Sync between leucine, biotin and citric acid to improve lipid production

by *Yarrowia lipolytica* on crude glycerol-based media

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August, 2020
Abstract

The use of biodiesel-derived glycerol as a carbon source for single cell oil (SCO) production is a biorefinery engineering strategy that aims to valorize the by-product waste and make the microbial lipid production process more cost-effective. This work aimed to improve the capacity of *Yarrowia lipolytica* to produce large amounts of lipids and replace genetic engineering by a metabolic approach based on the stimulation of the rate-limiting enzymes and reducing the activation energy thereby increasing the rate of lipids synthesis. The effects of biotin and leucine addition on the lipid content of *Y. lipolytica* have been investigated. The lipid content of *Y. lipolytica* was strongly influenced by the addition of biotin. In fact, an increase in biotin concentration from 25 µg/L to 200 µg/L practically increased the lipid concentration up to 15 g/L. Besides, to channel metabolic flux into lipid biosynthesis, the addition of citric acid as lipid precursor led to an increase in total catabolism activation and lipid accumulation to reach around 63% (w/w). The biochemical approach can be a useful target for improving the efficiency of lipid-producing yeast strain rather than genetic engineering.

Keywords: *Yarrowia*; citric acid; glycerol; biorefinery; lipid accumulation.
1 Introduction

Owing to the high productivity, justified by the shorter duplication time and higher lipid content compared to vegetable oils (up to 70% on dry weight) [1-2], it is clear that microorganisms accumulating lipids above 20% of the biomass on a dry basis represent an ideal source of lipids. Thus, concerns about energy supply have driven the development and the production of microbial-derived biodiesel. Currently, microbial lipids provide a unique platform for biodiesel, hydrocarbons production through the thermochemical processes such as pyrolysis. Latter, hydrocarbons are considered a potential source for biofuels [3-4]. In fact, oleaginous microorganisms contributed efficiently to fuel oils and responded sufficiently to global needs [5-6]. Accordingly, increasing lipid accumulation by oleaginous microorganisms has attracted significant attention as single cells oils (SCO) can serve as an important source of petroleum diesel replacement and other oleochemicals [7-10]. However, the current productivities of microorganisms-based processes are still very low, which has restricted their scale-up application. Although the extensive works carried out on lipid accumulation in oleaginous microorganisms, the efficient microbial production of lipids at the commercial stage is still challenging and even though the substantial improvements achieved in lipid production with Y. lipolytica, up to now, only high-value lipids are economically feasible and able to reach the market. Consequently, developing a bioprocess with higher lipid production from renewable carbon source with low cost is very promising since these bio-oils microbially produced do not compete with food feedstock production and do not require arable lands [11]. Generally, lipid overproduction has been performed through genetic modifications, however, the genetic engineering and the overexpression of one or few key genes involved in lipid biosynthesis results on
a modest increase in the lipid content [12] and engineered strains are usually recognized with their poor stability during scale-up.

Magdouli et al. stated that higher lipid production is a balance between metabolic and genetic approaches [2,10]. Unlike genetic modification, the metabolic approach relies on phenotypic screening and does not require specific knowledge of molecular targets in metabolic and catabolic pathways involved in the synthesis of lipid droplets. In addition to various efforts in strain improvement and cultivation optimization, it was proposed that the higher lipid productivity can also be achieved using various biochemical and metabolic engineering strategies to enhance cell growth and lipid accumulation. Thus, various metabolic triggers or enhancers can directly modulate cellular metabolism and can be applied to improve lipid productivity. Moreover, the application of cheap renewable sources in large-scale cultures with low concentrations and lower costs could be a valuable and practical approach for addressing the low productivity issue. Recently, numerous studies have shown that the lipid accumulation in oleaginous microorganisms is an adaptative response to particular environmental factors and the concentration and quality of lipids can vary as a result of changes in growth conditions [temperature and pH, dissolved oxygen or nutrient media characteristics [carbon source concentration, C/N ratio, macro and micro nutrients]] [13-16]. Besides, the examination of enzymatic activity and comparative genomics suggests that ATP: citrate lyase [ACL] may play a crucial role in directing excess carbon to be stored as lipids rather than carbohydrates in oleaginous yeasts [17-18]. Moreover, acetyl-CoA carboxylase [ACC] activity is reported to increase during the lipid biosynthesis [19-20]. ACC is a biotin-dependent enzyme that catalyzes the carboxylation of pyruvate to oxaloacetate and acetyl-CoA to malonyl-CoA. Additionally, leucine is a precursor to β-hydroxy-β-methylglutaryl CoA, an intra-mitochondrial
intermediate which is reported as a precursor of sterols and helps to the generation of acetoacetate and acetyl CoA. Their mechanism is presented in Fig. 1.

The activity of \( \beta \)-hydroxy-\( \beta \)-methylglutaryl CoA is reduced when yeast grows on media lacking or deficient in biotin. The decreased activity of acetyl CoA carboxylase has been reported to lead to reduced lipid content due to biotin deficiency [21-22]. Recently, the highest lipid content of 39% was produced by the culture of *Metschnikowia pulcherrima* without yeast extract but with a supplementation of biotin and nitrogen [23]. Besides, Yu et al. [24] have illustrated the pivotal role of many chemicals to enhance microalgal growth and accumulation of high-value bioproducts and Wahbi et al. [25] have patented many types of bio-modulators to increase biofuel production in microalgae. To the author’s knowledge, no study was reported in yeasts to increase lipid through modulators addition as done with microalgae and in crude glycerol based-media in particular.

