# Dissolved-oxygen feedback control fermentation for enhancing β-carotene in engineered Yarrowia lipolytica

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May 5, 2020

**Abstract**

β-carotene is an indispensable nutrient for human health and its global consumer demand is increasing. In order to promote the market supply and volumetric productivity of β-carotene using engineered Yarrowia. lipolytica strain. The fed-batch fermentation and DO-stat fed-batch fermentation were carried out. 73.5g/L biomass and 0.3g/L β-carotene were obtained in fed-batch. 94g/L biomass and 0.4g/L β-carotene were harvest during DO-stat fed-batch fermentation. The results of intracellular energy showed that more ATP and NADP+/NADPH were produced in DO-stat fed-batch fermentation as compared to that under fed-batch fermentation. Moreover, three kinetic models showed that the higher μm, Yx/s, and Yp/s values were achieved for DO-stat fed-batch fermentation. In this study, the DO-stat strategy was first reported for the β-carotene produce and the obtained results showed that it is a promising strategy in the industrialized β-carotene production.

**Key Words**

β-carotene, DO-stat fed-batch fermentation, Yarrowia lipolytica, kinetic models



# Introduction

β-carotene ($C_{40}H_{56}$), one of many carotenoids, is the precursor of vitamin A. β-carotene has become an essential ingredient in food additives, cosmetics, and pharmaceuticals.β-carotene has the function of enhancing immune functions, anti-oxidation, and anti-cancer activities (Hejazi, Holwerda, & Wijffels, 2004) (Bogacz-Radomska & Harasym, 2018). The global β-carotene market size is anticipated to reach USD 583 million by the end of 2024 (Nester, 2019). Currently, the primary sources of β-carotene include chemical synthesis, plant extracts, and microbial fermentation. Among these, the microbial fermentation is considered to be the best way to meet market demands, as it is environment-friendly and economical.

The most commonly used microorganisms to produce β-carotene are *Blakeslea trispora* (He et al., 2017), engineered *Yarrowia lipolytica* (Larroude et al., 2018),*Saccharomyces cerevisiae* (R. Wang et al., 2017), and*Escherichia coli* (Wu et al., 2017). β-carotene is a lipid-soluble compound and stored in the lipid bodies of engineered oleaginous yeast *Y. lipolytica* . Compared with the non-oleaginous *Saccharomyces* cerevisiae or*Escherichia* coli, *Y. lipolytica* is more suitable for the production of β-carotene. *Y. lipolytica* is generally recognized as a safe strain and a potential industrial host for the production of bio-ingredients (Beopoulos, Desfougéres, Sabirova, & Nicaud, 2010). An engineered *Y. lipolytica* strain to maximize β-carotene production and 1.47 mg/L of β-carotene was obtained after six days of cultivation in a flask (Jang, Yu, Jang, Jegal, & Lee, 2018). Another engineered *Y. lipolytica* strain was constructed by incorporating two genes, bi-functional phytoene synthase/lycopene cyclase (crtYB) and phytoene desaturase (crtI) from the red yeast

*Xanthophyllomyces dendrorhous* . After 72h cultivation, 31.1 mg/L β-carotene was obtained (Kildegaard et al., 2017). Although β-carotene-producing engineered *Y. lipolytica* has made significant progress, the fermentation process has not been systematically optimized.

The production of β-carotene in *Y. lipolytica* requires aerobic culture. When the glucose concentration in the culture medium is high, the Crabtree effect often occurs in aerobic conditions during most yeast fermentation, leading to the production of alcohol and acetate through substrate-level phosphorylation. Excessive alcohols and acids compete with β-carotene synthesis for the substrate of acetyl-CoA. The Crabtree effect eventually leads to a reduction in the yield of the target product (Li et al., 2019a). Fed-batch cultures provide a carbon source at a low level by feeding essential nutrients incrementally. Therefore, this culturing technique is used to overcome the Crabtree effect (Wen, Zhang, & Tan, 2006). Fed-batch strategies include constant dissolved oxygen value feeding (DO-stat) (Hu, Zheng, & Shen, 2010; B. Kim, Binkley, Kim, & Lee, 2018; Y. Wang et al., 2016), constant specific growth rate feeding (μ-stat) (Maghsoudi et al., 2012), constant PH feeding (pH-stat) (B. S. Kim, Lee, Lee, Chang, & Chang, 2004; Li et al., 2019b), and constant carbon source concentration feeding (Khatri & Hoffmann, 2006). Besides the Crabtree effect, oxygen in short supply is another obstacle in aerobic culture.

