MgCl_2 , and 25 mm malate. All the reagents except malate were mixed, and incubated at 30°C for 2 min and absorbance was measured at 340 nm (A340) for 3 min. Malate was then added to the mixture and A340 was measured for 3 min.

ACL activity was assayed as described by Sreer (1959) using 10 mm Tris/HCl buffer (pH 8.6), 10 mm MgCl_2 , 10 mm mercaptoethanol, 20 mm tripotassium citrate, 5 mm ATP, 0.3 mg/mL CoA, 5 unit's malate dehydrogenase, 0.2 mm NADH and 10 mm sodium azide. All the reagents except tripotassium citrate were mixed, incubated at 30°C for 2 min and spectrophotometrically analyzed at A340 for 3 min. Tripotassium citrate was then added to the mixture, gently stirred for homogenization and again A340 was measured for 3 min.

G6PD activity was assayed as described by Langdon (1966) using 50 mm Tris/HCl buffer (pH 8.0), 0.3 mm NADP^+ , 5 mm MgCl_2 , and 2.5 mm glucose-6-phosphate. All the reagents except glucose-6-phosphate were mixed, and incubated at 30°C for 2 min and A340 was measured for 3 min. Glucose-6-phosphate was then added to the mixture and A340 was measured for 3 min.

AMPD activity was assayed as described by Wynn *et al.* (2001) using 100 mm $\text{KH}_2\text{PO}_4/\text{KOH}$ (pH 7.0), 50 mm KCl, 4 unit's glutamate dehydrogenase, 7 mm oxoglutarate, 0.2 mm NADH and 50 mm AMP. All the reagents except AMP were mixed, and incubated at 30°C for 2 min and A340 was measured for 3 min. AMP was then added to the mixture and A340 was measured for 3 min.

NAD^+ -ICDH activity was assayed as described by Komberg (1955) using 82.2 mm Tris/HCl buffer (pH 8.0), 0.6 mm NAD, 3 mm MgCl_2 , and 5 mm isocitrate. All the reagents except isocitrate were mixed, and incubated at 30°C for 2 min and A340 was measured for 3 min. Isocitrate was then added to the mixture and A340 was

measured for 3 min. NADP -isocitrate dehydrogenase (NADP -ICDH) activity was assayed as described by Komberg (1955) using 82.2 mm Tris/HCl buffer (pH 8.0), 0.6 mm NADP^+ , 3 mm MgCl_2 and 5 mm isocitrate. All the reagents except isocitrate were mixed, and incubated at 30°C for 2 min and A340 was measured for 3 min. Isocitrate was then added to the mixture and A340 was measured for 3 min.

Analysis of Citrate and Isocitrate Concentrations in the Culture

The pH of the supernatant obtained after centrifugation was adjusted to 4.0 with the addition of phosphoric acid. The samples were then diluted to an appropriate concentration. The concentrations of citrate and isocitrate were analyzed by liquid chromatography using a high-performance liquid chromatographic system (Agilent 1100 series; Agilent Technologies, Santa Clara, CA, USA) equipped with an Ecosil C18 column (4.6×250 mm, $5 \mu\text{m}$). The mobile phase comprised a methanol/water/phosphoric acid (5/95/0.05) mixture. The flow rate was set at 0-8 mL/min, the column temperature at 30°C , the ultraviolet detector was at a wavelength of 210 nm, and the sample size was $5 \mu\text{L}$. The sample concentrations were compared with the known concentrations of citrate and isocitrate standards.

Results

Cell Growth and Lipid Accumulation Under Nitrogen-Limited Condition

The concentration of NH_4^+ was rapidly exhausted (within 12 h) when *Y. lipolytica* was cultivated on a high carbon-to-nitrogen ratio medium (Fig. 1). However, glycerol concentration remained high and the cells initiated a range of enzymes associated with lipid synthesis to convert the residual glycerol into fatty acids. The decrease in glycerol concentration after 12 h was greater than that within 12 h. The rapid decrease in glycerol concentration observed in *Y. lipolytica* could be attributed to lipid synthesis. The residual glycerol concentration of the medium was 31.2 g/L after five days of cultivation, which was less than a quarter of the initial glycerol concentration.