In this regard, this work aimed to increase the lipid content in yeast *Y. lipolytica* through biomodulators addition. Herein, the biomodulators include amino acids (i.e.leucine), vitamin (i.e biotin) and as well as citric acid. Accordingly, citric acid (CA) is defined as a key precursor in lipid accumulation and during the metabolic shift between growth and lipid production, a transient citrate excretion step occurred [26]. More often, CA has been reviewed to activate allosterically ACC enzyme [27]. Moreover, the metabolism of CA and lipid accumulation are competitive and both phenomena required a nitrogen limitation [28]. Despite the current knowledge on both mechanisms, many points arise as to how oleaginous microorganisms could coordinate both steps and metabolic shift from oxidative to CA production over lipid accumulation still need to be elucidated. Hence, the idea was to direct the carbon dissipation from CA production towards lipid accumulation. Blocking the activities of tricarboxylic-acid, glyoxylate cycle enzymes and CA transport
through mitochondrial is potential [29]. However, this approach remains complex and costly. Thus, a feed batch strategy is proposed in the current study to block CA production [feed-back inhibition] and strengthen the effect of biomodulators. Moreover, Blazek et al. (2018) reported a link between leucine signalling and lipogenesis in *Y. lipolytica* [30]. Biotin is reported to enhance the production of alkaloids and lipids [21]. Taken all together, the present study aimed to evaluate how low concentrations of bio-modulators supplied at an early stage combining with a dissolved oxygen shift strategy [31] can induce lipid accumulation in *Y. lipolytica*. The current study aims to enhance *de novo* lipid accumulation in *Y. lipolytica* through leucine-biotin mediated approach and the addition of CA as lipid precursor for high lipogenesis in a crude glycerol-based media. This work opens a new avenue towards economically viable microbial fuels and chemicals.
2 Materials and methods

2.1 Strain

The yeast *Y. lipolytica* SM7, isolated from the woody forest and selected as an oleaginous yeast in previous studies [31-33] was maintained in stock cultures on agar slants at 4°C containing [g/L]: 20 glucose, 5 yeast extract, 10 malt extract, and 20 agar, pH = 6.5. This strain was used in the current study and its higher potential to accumulate lipids was investigated.

2.2 Inoculum preparation

The yeast *Y. lipolytica* SM7 stored at 4°C was reactivated on potato dextrose agar Petri dishes for 24 h [31]. Subsequently, a sloop of reactivated microbial was transferred to Erlenmeyer flasks containing the growth medium (5% (v/v) inoculum). The culture medium used for inoculum was (GPY) composed of (g/L): Glucose 20, Peptone 20 and Yeast extract 10 named as at pH = 6.5. The inoculum preparation process is presented in Fig. 2. Experiments were conducted in mineral medium composed of: crude glycerol, 0.54 g/L NH₄OH, 1 g/L yeast extract, 3 g/L K₂HPO₄, 3 g/L NaH₂PO₄.H₂O, 0.5 g/L MgSO₄.7H₂O, 0.040 g/L ZnSO₄.7H₂O, 0.016 FeSO₄.7H₂O, 0.25 µg/L biotin, pH = 6.5, maintained at 28°C in flasks agitated at 180 rpm for 72 h.

2.3 Fermentation conditions

To evaluate the effect of modulators at large scale, fermentation was conducted in the 5-L fermenter (Biostat B plus, Sartorius) to improve lipid production in crude glycerol-based media. pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH = 4 and 7 (VWR, Canada). Before the sterilization cycle, the oxygen probe was calibrated to zero (using sodium thiosulfate water) and 100% (air-saturated water). Propylene glycol (Sigma-Canada) was used as an anti-foam agent. The
fermenter with the medium was then sterilized in situ at 121°C for 20 min. After the fermenter cooled down to 28°C, the DO probe was recalibrated to zero and 100% saturation by sparging N₂ gas and air, respectively, at an agitation rate of 250 rpm. The pH of the fermenter solution was adjusted to 6.5 with 4 N H₂SO₄. Thereafter, sterilized crude glycerol [83% w/v, c koi] and the mineral solution was transferred to the fermenter under aseptic conditions. Agitation at 250 rpm was carried out to mix the solution, after mixing, pre-culture of *Y. lipolytica* was added to the fermenter [31]. During fermentation, the airflow rate was kept constant at 2.5 L/min. The agitation rate was varied during fermentation to keep the DO above 30% saturation. The temperature was around 28 ± 2°C and was kept constant during fermentation.

### 2.4 Analytical methodology

#### 2.4.1 Biomass determination

Biomass was determined gravimetrically [34]. Briefly, the samples were centrifuged at 5,000 x *g* and the cells were washed twice to remove residual glycerol. Remaining pellets were frozen and lyophilized (VirTis Lyophilizer, USA) for 36 h. Cell dry weight was calculated by subtracting the weight of the biomass before and after lyophilization.