With cells growing, oxygen consumption exceeds the maximum oxygen transfer capacity, which becomes a limiting factor for cell growth (Van Hoek, 1998). Thus, a solution for microbes to receive an adequate amount of oxygen is by decreasing the specific growth rate and amount of oxygen consumption. Usually, DO-stat can be used to reduce the high rate of oxygen consumption because it can control dissolved oxygen at a constant value using fed substrate at a specific rate. Once the carbon source is exhausted in the logarithmic growth phase, the $O_2$ value rapidly increases due to cell death from hypoxia. If the carbon source was fed timely, the $O_2$ value decreases as the cells re-utilize carbon source and restore growth. Subsequently, a constant DO level can be maintained by continuous feeding and keep a balance between oxygen consumption and supply. The DO-stat strategy typically works well in a defined media where nutrient depletion results in cell death and a rapid elevating DO (Picotto et al., 2017). Many authors have used the DO-stat feeding strategy to achieve high yield. The DO-stat fed-batch fermentation strategy was used to produce tyrosine phenol lyase by recombinant *Escherichia coli* , the final biomass was 35.6 g/L, and the volumetric activity reached 12292 U/L after 30 h cultivation (Kawaguchi et al., 2019a). The DO-stat feeding strategy was a promising strategy together with the use of ammonium hydroxide for pH control to improve P(3HB) volumetric productivity (Cruz, Gouveia, Dionísio, Freitas, & Reis, 2019). This particular method has been widely used in the aerobic culture to produce highly valuable chemicals and biofuels.

Our laboratory has previously constructed an engineered β-carotene producing strain of *Y. lipolytica* . The purpose of this study was to develop a DO-stat culture strategy to improve biomass and β-carotene yield. The changes of ATP and $NADP^+$/NADPH during culture process was also explored. A simple kinetic model relating the cell growth to the limiting substrate (glucose) and major product (β-carotene) was constructed. This study established an effective method to increase the yield of β-carotene and provides a new vehicle for the industrialized β-carotene production.

# Materials and methods

### 2.1 Strains and culture conditions

Engineering *Y. lipolytica* stain YL-C11 (matA, leucine$^+$, uracil$^+$, xpr2-322, axpl$^-$, Δku70, Δsnf:: tHMG-carB-carRA-ggs1, Δgut2:: did2-ura3) was used in this study.

The rejuvenation of strain was carried out on YPD agar medium. Single colony was pre-cultured in YPD medium at 30, 180 rpm for 24h. Then draw 1 ml the pre-cultured solution to the 250 ml shake-flask containing 50 ml medium, which containing (100 ml) 3 g glucose, 1 g casein peptone, 1 g yeast, 0.3 g $(NH_4)_2SO_4$, 0.25 g $KH_2PO_4$, and 0.05 g $MgSO_4$. The seed was incubated at 30, 180 rpm for 48 h.

### 2.2 Fed-Batch fermentationandDO-stat Fed-batch fermentation

Fed-batch and DO-stat fed-batch fermentation were performed in a 5 L bioreactor (Bioflo 110, New Brunswick, USA) with working volume at 4 L. The medium was identical to the shake flask medium apart from glucose concentration. An aeration rate of 0.25-1.25 vvm, agitation speed was at 400-900 rpm, 30, and pH 5.5 was controlled with 15% ammonia, separately.

In fed-batch fermentation, the glucose was maintained at around 5 g/L. In the early stages of fermentation, when the value of $O_2$ was less than 15%, the aeration rate and agitation speed increased alternately until the aeration rate and agitation speed were reached a maximum of 1.25 vvm and 900 rpm.

In DO-stat fed-batch fermentation, when the aeration rate and agitation speed reached max, the pure glucose solution was pumped into the fermenter to maintained DO at 10-20%. $(NH_4)_2SO_4$ (5%) was added at $24^{th}$, $48^{th}$, and $72^{nd}$ hour to avoid pH rise.