The biomass of yeast cells reached a maximum level of 13.85 g/L at approximately 24 h and did not exhibit significant changes in the subsequent stage (Fig. 2). The increase in Total Fatty Acid (TFA) content of cells at the time of nitrogen exhaustion from the culture was significant, reaching a maximum level of 18.09% at approximately 72 h (Fig. 2). A slight decrease in TFA content was observed at 96 h, indicating that the fatty acids conserved other functional metabolites. The final TFA content of *Y. lipolytica* grown on glycerol was 16.55% at 120 h when cell biomass was 13.57 g/L.

Table 1: Fatty acid composition of *Y. lipolytica* grown on nitrogen-deficiency medium with glycerol as sole carbon source culture

Culture time (h)	Fatty acid composition (%)					
	C16:0	C16:1	C18:0	C18:1	C18:2	Other
12	14.88±0.11	14.37±0.11	2.37±0.05	31.10±0.15	33.09±0.11	4.19±0.04
24	14.61±0.12	16.39±0.13	1.92±0.03	36.69±0.11	26.45±0.09	3.94±0.03
48	16.68±0.10	18.31±0.13	3.92±0.02	35.77±0.13	20.21±0.12	5.11±0.03
72	15.47±0.08	20.04±0.11	4.70±0.02	36.60±0.10	19.96±0.13	3.23±0.05
96	13.26±0.04	21.52±0.10	3.25±0.03	35.56±0.12	21.06±0.12	5.35±0.02
120	12.15±0.03	22.70±0.10	2.52±0.04	37.29±0.13	22.43±0.10	2.91±0.01

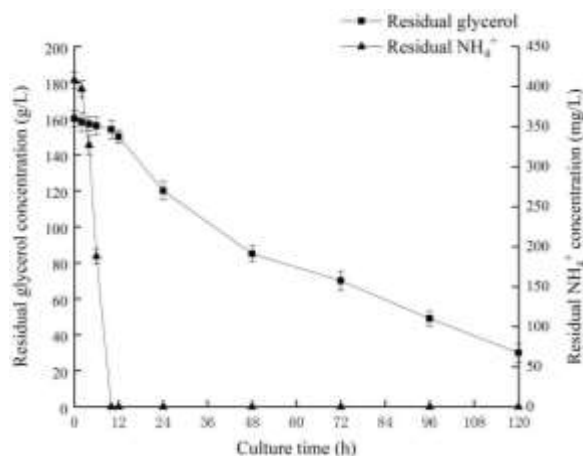


Fig. 1: The residual glycerol (■) and NH_4^+ (▲) concentration when *Y. lipolytica* grew in glycerol culture with limited nitrogen

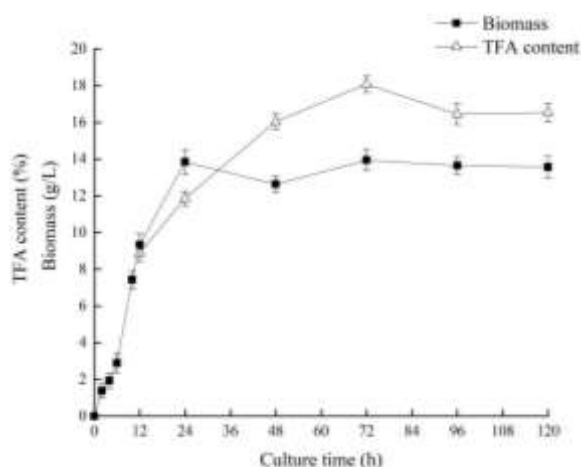


Fig. 2: The biomass (■) and TFA content (▲) of *Y. lipolytica* cultured in a nitrogen deficiency medium with glycerol as the sole carbon source culture

The fatty acid profiles of *Y. lipolytica*, including palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids, are shown in Table 1. Notably, the C18:2 content of the cell lipids decreased significantly during cell

growth, whereas C16:1 and C18:1 content increased. The C16:0 content decreased slightly during cultivation. The predominant fatty acids in *Y. lipolytica* were C16:0, C16:1, C18:1, and C18:2, which accounted for approximately 95% of the total lipid content of the cells.