#### 2.4.2 Glycerol consumption

For the measurement of glycerol and others organics acids in the broth, LC/MS/MS technique was employed [35]. The technical details of the LC/MS/MS instrument used for the analysis were: (a) for sugar estimation: Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in negative ion mode; Zorbax Carbohydrate [4.6 mm x 150 mm; 5 mm, Agilent] analytical column; 75%
acetonitrile; 0.1% NH₄OH; 25% water and 0.1% NH₄OH mobile phase and 10 mL injection volume. Glycerol and citric acid [all from Sigma] were used as the internal standards.

2.4.3 Determination of lipids and the fatty acid profile [FAME]

Lipids from previously lyophilized biomass were extracted according to the method of Bligh and Dyer (1959), with modifications. Resulting biomass was vortexed with methanol: chloroform for extraction of lipids, followed by centrifugation at 785 x g for 10 min for phase separation; the lower phase contained the lipids dissolved in chloroform was evaporated and dried at 60°C to measure the dry lipid mass. The extracted lipid fraction was esterified to obtain the fatty acid methyl esters. For fatty acid, the analysis was performed essentially as described earlier [31]. FAMEs were subsequently analyzed by GC–MS (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m x 0.25 mm, with a phase thickness of 0.25 µm. A 37-component FAME mix (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA) was used for qualitative analysis. 1,3-dichlorobenzene was used as an internal standard to quantify the fatty acid methyl esters. The results are expressed as a mean ± standard deviation. The results were compared by variance analysis (ANOVA) at 5% significance using Excel's Analysis ToolPak.
3 Results and discussion

3.1 Effect of C/N ratio on cell growth and lipid accumulation in Y. lipolytica

Recent studies have shown that Y. lipolytica, a potentially attractive organism, can grow at higher C/N ratio with higher glycerol concentrations [31-33], this fact seems to be strain-dependent and many strains belonging to Yarrowia species can tolerate up to 200 g/L of glycerol and can achieve efficient cell growth ranging from 16.5–26.5 g/L [36]. The selected strain used in this study can accumulate up 52 ± 4% in the presence of crude glycerol of 89 g/L and C/N = 75 at 66 h and a further increase of C/N will not increase the lipid content as well as biomass, Fig. 3.

The upper limit of lipid content in Y. lipolytica SM7 is 47.7% (w/w) in shake flasks at C/N = 75 and higher C/N ratio will lower the biomass content and the lipid content, for example for higher C/N = 200, lipid content and biomass decreased and reached around 7.37 g/L and 24.4% (w/w).

Meanwhile, at C/N = 120, 9.54 g/L of dry biomass and a lipid content of 43.2% (w/w) was obtained. As shown earlier, a higher C/N ratio above 200 will not be suitable to achieve the higher lipid content and the metabolism of the strain seemed to be inhibited by higher glycerol concentration. These observations agree with Karamerou et al. [15], who found that a glycerol concentration of 80 g/L and C/N = 182 resulted in lower biomass 4.62 g/L and a lipid content of 17.4% (w/w) in Rhodotula glutinis. Consequently, to trigger lipid accumulation, the C/N ratio should be adjusted according to the requirements of the cell. Thus, the amount of carbon in the medium must be higher than that required for growth and maintenance of the yeast so that there is a surplus for lipids synthesis.

However, this surplus cannot exceed since high glycerol concentration up to 150 g/L represses the
growth [32,37-38] and glycerol at 100 g/L will lead to lower consumption efficiency and more than
50% of glycerol will be left unconsumed (Fig. 4).

To increase the lipid productivity, metabolic alternatives can be suggested through the optimization
of the two phases: growth (biomass) and lipogenesis phases. The variation of physicochemical
parameters, such as temperature, pH and nutrient composition of the growth medium is crucial and
may impact positively the lipogenesis [15-16]. In the current study, the first phase will be devoted
to stimulating the growth of strain and biomass production within a higher oxygen supply (60%),
followed by the second phase of lipid accumulation that consists on applying lower oxygenation
stress (30%) or/and some modulators. Besides, the application of biological modulators under
nitrogen-limited conditions aimed to stimulate the production of lipids. The choice of the type of
stress, biomodulators type and quantity and their timing supply seems to be primordial to monitor
the efficacy of the process. Besides, a better fundamental knowledge about the requirements of
yeast (i.e. molecular and enzymatic systems implicated during the synthesis and the accumulation
of lipid) should be well understood to well monitor the process. To mention the fact that the
molecular mechanism that regulates lipid accumulation in Y. lipolytica remains a debate despite the
extensive studies carried out compared to microalgae in which the lipogenesis is well understood in
terms of ammonia nitrogen depletion and absorption. For instance, microalgae have both active and
passive ammonia nitrogen transport systems that are depending on ammonia concentration and
enzymatic (e.g. activity glutamine synthetase). Generally, ammonium nitrogen is directly converted
to amino acids by amination or transamination. Thus, free ammonia (NH3) enters the cell via passive
diffusion. With microalgae, the pH is the most critical parameter that impacts the nitrogen
adsorption and algal growth [39]. For instance, when pH is alkaline due to CO2 consumption, the
free ammonia can impact on the stability of the biological cell membrane and the enzymatic hydrolysis reaction and inhibit microalgal growth. In contrast, for Y. lipolytica, nitrogen adsorption, citric and lipid production are an energy-dependent (ATP utilizing) transport mechanisms. Generally, the lipid accumulation is initiated by a sharp decrease in adenosine monophosphate (AMP) concentrations. AMP is latter deaminized by AMP deaminase to produce inosine monophosphate (IMP) and ammonia, compensating for intracellular nitrogen depletion. Depending on the carbon substrate used in the culture medium, yeasts can perform de novo lipid accumulation process involving the formation of acetyl-CoA resulting from the inhibition of the Krebs cycle in sugar-based media. On the other side, yeast can perform ex novo route which is characterized by the incorporation of final products or intermediates of fatty acid β-oxidation into triacylglycerol molecules in hydrophobic carbon source based media. Collectively, yeast and microalgae are promising candidates for lipid production, however, the production cost of microalgae oils is still too high to be commercialized due to microalgae's slow growth rate, inefficiency in large scale photo-bioreactors, difficulty in contamination control especially for open ponds systems, and finally the high cost in downstream recovery [40-41]. Due to the current challenges, most attention is paid to yeasts-based biodiesel production.