### 2.3 Analytical methods

*2.3.1 Dry cell weight and cell density determination*

DCW was calculated as described previously (Wen, Zhang, & Tan, 2005).

2.3.2 *Γλυϲοϲε ανδ β-ϲαροτενε αναλψϲιϲ*

The glucose concentration of fermentation broth was measured by an SBA-40C bio-analyzer (Shandong Academy of Sciences; Jinan, China).

The β-carotene was extracted as described previously (Gao et al., 2017) and measured by HPLC (Agilent Technologies 1260 Infinity Series system, USA) using a C18 column at UV 450nm. Methanol, acetonitrile, and isopropanol (30:50:20) were used in the mobile phase at a flow rate of 1.0 mL/min at 30 .

*2.3.3 Analysis of ATP and NADP/NADPH*

The ATP and $NADP^+$/NADPH were measured by an assay kit (Beyotime, Nanjing, China) according to the manufacturer's protocols.

*3. Kinetic model*

The logistic equation was used to fit the growth kinetics of YL-C11. The Luedeking-Piret equation was used to describe the β-carotene synthesis and glucose consumption. The detailed equation forms in result 4.

*4. Statistics Analysis*

All experiments were repeated three times. Data in ATP and NADPH/NADPH were analyzed using ANOVA, followed by Duncan's multiple range test to determine the significant difference between the means using SPSS software. Origin software was used for the statistical analysis and graphs construction.

## Results

### 1. Fed-batch fermentation

Fed-batch experiments were performed with glucose maintained at around 5 g/L to determine the growth characteristics of YL-C11 (Figure 1). Cell growth increased rapidly during the 12-$42^{th}$ h fermentation. After 42 h fermentation, cell growth entered the stationary period. Although cell growth was in a stationary phase, the synthesis of β-carotene continued. It was not until the $96^{th}$ h of fermentation that the synthesis of the β-carotene trend finally stopped. Glucose (1200 g) was consumed during fermentation. The DO value decreased rapidly in the first 28 h of fermentation and reached 0% when the fermentation time extended from 28 to 120 h. Cell biomass reached 73.5g, and β-carotene concentration reached 0.3g/L at the end of

fermentation. The results showed that the biomass and β-carotene concentration were at a low level, and there is potential for improvement.

**2. DO-stat fed-batch fermentation**

We conducted DO-stat fed-batch fermentation to increase the yield of β-carotene (Figure 2). Glucose (1350 g) was used to maintain DO value at 10%-20%. The medium for the fed-batch phase contained 8 g/L glucose, which was depleted completely after 20 h. DO-stat fed-batch fermentation was implemented at this time until the end of fermentation. The increase of biomass tended to be stable after 96 h fermentation, which demonstrates the cell growth entered the stationary period. 94 g/L biomass and 0.4 g/L β-carotene concentration were harvested at the end of DO-stat fed-batch fermentation, separately. 8 g ammonium sulfate was fed at $24^{th}$, $48^{th}$, and $72^{th}$ h to avoid pH rise.

Application of DO-stat fed-batch fermentation resulted in a 1.3-fold higher production of biomass and β-carotene content when compared to fed-batch fermentation. More glucose were consumed in DO-stat fed-batch fermentation.

**3.More ATP and $NADP^+$/NADPH were produced in DO-stat fed-batch fermentation**

The intracellular levels of ATP content and $NADP^+$/NADPH were determined to explore the effect of feeding strategy on the generation of energy ATPs and $NADP^+$/NADPH.

ATP was gradually diminished during fed-batch fermentation. The highest level of ATP appeared at $24^{th}$ h (Figure 3). At the $48^{th}$ h, the ATP content was dropped to 0.81 nM/mg protein. The ATP content was only 0.43 nM/mg protein at the $72^{nd}$ h.

More ATP were generated in the DO-stat strategy when compared to the fed-batch strategy. The ATP content was 6.80 nM/mg protein at $24^{th}$ h during DO-stat fed-batch fermentation. ATP content gradually decreased as the fermentation progresses and dropped to 2.33 nM/mg protein at the $72^{nd}$ h.

