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RESEARCH PAPER

Oxygen Uptake Rate Controlling Strategy Balanced with Oxygen Supply for Improving Coenzyme Q_{10} Production by *Rhodobacter sphaeroides*

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Abstract The effects of different oxygen uptake rates (OUR) on the physiological metabolism of *Rhodobacter sphaeroides* were investigated systematically in 50 L fermenters due to the significant influence on industrial coenzyme Q_{10} production under oxygen supply limitation. Meanwhile, the seriously decreased oxygen transfer rate caused by the increased broth viscosity was successfully prevented with OUR-directed continuous ammonium sulfate feeding in the late fermentation phase. The statistical analysis results showed that controlling OUR constantly at 45 ± 2.2 mmol/L/h by the oxygen supply level adjustment and the continuous ammonium sulfate feeding could greatly enhance Q_{10} production. This OUR-Stat controlling strategy successfully achieved the maximal coenzyme Q_{10} production (2584 ± 82 mg/L), which was 15.4% higher than that of the control. The highest specific CoQ_{10} content was 25.9 mg/(g DCW)), and the yield of CoQ_{10} to glucose consumption was up to 19.37 mg/g. These results demonstrated that the optimal OUR-Stat controlling strategy would be effective and economical for improving the industrial CoQ_{10} production.

Keywords: coenzyme Q_{10}, OUR controlling strategy, *Rhodobacter sphaeroides*, oxygen supply, balance

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1. Introduction

Coenzyme Q_{10} (CoQ_{10}, 2,3-dimethoxy-5-methyl-benzoquinone with a side chain of ten monosaturated isoprenoid units), is an obligatory cofactor in the aerobic respiratory electron transfer system for energy generation in the plasma membrane of prokaryotes and the inner mitochondrial membrane of eukaryotes [1,2]. It plays a key role in the electron transfer system as the electron donor/acceptor between complex I/II and complex III [3,4]. As the important ubiquinone homolog found in human organs, it can boost energy, enhance immune system, and act as an antioxidant [5-7]. Therefore, CoQ_{10} is beneficial in treating human conditions, such as cardiomyopathy, diabetes, Parkinson's and Alzheimer's disease [8], and it can also reduce the risks of myopathy associated with statin drugs [9,10]. Recently, CoQ_{10} has been widely used for pharmaceuticals, cosmetics, food supplements, *etc.* because of its various physiological activities [11]. Additionally, it has received much attention due to the increasing need in the world market [12,13].

For the high cost of chemical synthesis owing to the complicated structure, nowadays, the commercial production of CoQ_{10} biologically synthesized from microorganisms has attracted the increasing attention [14]. Numerous natural strains, such as *Agrobacterium tumefaciens, Paracoccus denitrificans, Cryptococcus laurentii, Trichosporon sp., Sporobolomyces salmonicolor, and Rhodobacter sphaeroides*, were screened and used for CoQ_{10} production [15,16]. Meanwhile, construction of genetically engineered microorganism and metabolic modification have been implemented for improving CoQ_{10} production [3]. *R. sphaeroides* is widely used in CoQ_{10} production because of its high biosynthesis ability [17]. *R. sphaeroides* is a photosynthetic bacterium

Table 1. CoQ_{10} production in different strain types from the previous reports

Strain	CoQ_{10} (mg/L)	Qp (mg/(g DCW))	Strain type	Reference
Paracoccus denitrificans ATCC 19367	27.6	0.86	Wild type	Choi *et al.* 2007 [1]
Agrobacterium tumefaciens ATTC4452	87.6	1.9	Wild type	Ha *et al.* 2007 [49]
Spingomonas sp. ZUTEO3	441.6	---	Wild type	Qiu *et al.* 2012 [16]
Rhodobacter sphaeroides Co-22-11	347.0	2.5	Chemical mutants	Choi *et al.* 2005 [18]
R. sphaeroides KY8598	770.0	8.7	Chemical mutants	Choi *et al.* 2005 [18]
R. sphaeroides	350.0	8.7	Chemical mutants	Jeya *et al.* 2010 [51]
R. sphaeroides RspSRIDL	93.0	7.2	Recombinant mutants	Lu *et al.* 2015 [19]
R. sphaeroides	600.0	8.6	Recombinant mutants	Zhu *et al.* 2017 [20]
A. tumefaciens KCCM10413	638.0	9.7	Recombinant mutants	Ha *et al.* 2009 [34]
R. sphaeroides RspMQd	138.5	12.9	Recombinant mutants	Lu *et al.* 2014 [48]
Escherichia coli GD-51	433.0	11.7	Recombinant mutants	Dai *et al.* 2015 [15]