3.2 Effect of modulators to enhance lipid accumulation

The biotin trigger has been tested and optimized on various species, including chlorophytes [42-43] and some of the baker’s yeasts [44] and filamentous fungi of Claviceps species [21]. In the latter case, the production was principally aimed at the biological production of alkaloids. However, Desai et al. [21] observed a concomitant production of alkaloids and lipids after supplying biotin to the culture. The present study aimed to evaluate how low concentrations of bio-modulators can induce
triacylglycerols (TAG) accumulation and their lower concentrations at an early stage of lipid accumulation associated with a dissolved oxygen shift could enhance efficiently the growth and the lipid accumulation scenario in *Y. lipolytica*.

The supplementation of cultures with low doses of biotin (50, 100 and 200 µg/L) at an early and late stage of lipid accumulation (16 h and 60 h), was evaluated in the 5-L fermenter. Briefly, batch cultures of *Y. lipolytica* were grown in crude glycerol medium until just before nitrogen depletion at 16 h. As expected based on our previous work [31], the cultures that did not receive any biotin had a maximum of growth rate until 12 h of 0.15 h⁻¹, and biomass tends to increase until reaching stationary growth. The original medium supplemented with 25 µg/L of biotin showed a lipid content of 52.6 ± 1.3 (w/w) with maximum lipid concentration of 13.5 ± 0.5 g/L at 66 h and biomass 25.1 ± 0.4 g/L. The effect of biotin addition on different growth stages is presented in Table 1. The biotin addition at an early stage showed improvement in lipid quantities. However, the biomass did not increase and remained almost stagnant at 26 g/L. Even though cell dry weight did not increase as much in biotin supplemented media, lipid contents in all biotin supplemented cultures increased. Thus, the addition of 50 µg/L of biotin at an early stage increased the lipid content up to 14.0 ± 0.3 g/L, and the increase of biotin concentration led to around 15.5 and 14.9 ± 0.5 g/L in the presence of biotin concentration 100 and 200 µg/L, respectively. This slight decrease can be attributed to yeast requirements; thus, biotin is involved in both amino acid metabolism and lipid synthesis and variations in its level may impact the production of intermediates and fatty acids [45-46]. Previous reports showed that the yeast is more active at lower biotin concentration and the genes involved in biotin uptake and biosynthesis are highly expressed acids [47-48]. In *E. coli*, only a few hundred molecules of biotin per cell are sufficient for growth [49] and some enzymatic pathways
are expressed at very low levels (< 350 molecules/cell [50]. Although biotin is widely recognized to be involved in the regulation of several biological processes in both prokaryotic and eukaryotic systems, the molecular details surrounding its exact cellular function in relation with lipid synthesis remain elusive.

In the later stage of accumulation at 60 h, the addition of biotin did not improve the lipid concentration and the maximum value was obtained in the case of 100 µg/L, where only 13.8 ± 0.4 g/L was reported. Thus, the biotin factor acted preferentially at an early stage and the active phase of lipid accumulation was between 16 h and 60 h. The action of biotin could be also attributed to an effect on intermediary metabolism, correlating with the activity of fatty acid synthases and acetyl-CoA carboxylases, besides, cells require more time for biotin uptake since the entry of biotin into cells is performed by passive diffusion [51]. Likewise, microbial transport systems of biotin are dependent upon, or stimulated by, the presence of an energy carbon source, which is in the present case glycerol, and only 9.2 ± 0.3 g/L are available in the culture medium at 60 h compared to 49.7 ± 0.9 g/L at 16h. The growth seemed to be less affected by biotin addition which was probably due that biotin acts preferentially during the lipogenic phase and if the biotin is required for growth and cell wall synthesis, the strain will use the exogenous biotin supplied in the medium, otherwise, it will synthesize biotin according to its needs.