More $NADP^+$/NADPH was generated during the fed-batch fermentation (Figure 4). $NADP^+$/NADPH content was the least at $24^{th}$ h in fed-batch fermentation. At $48^{th}$ h, the $NADP^+$/NADPH content reached 15.29 nM/mg protein. The $NADP^+$/NADPH content was 17.49 nM/mg protein at $72^{nd}$ of fed-batch fermentation.

More $NADP^+$/NADPH were generated in DO-stat fermentation when compared to fed-batch fermentation. $NADP^+$/NADPH content was least at $24^{th}$ h. $NADP^+$/NADPH content was increased to 52.86 nM/mg protein at the $48^{th}$ h. $NADP^+$/NADPH content reached 101.36 nM/mg protein at the the $72^{nd}$ h during DO-stat fed-batch fermentation.

**4. Fermentation kinetics of YL-C11**

Establishing a preliminary kinetic model to investigate cell growth in all kinds of fermentation environments would be well utilized in directing scale production. Although the feed will slightly increase the volume of the fermentation broth, the volume of the fermentation broth was considered to be constant due to sampling in this study.

**4.1Kinetics of cell growth**

Figure 5 shows the time courses of biomass separately derived from the model equations and the experiments. Figures 5a and 5b show the fitting results from the fed-batch fermentation and DO-stat fed-batch fermentation, respectively. The cell growth showed an "S" pattern. The cell growth curve of YL-C11 was consistent with the logistic equation, which was presented as follows:

$\frac{\mathrm{dX}}{\mathrm{dt}} = \mu_m(1 - \frac{X}{X_m})X$ (1)

Where X is the biomass concentration (g/L), t is fermentation time (h), dX/dt is cell growth rate [g/(L h)]. $\mu_{max}(h^{-1})$ is maximum specific growth rate, and $X_m$ is the maximum microorganism concentration (g/L).

The simulated kinetic parameters were listed in Table 1. The models fit the experimental data well with $R^2$ values of 0.9315 for fed-batch fermentation and 0.9526 for DO-stat fed-batch fermentation, indicating the adequacy of the logistic equation model to fit experimental data. The $\mu_m$ value in DO-stat fed-batch fermentation is 0.49, which is higher than that in fed-batch fermentation (0.113). This result suggests that DO-stat fed-batch fermentation resulted in a higher cell growth rate, thus, more biomass was obtained.

### 4.2 Κινετιςς οφ β-ςαροτενε σψντηεσις

There are three fermentation forms: I, growth-related type; II, growth part-related type; III, non-growth-related type. In general, the formation of weak organic acids, such as citric acid, lactic acid, and succinic acid, has been well simulated by the Luedeking-Piret model that consists of a growth-associated part and non-growth-associated part. In this case, we employed this model to describe the kinetics of β-carotene production.

$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X (2)$

Where P is the product concentration, X (g/L) is the concentration of cells, t is the fermentation time, α and β are the coefficients. α (g/g) denotes the parameters for product formation constant (associated with the cell growth rate) and β (g/g) denotes the parameters for product formation constant (related to the number of cells). As for three fermentation types: I: α [?] 0, β = 0; II: α [?] 0, β [?] 0; III: α = 0, β [?] 0.

Figure 6 depicts the time courses of β-carotene content separately derived from the model equations and the experiments. Figure 6a and Figure 6b show the fitting results for the fed-batch fermentation and DO-stat fed-batch fermentation, respectively. β-carotene synthesis of engineered *Y. lypolitica* belongs to a partial coupling with cell growth (fermentation type II) because the rate of product formation is related to both the growth rate and the number of cells. A mathematical model was proposed by Luedeking and Piret, which properly described the mechanism of β-carotene synthesis.

The simulated kinetic parameters were listed in Table 1. It shows that a relatively accurate result could be observed between experimental and simulation data. The value of α was $4.74\times10^{-4}$ for fed-batch fermentation and -0.6 for DO-stat fed-batch fermentation. The value of β was $-2.86\times10^{-6}$ for fed-batch fermentation and 0.05 for DO-stat fed-batch fermentation.

A higher α value was obtained for the fed-batch fermentation, indicating the β-carotene synthesis was mainly affected by cell growth rate at fed-batch fermentation. A higher β value was obtained for DO-stat fed-batch fermentation, which indicates the β-carotene synthesis was affected mainly by the number of cells during DO-stat fed-batch fermentation.