Among them, the proportion of C18:1 was the largest, between 30-40%, and the proportion elevated with the increase of culture time. The proportion of C16:0 is about 15%, which has little change over time. The proportion of C16:1 was between 10-25% and elevated with the increase of culture time. The proportion of C18:2 was the highest at 12 h, up to 33.09%, but decreased with the increase of culture time and remained at about 20% in the later stage of growth.

The Onset of Lipid Accumulation Under Nitrogen-Limited Condition

The cells immediately initiated the relevant enzymes to compensate for nutrition defects when the nitrogen source became limited. AMPD activity increased by approximately twofold ($31.33 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ at 4 h and $57.25 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ at 10 h) immediately after nitrogen in the cultures became limited and maintained a high level of approximately $50 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ during the later growth period (Fig. 3). In addition, AMPD activity was maintained at a high level, with no significant decreases being observed after 12 h.

NAD^+ -ICDH activity of *Y. lipolytica* cultures exhibited significant decreases ($29.98 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ at 4 h and $12.29 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ at 10 h) following nitrogen limitation. NAD^+ -ICDH activity exhibited contrasting trends to those of AMPD activity. NAD^+ -ICDH activity increased after 12 h, although the increase was not significant and it did not exceed $60 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ (Fig. 3).

ACL Provides Acetyl-CoA for Lipid Accumulation

From the beginning of the investigation of the mechanism of oil overproduction in oleaginous microorganisms, ACL was deemed to provide acetyl-CoA for fatty acid synthesis. A significant decrease was observed in ACL activity before nitrogen depletion from 4-10 h (Fig. 4). However, ACL activity increased immediately after *Y. lipolytica* was subjected to nitrogen-limiting conditions at 10 h.

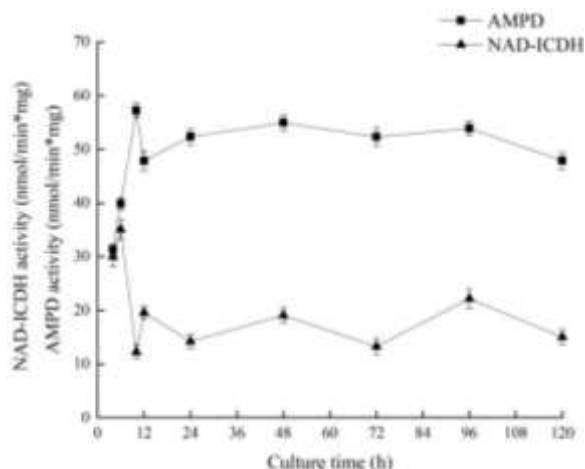


Fig. 3: The activities of AMPD (■) and NAD-ICDH (▲) of *Y. lipolytica* grown in glycerol culture with nitrogen as the limited nutrition

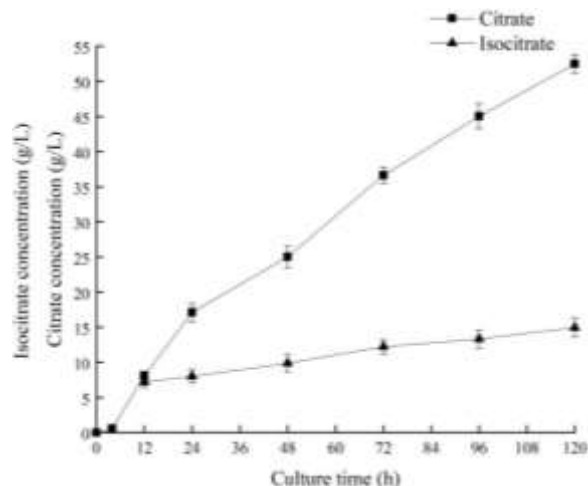


Fig. 6: The citrate (■) and isocitrate (▲) concentration in the fermentation culture when *Y. lipolytica* grew a high carbon-nitrogen ratio medium with glycerol as a carbon source