The analysis of glycerol consumption indicated no statistical difference between the control treatments (Y_{X/S} = 0.32 g/g), and those that received biotin addition (Y'_{X/S} = 0.32 g/g). This was due to the delay or arresting of the cellular division, and this phenomenon is assumed to be the result of a fundamental metabolic shift from growth metabolism to lipid accumulation metabolism after nitrogen depletion [31].
The addition of up to 200 µg/L biotin did not affect the lipid accumulation activity of *Yarrowia*. Here, the addition of biotin resulted in a single fold increase of lipid yield over the biotin-free control, which suggested that biotin can enhance fatty acid synthesis in agreement with nitrogen depletion and oxygen limitation [concentration of dissolved oxygen DO = 30% of saturation].

The results provided evidence that the timely addition of biotin, when coupled with nitrogen depletion, can induce significant lipid accumulation. Therefore, the effect of the biotin on lipid production is explained by the stimulation of genes involved in the lipid production pathway, such as ACC1 genes [52]. Generally, biotin participates in carboxylase reactions and various enzymes are activated in the presence of biotin, such as ACC and pyruvate carboxylase which contained biotin. Their mechanism is clarified by the work of the Lynen group [45-46]. Generally, biotin is reported to function as the prosthetic group of various carboxylases [45-50]. Meanwhile, the only biotin-enzymes functioning in yeast growing in a synthetic medium containing sugar and ammonium salt are pyruvate carboxylase and acetyl-CoA carboxylase catalyzing the carboxylation of pyruvate to oxaloacetate and acetyl-CoA to malonyl-CoA.

More often, it was hypothesized that higher lipid productivity might be achieved by adding lower concentrations of leucine near nitrogen depletion. A recent study revealed that leucine auxotrophy caused a 2.5-fold decrease in cell fatty acid content and that *leuA* gene expression restored its level in *M. circinelloides* strain R7B [53]. Moreover, acetyl-CoA generated from the endogenous leucine metabolic pathway was postulated to be another rate-limiting step during fatty acid synthesis in *M. circinelloides* [53]. Interestingly, Kamisaka et al. [54] have elucidated a correlation between leucine biosynthesis and oleaginicity. Thus, in that work, the complementation of leucine in *Saccharomyces cerevisiae* increased lipid accumulation in comparison with auxotrophic strains.
In this regard, in an attempt to increase biomass production prior to inducing lipid accumulation, the culture was also supplemented with leucine [60 µg/mL] at 16 h, just prior to nitrogen depletion, to induce TAG accumulation. The results are shown in Table 2.

As indicated earlier, the leucine enhanced the lipid accumulation and lipid content reached around 14.7 g/L which is better than the control experiment, besides, biotin and leucine, supplemented together enhanced lipid synthesis and the positive effect of leucine was verified. This suggests that the leucine metabolic pathway is participating in acetyl-coenzyme A [acetyl-CoA] generation which may be critical during fatty acid synthesis [55-57]. The key two-carbon metabolite for lipid biosynthesis, acetyl-CoA, can be produced via degradation of branched-chain amino acids, e.g, leucine, in addition to other metabolic pathways presented in Fig. 1. Interestingly, a recent comparative genome analysis of non-oleaginous and oleaginous species identified a theoretical pathway for leucine degradation that was specific to oleaginous strains and might provide acetyl-CoA for lipid biosynthesis [18]. Besides, a higher concentration of leucine up to 120 µg/mL showed a decrease of the lipid concentration only 12.7 ± 0.3 g/L, and the biomass increased comparatively to control experiment up to 30 g/L, this fact may be due to that higher concentration of leucine concentration can induce leucine biosynthesis and block leucine biodegradation, therefore, the strain will use the leucine for growth and protein synthesis and not synthesizes lipid.

This may provide a proof that operating leucine metabolic pathway is required for the accumulation of lipids, suggesting that if the strain is endogenously producing leucine, this latter is degraded for the generation of the corresponding acetyl-CoA, which is incorporated into fatty acid biosynthesis, more often, the strain may present smaller amount of leucine coming mainly from its biosynthesis which is used for lipid synthesis. This fact goes in line with the latter observation in which, the
selected strain synthesizes biotin during fatty acid biosynthesis as well as leucine which are both indispensable for lipid synthesis and their overproduction via supplementation may have a crucial role in the present study. As shown in Table 2, leucine and biotin act in synergy and up to 16.8 g/L of lipid was obtained. The essential vitamin biotin is a covalent and tenaciously attached prosthetic group in several carboxylases that play important roles in the regulation of energy metabolism. Thus, the biotin activates the methylcrotonyl-CoA carboxylase (MCCase) enzyme involved in leucine catabolic pathway named as branched-chain catabolism (BCC) which provides more acetyl-CoA as shown in Fig. 1. Blazek et al. (2018) have observed a link between leucine signalling [30] and lipogenesis in Y. lipolytica [56]. A comparative study on the effects of leucine and isoleucine supplementation on lipogenesis showed that only leucine promote lipogenesis and Y. lipolytica possesses degradation pathways for leucine to produce acetyl-CoA fatty-acid precursors [57]. Thus, leucine is found to act as an intracellular signal to favor lipid production. Moreover, MCCase is a biotin-dependant enzyme that enhance the amino acid leucine catabolism. The ultimate product of catabolism is acetyl-CoA which has a wide array of potential fates especially lipogenesis. Consequently, next to a carbon source, the most prerequisite elements for lipid production are biomodulators, which play a pivotal role during lipid synthesis.