### 4.3 Kineticsof glucose consumption

Figure 7a shows the nonlinear fitting of experimental data from DO-stat fed-batch fermentation substrate consumption. The classical kinetic model suggested by Luedeking and Piret was chosen to describe the substrate consumption as follows:

$-\frac{dS}{dt} = \frac{1}{Y_{\frac{X}{S}}} \frac{dX}{dt} + \frac{1}{Y_{\frac{P}{S}}} \frac{dP}{dt} + mX (3)$

Where $Y_{x/s}$ (g/g) and $Y_{p/s}$ (g/g) represent the substrate yield for biomass and product, respectively, and m represents the maintenance coefficient. The whole formula (3) represents glucose used to generate biomass, product, and cellular maintenance energy. The simulated kinetic parameters were listed in Table 1. The equation fits the experimental data from the consumption of the substrate with an $R^2$ of 0.9558. The $Y_{x/s}$ and $Y_{p/s}$ are 0.22 and 0.14, respectively. It is difficult to fit the data of glucose consumption during fed-batch fermentation because the change in glucose concentration was small. The $Y_{x/s}$is 0.06 in fed-batch fermentation that directly calculated by $Y_{x/s}$= [?]X/[?]S. The $Y_{p/s}$ is $2.5\times10^{-4}$ in fed-batch fermentation solved by $Y_{p/s}$= [?]P/[?]S. It is evident that the parameter $Y_{x/s}$ and $Y_{p/s}$ are higher for DO-stat fed-batch. It demonstrates that DO-stat fed-batch fermentation led to higher biomass and product yield for the substrate.

Figure 7b depicts the glucose consumption in different growth periods at fed-batch fermentation and DO-stat fed-batch fermentation. We divided the glucose consumption into three phases: the lag phase, the logarithmic phase, and the stationary phase. In the lag phase, 1.5 g of glucose were consumed in one liter of fermentation broth at both fermentations. In the logarithmic phase, one liter of fermentation broth consumed 123.5 g and 262 g of glucose for the fed-batch fermentation and the DO-stat fed-batch fermentation, respectively. In the stationary phase, 175 g and 74 g glucose were consumed in one liter of fermentation broth for the fed-batch fermentation and the DO-stat fed-batch fermentation, respectively. These results clearly show that more glucose consumption was achieved in the logarithmic phase when DO-stat fed-batch fermentation strategy was implemented.

# Discussion

Oxygen plays a pivotal role in aerobic fermentation. Aerobic microorganisms generally require large amounts of oxygen to generate NAD(P)H or $FADH_2$ and ATP for metabolism. Several studies also showed that the dissolved oxygen levels directly affect the transcription level of genes and synthesis of different enzymes and results in the changes of cell metabolism, product yield and productivity (Song et al., 2013) (Martinez, Bennett, & San, 2010).

The DO level was maintained at 10%-20% during DO-stat fed-batch fermentation in this study, and the DO level was significantly higher than that of fed-batch fermentation. We found that different dissolved oxygen level affects the metabolism of YL-C11. Low DO resulted in less ATP and the reducing power [NAD(P)H] that required for cell maintenance and growth in fed-batch fermentation. Using the DO-stat method revealed that biomass was enhanced than that of in fed-batch fermentation. The glycolytic, hexose monophosphate, and tricarboxylic acid cycle (TCA cycle) pathways not only are used as the hub to convert carbohydrates, proteins, and lipids but are the essential pathways to produce energy during the metabolism. In DO-stat fed-batch fermentation, the above three pathways were strengthened with adequate oxygen delivery. The oxidative phosphorylation and substrate-level phosphorylation was enhanced simultaneously. We set the oxygen level at 10%-20% for DO-stat fed-batch fermentation. That lead to more ATP and $NADP^+$/DADPH were generated, thereby resulting in the boosted of biomass and β-carotene concentration. Several studies have confirmed that high oxygen levels could improve the metabolic flux of aerobic microorganisms; thus, more biomass and target product was obtained. (Kawaguchi et al., 2019b) (Xu et al., 2009) (Song et al., 2013). Moreover, the transcriptional level of related genes in the β-carotene biosynthesis pathway was higher in DO-stat fed-batch fermentation (Supporting Information, Figure 1).