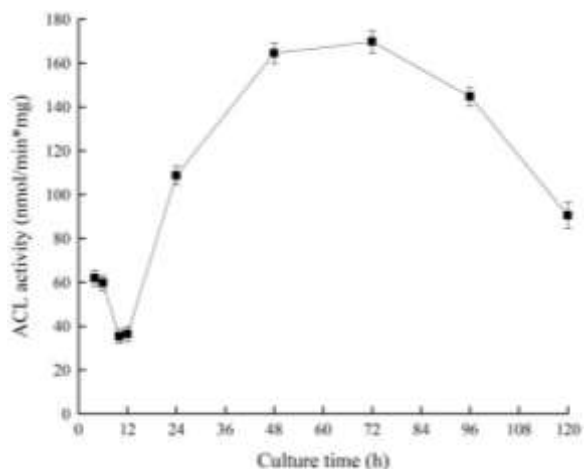


Fig. 4: The ACL activity when *Y. lipolytica* cultured in nitrogen deficiency medium with glycerol as a carbon source

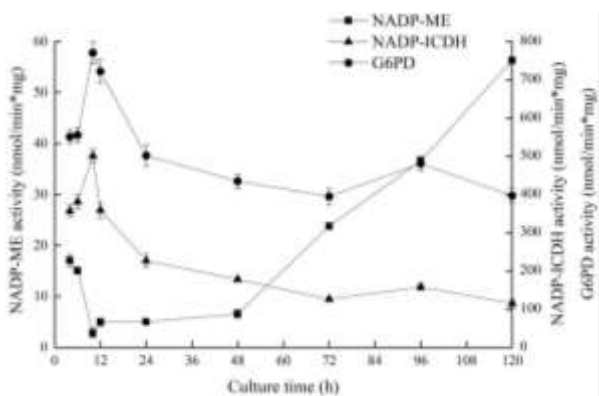


Fig. 5: The activities of NADP-ME (■), NADP-ICDH (▲), and G6PD (●) of *Y. lipolytica* cultured in glycerol medium with a deficient nitrogen source

ACL activity was $169.8 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at 72 h, which was approximately fourfold higher than that before nitrogen depletion. However, ACL activity decreased after 72 h and decreased further to half of the highest activity level at 120 h. This observation is consistent with the change in TFA content, indicating an association between ACL and lipid biosynthesis. This correlation also requires the participation of NAD-ICDH, because the reduced activity of NAD-ICDH leads to a high level of citric acid concentration, thus providing more substrates for ACL.

Supply of NADPH for Fatty Acid Synthesis

In addition to acetyl-CoA, the synthesis of fatty acids requires another compound with reductive power, NADPH. Therefore, we detected the activities of NADPH-producing enzymes G6PD, NADP-ICDH, and NADP-ME (Fig. 5). G6PD and NADP-ICDH activities increased when nitrogen in the cultures was exhausted and then decreased rapidly during the subsequent stage of cell growth. The highest G6PD activity was $771 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, which rapidly decreased at 24 h and returned to its initial activity level before nitrogen depletion. G6PD activity was $502 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at 24 h and $397 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at 120 h, which was almost half of the highest activity level. NADP-ICDH activity exhibited a trend similar to that of NADP-ME, with the highest activity being $501 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at 24 h and the final activity was $115 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at 120 h. Conversely, NADP-ME activity decreased rapidly after nitrogen was exhausted, although it increased gradually after 48 h to $56.33 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, which was thought to be insignificant as it was inconsistent with lipid accumulation.

Citrate and Isocitrate Secretion in the Culture Medium

The cleavage of citrate is thought to provide the precursor for lipid biosynthesis, acetyl-CoA; therefore, citrate and isocitrate concentrations were determined. Citrate concentration increased continuously and no significant change was observed in isocitrate concentration, probably because of the equilibrium maintained between citrate and isocitrate. The concentration of citrate reached 52.5 g/L at 120 h (Fig. 6).