3.3 Effect of citric acid production to enhance lipid production in Y. lipolytica

The addition of citrate is reported to activate allosterically ACC1 enzymes [18]. ACC 1 is allosteric requiring tricarboxylic acid intermediates such as citrate for their activation and are inhibited by long-chain saturated fatty acyl-CoA [58-60]. Herein, the nitrogen depletion occurred at 16 h announcing the lipogenesis phase. The nitrogen concentration of 90 mg/L was kept constant along with the fermentation. In fact, the nitrogen exhaustion activates AMP deaminase which is
responsible for the decrease in AMP concentration and increases cellular ammonium concentration [61]. The decrease in AMP concentration inhibits isocitrate dehydrogenase, blocking the citric acid cycle at the isocitrate level. Aconitase mediates the accumulation of citrate in mitochondria, with an exit from the mitochondria-mediated by the citrate/malate cycle [61-62]. This reaction provides large amounts of acetyl-CoA for fatty acid synthesis. Thus, acetyl-CoA is provided by the cleavage of citrate coming from the mitochondria by ATP-citrate lyase [ACL] in the cytosol. Later, ACL cleaves the citrate to give oxaloacetate and acetyl-CoA. Both CA production and lipid accumulation are competitive and they are induced by lower nitrogen concentration [28,63]. Fig. 5 showed the kinetic growth of Y. lipolytica in the optimized process [e.g. 89 g/L of crude glycerol and C/N = 75], Y. lipolytica produced low concentrations of CA 7.8 ± 0.7 g/L and this concentration seemed to be dependent on glycerol concentration. Thus, the variation of glycerol concentration from 75 to 100 g/L, increased the CA production around from 2.5 to 14 g/L [32]. The increase of CA concentration in cells at higher glycerol concentration contributed to low levels of lipid accumulation 12.8 g/L at 72 h within cells which justify that both phenomena are competitive and more knowledge is required to understand this competitiveness. Likewise, Papanikolaou et al. [2002] assumed that ATP-citrate lyase was inactive in the presence of excess carbon, resulting in low levels of lipid accumulation [64-66].

Hence, the idea herein was to inhibit the production of CA. Once CA is available, the metabolic pathway of CA production is blocked and the cells avoided CA production since this latter is available in the growth media. In this manner, the strain maintains nitrogen levels optimal for high lipogenesis and prevented extracellular carbon flux, promoting easier lipid accumulation [30]. For this purpose, the addition of 30 g/L of CA to the culture was investigated, the time was chosen according to the
quantities detected in the culture [0.50 g/L] of CA at 36 h. Thus, the feed of the current solution with citric acid at the lipogenic phase was assumed to repress the CA metabolism towards lipid accumulation and provide more acetyl Co during the lipogenic phase as well as reinforcing the biotin effect. Hence, a higher C/N ratio of 75, combined with a buffered pH, lower oxygenation rate at 30% of dissolved oxygen and higher biotin supplement will have a significant effect [p < 0.05] on lipid accumulation. The p-values of the models were 0.0002 indicating that the models were significant. Usually, a model term is considered to be significant when its value of “p-value” is less than 0.05.

In the control experiment, the final biomass reached around 25.1 g/L. This concentration increased sharply to 28.7 ± 0.5 g/L at 72 h when the culture is supplemented by citric acid and biomodulators. Fig. 6 represents the growth of *Y. lipolytica* on crude glycerol with different biochemical modulators [citric acid (30 g/L) and biotin (100 µg/L)]. As shown in Fig. 6, *Y. lipolytica* simultaneously consumed crude glycerol and CA. Once CA is introduced to the media, its concentration shows a sharp increase and concentration raised up from 2g/L to 3 g/L following the supply of CA at 16h. The exogenous source of CA supplemented to the growth media as well as the endogenous CA produced during growth media and the lipogenesis phase will constitute a potential CA pool and justify the sharp increase. The CA exogenously supplied enhances the production of CA compared to control experiment where the concentration of CA was not exceeding 3g/L during lipogenic phase (Fig. 5). Latter, a slight decrease observed is due to the consumption of CA by *Y. lipolytica*. This phenomenon seemed to be not frequent and the simultaneous utilization of two different substrates is not typical of microorganisms, which first assimilates one of the two available substrates, whereas the assimilation of the other substrate starts only after the first substrate is fully consumed from the medium. Herein, *Yarrowia* was assimilating citric acid [3 g/L], followed by glycerol [0.5 g/L]. This
simultaneous consumption can be attributed to metabolic signaling since some sugars can produce signals which modify the conformation of certain proteins that, in turn, directly or through a regulatory cascade affect the expression of the genes subject to catabolite repression. These genes are not all controlled by a single set of regulatory proteins [66-68]. The glycerol consumption rate was decreased principally because the cells consumed both substrates at low rates. Thus, the strategy of the CA feeding was efficient to create a pool of CA required during metabolism shift towards lipid production. More often, the influence of CA on the production phase of lipid production has not been described until date. Iske et al. [69] have determined the effect of CA on the growth rate of the bioprocess and they found that the addition of initial concentrations of CA at 30 g/L into the culture had no remarkable influence on the growth rate. Nevertheless, with increasing concentrations of CA, a significant decline of the growth was observed, which is not the case in the present study, where growth was activated and lipid production was more promoted. Herein, the high-lipid production is due to the continuous production of acetyl-CoA, mainly achieved under nitrogen-limited condition leading to a citrate accumulation in the mitochondria. Subsequently, the activity of AMP deaminase responsible for the cleavage of AMP to IMP and ammonia increased considerably. The decrease of AMP inhibits the activity of isocitrate dehydrogenase, which catalyze the conversion of isocitrate into α-ketoglutarate. Therefore, isocitrate is converted back to citrate by the enzyme aconitase, which leads to citrate acid accumulation. The formed citric acid is transported to cytosol, further converted to acetyl-CoA by ACL, and eventually goes to the β-oxidation pathway to produce fatty acids [61-62]. Thus, citric acid can be used as a carbon source for the fatty acid pathway rather than being a major by-product. The
addition of CA directs this pool of acetyl-CoA to fatty acid and lipid production under nitrogen-limited conditions.