The main aim of the present investigation was to achieve high cell concentration, thus increase β-carotene concentration. A series of the kinetic models were constructed to elaborate on the relationship among cell growth, substrates and products. The kinetic parameters were determined from different feeding strategies in this study. These simulations provided an insight into the operational protocol that may be implemented to obtain the best results.

The fed-batch fermentation produced of 73.5 g/L cell biomass and productivity of 0.61 g/L/h. In contrast, The DO-stat fed-batch fermentation produced 94g/L cell biomass and productivity of 0.78 g/L/h. The fermentative capacity was strongly affected by the specific growth rate of aerobic cultures (PIM VAN HOEK, 1998). The simulation results showed that the higher $\mu_m$, $Y_{x/s}$, and $Y_{p/s}$ values were achieved for DO-stat fed-batch fermentation. In addition, the DO-stat feeding strategy extended the logarithmic period, and more glucose was consumed in this period. As a result, more biomass was obtained for the DO-stat fed-batch fermentation. The improved growth can be attributed to that the Crabtree effect was blocked with DO-stat feeding strategy. The Crabtree effects are prevented from occurring during DO-stat fed-batch fermentation, which is one of the critical reasons that longer logarithmic phase, higher biomass, and glucose utilization were obtained. Thus, DO-stat fed-batch fermentation is an excellent approach to produce β-carotene. Although further research is needed to improve this kinetic model further, we could use this kinetic model to understand

the relationship between cell growth, substrate consumption, and product synthesis for different feeding strategies.

## Conclusion

Oxygen plays an important role in aerobic fermentation. Adequate oxygen allows aerobic microorganisms to have good conditions for its growth. DO-stat fed-batch fermentation was successfully applied to increase dissolved oxygen in broth and the volumetric productivity of β-carotene. The kinetic model could make a significant contribution to the industrialized β-carotene production.

## Acknowledgement

This research was supported by the National Natural Science Foundation (31972089) and National Key Research and Development Program of China (2017YFD0400702).

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# Tables

Table 1. Kinetic parameters of cell growth, β-carotene production, and glucose consumption

| | Parameters of cell growth | Parameters of cell growth | Parameters of cell growth | Para |
|---|---|---|---|---|
| | $X_m$ | $\mu_m$ | $R^2$ | α |
| Fed-batch fermentation | 71.18 | 0.113 | 0.9315 | 4.74 |
| DO-stat fed-batch fermentation | 94.73 | 0.49 | 0.9526 | -0.60 |

# Figure legends

Figure 1. The time-course of dry cell weight, dissolved oxygen, glucose concentration, and β-carotene concentration during fed-batch fermentation for YL-C11. The glucose concentration was fed at around 5 g/L.

Figure 2. The time-course of dry cell weight, dissolved oxygen, glucose concentration, and β-carotene concentration during DO-stat fed-batch fermentation for YL-C11. The DO-stat fed-batch induction was initiated at 22 h, and the DO was controlled around 10-20%.

Figure 3. More ATP was determined in DO-stat fed-batch fermentation. The ATP of both fermentations was extracted and assayed, as described in the text. Error bars indicate ±SD from at least duplicate. (* denotes $p < 0.05$) (ANOVA).

Figure 4. More $NADP^+$/NADPH was determined in DO-stat fed-batch fermentation. The $NADP^+$/NADPH of both fermentations was extracted and assayed, as described in the text. Error bars indicate ±SD from at least duplicate. (* denotes denotes $p < 0.05$) (ANOVA).

Figure 5. Simulations of kinetic models and experimental results of biomass at fed-batch and DO-stat fed-batch fermentation (a: fed-batch fermentation; b: DO-stat fed-batch fermentation; Solid line represents fitted data and circles represent experimental data).

Figure 6. Simulations of kinetic models and experimental results β-carotene synthesis at fed-batch and DO-stat fed-batch fermentation (a: fed-batch fermentation; b: DO-stat fed-batch fermentation; Solid line represents fitted data and circles represent experimental data).

Figure 7. Simulation of kinetic models and experimental results of glucose concentration (a) (Solid line represents fitted data and circles represent experimental data) and glucose consumption in different periods (b).

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