Discussion

Microorganisms have evolved a set of mechanisms to deal with harsh environments after exposure to nutrient-limited conditions (Ratledge and Wynn, 2002; Ratledge, 2004). When microorganisms grow in a medium with unbalanced nutritional conditions, especially a nitrogen-limited medium with an excess of carbon, the signal of the harsh environment initiates the development of a mechanism to absorb more nutrients to cope with severe conditions (Abreu *et al.*, 2022). Oleaginous microorganisms usually absorb chemical units richer in carbon content, which is stored in the formation of fatty acids for future use in hostile environments to which they may be exposed (Liu *et al.*, 2021). To promote the initial fatty acid synthesis mechanism in oleaginous microorganisms, cells are usually cultured in a medium with high carbon and low nitrogen sources. *Y. lipolytica* senses the signal of a harsh environment when the nitrogen source in the culture is depleted and initiates a series of related enzymes and signal molecules to compel the cells to continue absorbing the carbon source and convert it into fatty acids (Jia *et al.*, 2022). The investigation of fatty acid synthesis mechanism in oil production microorganisms has been widely carried out and we attempt to picture a network of fatty acid biosynthesis in *Y. lipolytica* (Fig. 7).

Many studies have been conducted on oleaginous yeasts and filamentous fungi to elucidate the biochemical processes of fatty acid accumulation. Previous studies have found that AMPD activity increases when cells are exposed to nitrogen-depleted conditions (Boulton and Ratledge, 1983). AMPD catalyzes the cleavage of AMP to produce ammonium ions to supplement the insufficient nitrogen sources to ensure continuous synthesis of the necessary proteins and nucleic acids by the cells (Chang *et al.*, 2019; Shi *et al.*, 2022). In oleaginous yeasts, AMPD activity rapidly increases during the initial stage of nitrogen depletion (Ratledge and Wynn, 2002). The reduction in AMP concentration caused by an improvement in AMPD activity is closely associated with the initiation of subsequent lipid accumulation mechanisms (Dourou *et al.*, 2018). In this study, AMPD activity in *Y. lipolytica* increased rapidly at 10 h and almost immediately after nitrogen depletion.

Subsequently, AMPD activity was maintained at a high level, which promoted fatty acid production by *Y. lipolytica* (Fig. 3). Nevertheless, the specific mechanism by which AMPD senses the signal of nitrogen depletion and regulates its activity is unclear. The increase in AMPD activity has been shown to be the first biochemical event of lipid synthesis in oil-rich microorganisms (Ratledge and Wynn, 2002) and our study confirms the mechanism in *Y. lipolytica*. The declined concentration of AMP caused by an elevation in AMPD activity in cells leads to a decrease in NAD⁺-ICDH activity, which is heavily dependent on AMP concentrations (Tang *et al.*, 2015). However, in non-oleaginous microorganisms, NAD⁺-ICDH activity is not strongly correlated with AMP concentration, which is one of the disparities between oil-producing and non-oil-producing microorganisms (Botham and Ratledge, 1979). Therefore, the tricarboxylic acid cycle is inhibited, which prevents the generation of energy for cells through carbon flux into the tricarboxylic acid cycle. Isocitrate accumulated due to a decrease in NAD⁺-ICDH activity is converted to citrate by aconitase, resulting in high citrate levels in the mitochondria.

Generally, the requirement of NADPH for fatty acid synthesis is provided by ME. A previous study identified the crucial role of ME in fatty acid biosynthesis in oil-rich microorganisms (Wynn *et al.*, 2001). The addition of sesamol, an inhibitor of ME, results in a decrease in lipid content in the oleaginous microorganism *Mucor circinelloides* (Wynn *et al.*, 2001). Owing to the key role of ME in oleaginous fungi, increasing attention has been paid to its role in oleaginous yeasts. However, the ME in *Y. lipolytica* can use NAD⁺ or NADP⁺ as a cofactor, although the K_m value of NAD⁺ is relatively low, indicating that NAD-ME activity in *Y. lipolytica* is high (Beopoulos *et al.*, 2009a). The ME in *Y. lipolytica* occurs in the mitochondria; therefore, homologous overexpression of ME cannot significantly improve its lipid content (Beopoulos *et al.*, 2011). In addition, heterologous cytoplasmic NADP-ME does not considerably increase its fatty acid content in *Y. lipolytica* (Zhang *et al.*, 2013), suggesting that NADPH produced by ME in *Y. lipolytica* was not used for fatty acid production. ME activity does not match the fatty acid accumulation curve. Consequently, an ancillary method for NADPH production is necessary. NADPH required for lipid synthesis can be generated by the PPP. Cytosolic NADP-ICDH may be an alternative pathway for NADPH generation during lipogenesis. However, in our study, NADP-ICDH activity did not increase to a level sufficient to produce NADPH for lipid synthesis, whereas G6PD activity was sufficiently high to supply the necessary reducing power for fatty acid synthesis (Fig. 5).