During the lipogenic phase, $K_La$ was kept between 50-60 $h^{-1}$ and OTR varied between 0.2 to 0.3. During fed-batch fermentation, CA was added at 36 h. The supplementation of the medium with CA at 30 g/L led to a decrease of $K_La$ reaching 21.1 $h^{-1}$, however manual adjustment of agitation was required to keep the DO around 30% [i.e. agitation 420 rpm and air flow rate 2.5 L/min].

The combined effect of biomodulators (e.g. leucine) and CA, which were reviewed to work simultaneously [30] increased the lipid concentration to 18.3 g/L and lipid accumulation up to 63.7 ± 0.9% (w/w). These results were advantageous compared to the control experiment where only 52.7% (w/w) were obtained. Blazek et al. (2013) confirmed that lipid accumulation phenotypes are dependent on leucine-mediated signalling and that high lipogenesis can be uncoupled from nitrogen starvation and entails a reduction in citric acid cycling [30]. Besides, as biotin is involved in both amino acid metabolism and lipid synthesis (Fig1), the present study, showed that biotin acts in synergy with leucine and citric acid. These results are promising compared to other Yarrowia species such as Y. lipolytica TISTR 5151 that accumulate around 50.8% (w/w) [70] and Y. lipolytica MUCL 28849 which accumulate 34.6% (w/w) [71]. The feasibility of this biomodulator-based approach should be assessed using a techno-economic analysis to compare costs associated with biotin addition compared to carbon source addition. Beyond further optimization of modulators supply, additional optimization can perceptibly be achieved via metabolic engineering tools.
3.4 Fatty acid composition

The fatty acid profile is essential to assess the biodiesel quality. In the current study, in the presence of CA and biomodulators, C16 and C18 were the mainly fatty acids present (Table 3) and the concentration of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in *Y. lipolytica* was predominating the total lipid pool. *Y. lipolytica* showed that the major fatty acid detected was oleic acid [C18:1]. This was 33–45% of the fatty acid pool during biomass and lipid production phases. Also, palmitic [C16:0] and linoleic [C18:2] acids have also been detected in high content in the cells [14–20%]. The predominance of palmitic and linoleic acids is propitious for biodiesel synthesis [72-73]. However, in the current study, *Y. lipolytica* produced more stearic acid 12.98%, compared to the control experiment where 8.30% of stearic acid was obtained. These results agree with previous reports [31,74]. Several studies indicated that oleaginous yeasts produce lipids with a similar composition to plant oils with a predominance of SFA and MUFA [20,73]. Fatty acids speciation data exhibited by *Y. lipolytica* agrees with previously reported values of C16:0, C18:1 and C18:2, C18:1 being the main species present in oleaginous yeasts [72-74]. This study presented a significant advantage in fatty acid composition for further potential biodiesel production because polyunsaturated fatty esters have low cetane number, low melting points, and reduced oxidative stability which restricts their use in diesel fuel [75]. In general, the FA composition of lipids produced by *Y. lipolytica* agrees with the FA profile of *Rhodosporidiobolus fluvialis* DMKU-RK253, *R. glutinis* R4, vegetable oils and *Chlorella vulgaris*, [1, 76-79] (Table 3). Therefore, this research provides new insight into fatty acid production by *Y. lipolytica*. Taken together, our results demonstrate that *Y. lipolytica* could be a promising feedstock for third-generation biodiesel. From a technological point-of-view, it is much easier to use this strain to produce biodiesel by only supplementing biochemical
targets products. To sum up, SCO production based on crude glycerol with an advanced biochemical approach can yield promising breakthroughs in low cost and effective synthesis of fatty acids.

4 Conclusion

The influence of various modulators concentrations on growth and lipid accumulation in Y. lipolytica was explored through the strategic addition of biotin and leucine to enhance growth and lipid accumulation rates as compared to traditional growth regimes which usually supply elevated concentrations of carbon source up to 89 g/L. These data indicated that the type and strategy [e.g., timing, concentration] of modulators had a significant influence \[p \text{ value } < 0.05\] lipid production in Yarrowia and at an early stage of lipid accumulation, the strain was accumulating up to 15.5 g/L. An optimized two-phase growth with the biochemical approach of biotin and citric acid is an effective strategy to increase its fatty acid biosynthesis and provide pathway precursors for the production of targeted products. This could offer a promising strategy for optimizing productivity and reducing resource costs. Moreover, this strain can be regarded as a valuable tool for generating large amounts of lipids owing to the recent identification of additional putative rate-limiting steps required during the lipid synthesis and regulatory elements involved in lipid accumulation.