Qp: specific CoQ_{10} content.

selected from the mutant strains based on the color change, which indicated a reduction in the carotenoid production by a lighter color [18]. Lots of researches were focused on the balance of metabolic flux pathway to increase the production, and a recent study reported the production was as high as 12.9 mg/(g DCW) (Table 1) [19]. Although many researchers put more efforts to increase the 2-methyl-D-erythritol-4-phosphatepathway(MEP) pathway flux, almost no positive result was achieved on increasing CoQ_{10} production by the industrial strains, which was probably due to the accumulation of toxic intermediates [20].

However, further improvements were achieved in this producing-strain by the chemical mutagenesis and radiation mutagenesis combined with several indirect phenotypes screening [21]. Meanwhile, the process controlling technology also played significant roles in improving the industrial CoQ_{10} production [17]. Many researches have revealed that oxygen supply levels have a critical effect on CoQ_{10} biosynthesis. Kien *et al.* enhanced the production through aeration shift from the adequate aeration to limited aeration in active cellular metabolism at the early growth phase [22]. Skate *et al.* investigated the agitation-aeration conditions optimization directed by the oxidation-reduction potential for CoQ_{10} production under limited oxygen supply [23].

All these results demonstrated that the suitable oxygen limitation and the redox respiration-chain state have a close correlation with CoQ_{10} biosynthesis. Moreover, the defection of genes *sdhB* (encoding the catalytic subunit of redox respiration chain components) could significantly promote CoQ_{10} content especially under low oxygen supply conditions, while the cell growth would be greatly inhibited. All these results confirm that it is a perplexing and challengeable problem in controlling the oxygen supply level during CoQ_{10} fermentation process [24,25].

As for controlling the oxygen supply level in the aerobic fermentation, it is well known that the dissolved oxygen tension and oxidation-reduction potential play a significant role in the limited aerobic fermentation [26,27]. However, the dissolved oxygen tension reflected the balance state between the oxygen transfer rate and the oxygen uptake rate of microorganisms in the fermentation broth [24], but it could hardly reflect the oxygen supply capacity and the physiological metabolism when process conducted the oxygen limit conditions [28]. Oxygen uptake rate (OUR) is one of the pivotal physiological parameters, which correlated with other parameters such as carbon dioxide elution rate (CER), respiratory quotient (RQ), and dissolved oxygen tension (DO), *etc*.. And it has been proved to be an effective and convenient on-line controlling parameter for aerobic fermentation process [29,30], especially under the oxygen limited conditions [31]. OUR was affected by not only agitation, aeration, and pressure, but also the nutrient condition, broth viscosity, and cell physiological metabolic state [32]. Therefore, to better identify the process-specific factors and understand the physiological responses to the physical conditions, the mutual influences and interactions of the various physical and physiological parameters need to be analyzed in detail [33].

In large-scale CoQ_{10} fermentation process, the increased broth viscosity always appears in the late phase with the increased cell density and the exopolysaccharide. These factors seriously affect the mass transfer and the oxygen transfer rate, and thus inhibit the cell's respiratory metabolic activity. Ha *et al.* [34] revealed that the exopolysaccharide accumulation could be inhibited by the maintained sucrose concentration at a lower level during the fed-batch fermentation by *A. tumefaciens*. However, in the industrial fermentation by *R. sphaeroides* with glucose as the sole carbon source and maintained at a lower concentration, the exopolysaccharide could also accumulate at the late fermentation phase, and greatly repress OUR and CER of *R. sphaeroides*. There must be another main carbon flux to

stimulate the exopolysaccharide biosynthesis. Therefore, the reasons and solutions for reducing the increased broth viscosity remain to be elucidated.