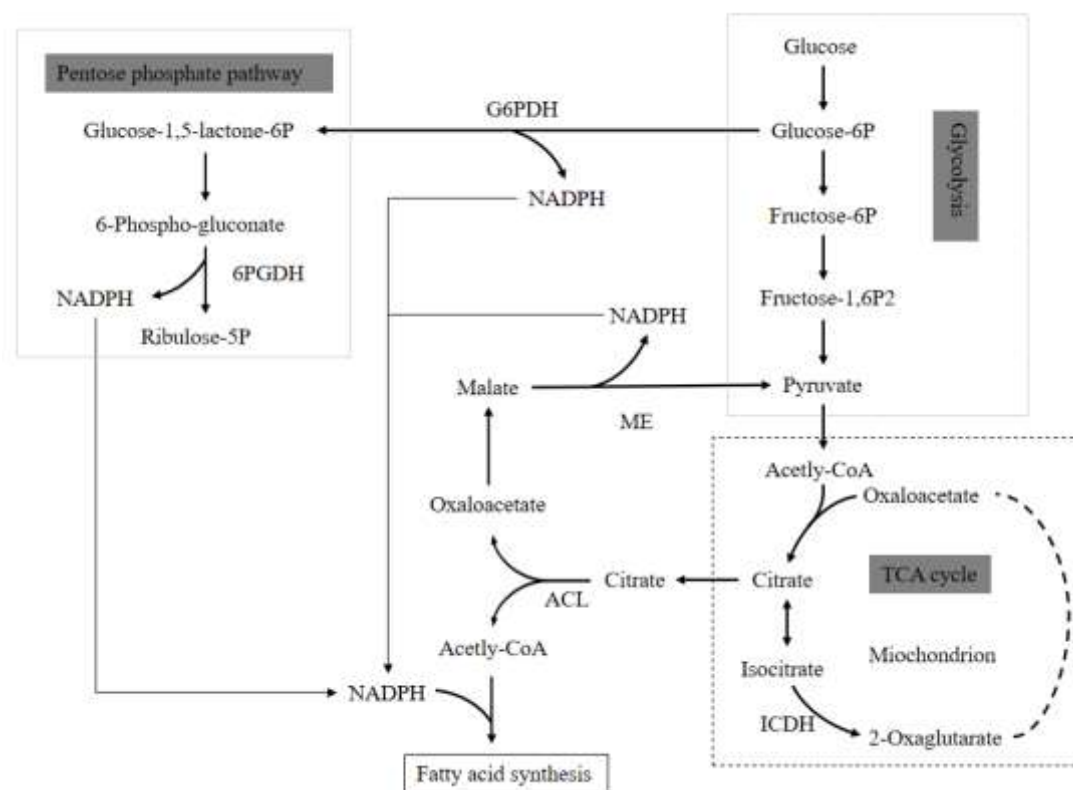


Fig. 7: The biochemical pathway of lipid accumulation in *Y. lipolytica*

The production of NADPH by PPP as reducing power for fatty acid synthesis among oleaginous yeasts may not be special to *Y. lipolytica*. Liu *et al.* (2009) have performed a proteomic study of the enhancement of the activity of 6PDG during fatty acid production in *Rhodospiridium toruloides* and *Lipomyces starkeyi* (Liu *et al.*, 2009, 2011). A review by Ratledge researched the stoichiometries of the transformation of glucose to triacylglycerols, providing a theory that PPP is not the sole provider in oleaginous microorganisms *Y. lipolytica* that lack a cytosolic ME. Therefore, another cytosolic enzyme ICDN may be a provider of NADPH (Ratledge, 2014).