Acknowledgements

The authors are thankful to the Natural Sciences and Engineering Research Council of Canada for their financial support.
Figure Captions List

Figure 1  The diagram of inoculum preparation process under optimal conditions. The crude glycerol solution was used without any aseptic techniques.

Figure 2  Overview of metabolic pathways for fatty acid synthesis in the presence of glycerol as carbon source, underlined enzymes are activated in the presence of biotin [pyruvate carboxylase, acetyl-CoA carboxylase, and methylcrotonyl-CoA carboxylase].

Figure 3  Effect of C/N on the growth and lipid production in Y. lipolytica, cultured in shake-flasks (Culture conditions: T = 28°C, pH = 6.5, 180 rpm, 72 h).

Figure 4  Effect of initial glycerol concentration on the growth of Y. lipolytica (Culture conditions: T = 28°C, pH = 6.5, 180 rpm, 72 h).

Figure 5  Batch bioreactor fermentation at C/N 75. Crude glycerol was used as a carbon source (89 g/L).

Figure 6  Effect of citric acid and biotin on the lipid by Y. lipolytica SM7, Conditions: T = 28°C, pH = 6.5, DO = 30%, 440-480 rpm, 100 h [duplicates].
References


J. E. Cronan, The biotinyl domain of *Escherichia coli* acetyl-CoA carboxylase. Evidence that the "thumb" structure is essential and that the domain functions as a dimer, J Biol. Chem. 276 (2001) 37355–37364.


<table>
<thead>
<tr>
<th>Biotin concentrations (µg/L)</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid DCW Lipid DCW Lipid DCW</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early stage</td>
<td>14.0 ± 0.3</td>
<td>26.4 ± 0.4</td>
<td>15.5 ± 1.1</td>
</tr>
<tr>
<td>Late stage</td>
<td>13.5 ± 0.5</td>
<td>25.9 ± 0.3</td>
<td>13.8 ± 0.9</td>
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</table>

DCW: dry cell weight (g/L), Lipid (g/L), The values are means ± standard deviations of three independent experiments.
<table>
<thead>
<tr>
<th>Biomodulators</th>
<th>DCW (g/L)</th>
<th>Lipid concentration (g/L)</th>
<th>Lipid content (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With Leucine</td>
<td>26.7 ± 0.6</td>
<td>14.7 ± 0.9</td>
<td>54.9 ± 0.5</td>
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<tr>
<td>With Biotin</td>
<td>25.8 ± 0.6</td>
<td>15.5 ± 1.1</td>
<td>60.1 ± 0.7</td>
</tr>
<tr>
<td>With Biotin+leucine</td>
<td>27.4 ± 0.5</td>
<td>16.8 ± 0.5</td>
<td>61.3 ± 0.9</td>
</tr>
</tbody>
</table>

DCW: cell dry weight (g/L), The values are means ± standard deviations of three independent experiments.
Table 3: Fatty acids composition of lipid produced by different oleaginous microorganisms and vegetable oils

<table>
<thead>
<tr>
<th></th>
<th>C16:0</th>
<th>C16:1</th>
<th>C18:0</th>
<th>C18:1</th>
<th>C18:2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y. lipolytica SM7</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.5</td>
<td>0.97</td>
<td>12.98</td>
<td>45.0</td>
<td>20.1</td>
</tr>
<tr>
<td><strong>Y. lipolytica SM7</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.5</td>
<td>0.93</td>
<td>8.30</td>
<td>33.0</td>
<td>14.0</td>
</tr>
<tr>
<td><strong>R. fluvialis DMKU-RK253</strong></td>
<td>18.8</td>
<td>0.00</td>
<td>6.20</td>
<td>35.8</td>
<td>34.2</td>
</tr>
<tr>
<td><strong>R. glutinis R4</strong></td>
<td>16.78</td>
<td>1.81</td>
<td>1.35</td>
<td>61.60</td>
<td>11.64</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>11.60</td>
<td>1.00</td>
<td>3.10</td>
<td>75.00</td>
<td>7.80</td>
</tr>
<tr>
<td>Jatropha Oil</td>
<td>14.90</td>
<td>1.00</td>
<td>6.10</td>
<td>40.40</td>
<td>36.20</td>
</tr>
<tr>
<td>Palm Oil</td>
<td>36.70</td>
<td>0.10</td>
<td>6.60</td>
<td>46.10</td>
<td>8.60</td>
</tr>
<tr>
<td><strong>C. vulgaris</strong></td>
<td>10.66</td>
<td>2.76</td>
<td>5.17</td>
<td>24.42</td>
<td>7.17</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Crude glycerol media supplied with 100µg/L of biotin; 60 µg/L of leucine and 30g/L of citric acid at an early stage (16h)

<sup>b</sup>: Control media without biomodulators supply
FIG 2
FIG 3

[Graph showing biomass, lipid quantity, and lipid content as a function of C/N ratio.]
FIG 5
FIG 6