The objective of this study was to enhance CoQ_{10} production by optimizing the oxygen supply levels and ammonium sulfate feeding. To this aim, the physiological characteristics of *R. sphaeroides* during CoQ_{10} fermentation was investigated in a 50 L fermenter with a multi-parameter monitoring system [29]. Additionally, the effects of OUR levels on the metabolic state and the exopolysaccharide accumulation directed by the nitrogen deficiency were also investigated.

2. Methods

2.1. Microorganism and culture conditions

The industrial strain was screened and obtained by N-methyl-N′-nitro-N- nitrosoguanidine mutagenesis of wild-type *R. sphaeroides* (ATCC BAA-808) [35].

The seed medium was the following (g/L): glucose 10.00, yeast extract 1.00, NaCl 1.00, ferric citrate 0.003, K_2HPO_4 0.90, KH_2PO_4 0.60, NH_4Cl 1.00, $MgSO_4$ 0.10, and pH 7.0.

The formula of the fermentation medium was as follows (g/L): glucose 20.00, yeast extract 8.00, NaCl 1.00, corn steep powder 14.20, ferric citrate 0.003, $CaCl_2$ 0.10, $MgSO_4$ 0.10, and pH 7.0. Feed medium (FM) for the fed-batch fermentation contained 300 g glucose per liter, the pH was controlled at 6.9 ± 0.1 with NaOH. The phosphate ion concentration was controlled at 150 mg/L by addition of 10% potassium dihydrogen phosphate solution during the fermentation process.

2.2. Culture in 50 L fermenter

Seed culture was grown at 32°C in 500-mL Erlenmeyer flasks containing 100 mL of seed medium inoculated with cells from fresh slant with shaking at 260 rpm for 28 h. 1.5 L of the harvested seed culture was then inoculated into the 50 L turbine-agitated bioreactor (Shanghai Guoqiang Inc., China) with a working volume of 30 L. It was also equipped with devices to monitor many on-line measurable parameters [29]. The aeration rate and pressure were kept at 0.8 vvm and 0.05 MPa throughout the experiments, respectively. The residual glucose concentration was kept at 5.0 ± 0.1 g/L by continuously feeding the feed mediums during the fermentation process. To investigate the effects of different oxygen supply levels on CoQ_{10} production, the agitation speed was controlled at 340, 450, 550, and 640 rpm, respectively.

2.3. OUR-stat directed ammonium sulfate feeding strategy

OUR-stat directed scale up strategy with consecutive feeding ammonium sulfate was implemented in a 50 L bioreactor. The agitation speed, aeration rate, pressure, and temperature was set at 550 rpm, 1.0 vvm, 0.05MPa and 32°C throughout the experiments. The optimal OUR was controlled at 45 ± 2.6 mmol/L/h online by adjusting the ammonium sulfate solution feeding rate at the late phase after 70h.

2.4. Determination of dry cell weight (DCW) and on-line capacitance

To determine the dry cell weight (DCW), 10 mL of sample was centrifuged at 4000 g for 10 min, and then the cell cluster was washed twice with water and dried to a constant weight for 24 h at 105°C. The viable cell density sensor (Hamilton Company, Switzerland) was used to measure the broth capacitance on-line. In this study, the selected frequency was 680 kHz and the low pass was 10, thus the correct and flat capacitance curve would be observed [36].