Citrate plays an important role in metabolism because it regulates the activities of a variety of enzymes, including phosphofructokinase and pyruvate kinase (Makri *et al.*, 2010). Thus, intracellular citrate concentration may be a key factor influencing fatty acid synthesis (Moeller *et al.*, 2011; Hambalko *et al.*, 2021). Citrate concentration not only affects the activity of acetyl-CoA carboxylase, which initiates fatty mobilization, but may also delay the tricarboxylic acid cycle and even have a certain impact on glycolysis (Souza *et al.*, 2014). Citrate links lipid metabolism and glycolytic pathways. However, the specific mechanism underlying the effect of citrate has not been elucidated.

Citrate accumulation in cells occurs in two ways: Through the secretion of citrate by cells and activation of ACL activity in some way (Beopoulos *et al.*, 2009b). ACL can cleave excess citrate to produce acetyl-CoA, a precursor for fatty acid synthesis (Blazcek *et al.*, 2014). ACL exists in oil-producing yeast, but not in non-oil-producing yeast (Ratledge and Wynn, 2002). Therefore, ACL is considered one of the crucial enzymes associated with fatty acid accumulation in eukaryotes. A comprehensive exploration of ACL activity may considerably improve lipid content in oleaginous yeasts. Overexpression of homologous ACL does not increase fatty acid production; however, downregulation of the ACL gene reduces lipid production by 19-30% (Liu *et al.*, 2013). The affinity of ACL for citrate also affects lipid synthesis. The K_m value of ACL for citrate in *Lipomyces starkeyi* is 0.07 mM and its lipid content can be as high as 65% (Boulton and Ratledge, 1983). The K_m value of ACL in *Aspergillus niger* is 2.5 mM and its lipid content is relatively low; therefore, it can be used to commercially produce citrate (Pfitzner *et al.*, 1987; Lu *et al.*, 2021). Overexpression of heterologous ACL with a lower K_m value can increase lipid production by up to 200% in transformants with the highest ACL activity (Zhang *et al.*, 2014). The overexpression of ACL with a low K_m value can increase the lipid content in non-oleaginous yeasts

by 15.4% (Tang *et al.*, 2013). Modification of the ACL gene enhances microbial lipid production, although the gene cannot solely improve microbial lipid production (Zhang *et al.*, 2014). The mechanism of microbial lipid production is complex and involves several enzymes. In our study, we observed that substantial amounts of citrate were excreted from *Y. lipolytica* cells, which may explain why the lipid content in *Y. lipolytica* was relatively high. Similarly, ACL activity tended to decline during the later stages of cell growth, which may be because the cells no longer needed to carry out metabolic activities that synthesize lipids after achieving maximum growth. Furthermore, ACL activity decreased, leading to the accumulation of storage lipids.

Conclusion

The biochemistry of fatty acid biosynthesis prompted by nitrogen limitation in oleaginous fungi has been extensively studied; however, the mechanisms underlying fatty acid biosynthesis in *Y. lipolytica* remain unclear. We investigated enzyme activity related to fatty acid overproduction in *Y. lipolytica* grown on glycerol. Our results revealed that ACL produced acetyl-CoA, a precursor for fatty acid biosynthesis through citrate cleavage. The reducing power NADPH was not provided by ME, which has been regarded as the prime NADPH producer in oleaginous fungi; NADPH was mainly produced by G6PD of the PPP.

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Author's Contributions

Ruixue Wang: Participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.

Feifei Xin, Yufei Chang and Wenrui Dang: Participated in part of the experiment.

Huaiyuan Zhang: Contributed to the study designed, the interpretation of the results, and manuscript preparation.

Yuanda Song: Contributed to the guidance of experimentally designing and ameliorating the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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