2.5. Determination of total sugar, exopolysaccharide, and ammonium ion concentration

The residual sugar concentration was measured with the anthrone method [33]. To determine the amount of exopolysaccharide, 30 mL of broth mixed with 1 M NaOH solution for 20 min, and then centrifuged at 4,000 g for 15 min. Then, the supernatant was hydrolyzed under 6 M HCl solution at 100°C for 18 h in a sealed glass vial. The hydrolyzed EPS was dried using a speed vacuum concentrator and analyzed with HPLC [34]. The results showed that glucose was the main component in the hydrolysate component, demonstrating that the viscosity cohesive material was an exopolysaccharide. The exopolysaccharide concentration was determined and quantified by determining the total residual sugar in treated solution. All data given below were the mean values based on triplicate experiments. The concentrations of ammonium-nitrogen (NH_2-N) in broths were quantified by the method described by Kanda [37], in which using o-phenylphenol as a substitute for liquid phenol.

2.6. Detection of coenzyme Q_{10} concentration

To analyze the titers of $CoQ_{10,}$ 1 mL of broth was added into 5 mL of methanol solution. After vortexing vigorously, the mixture was sonicated for 20 min. Cell debris was removed by centrifugation and 20 μL of supernatant solution was used for HPLC detection (Agilent model 1100 instrument equipped with a Waters C_{18} column) and a refractive index detector (Agilent). A solvent mixture of ethanol and methanol (9:1, v/v) was used as the mobile phase at a flow rate of 0.6 mL/min [3].

2.7. Measurement of organic acid and amino acids

To analyze the intracellular organic acids and amino acids of metabolism, the quick filtration and washing were

carried out for collecting the biomass from 3 mL of broth, which was then frozen in liquid nitrogen. The cells were sonicated to extract the intracellular metabolites. HPLC system (Agilent 1100, USA) and the reference method were used for organic acid and amino acids determination [38], all the off-line data were measured with three parallels.

2.8. Determination of oxygen uptake rate

OUR was determined from the exhaust gas analysis, the mass fractions of oxygen and carbon dioxide in the inlet and the outlet flows [39]. By using these quantities and a nitrogen (inert) balance, OUR and CER were determined from the following balance Eq. (1):

$$OUR = \frac{F_{in}}{V}\left[C_{O_2 in} - \frac{C_{inert in}\cdot C_{O_2 in}}{1-(C_{O_2 out}+C_{CO_2 out})}\right]\cdot\frac{273}{273+t_{in}}\cdot P_{in}\cdot\frac{1}{1+h}\times 10^{-5} \quad (1)$$

Where F_{in} is the aeration rate, V is broth volume; $C_{inert\ in}$\ $C_{O_2\ in}$\$C_{CO_2\ in}$ are input mass fraction of nitrogen, oxygen and carbon dioxide, $C_{O_2\ out}$\$C_{CO_2\ out}$ are output mass fraction of nitrogen, oxygen and carbon dioxide, P_{in} is the intensity of pressure of input gas, t_{in} is the temperature of input gas, h is the humidity of input gas.

3. Results and Discussion

3.1. Physiological parameters acquisition and process analysis

Fig. 1 illustrates the profiles of OUR, DO, and DCW during CoQ_{10} fed-batch fermentation process. With the cells growth and OUR increase, the dissolved oxygen limitations encountered at about 22-26 h, and DO kept constant afterwards at nearly zero level. Even with the alterations of the oxygen supply conditions by manipulating agitation speed, DO had almost no change, while OUR was increased with the improved oxygen supply. OUR was increased to 60-64 mmol/L/h and kept constant with the agitation speed and aeration rate were kept at 600 rpm and 2,500 L/h, respectively, during the former CoQ_{10} cultivation stage until 73 h. OUR and CER greatly decreased from 60 mmol/L/h to 20 mmol/L/h at the late stage of the fermentation even with the constant agitation and aeration conditions. In this phase, CoQ_{10} biosynthesis rate was significant inhibited, and DO was still kept constantly at zero level. Meanwhile, the broth viscosity increased seriously during this period.

It's well known that DO reflects the dynamic balance between OTR and OUR [40]. From the mass balance, the dissolved oxygen concentration changes in batch fermentation could be established by Eq. (2) [41]:

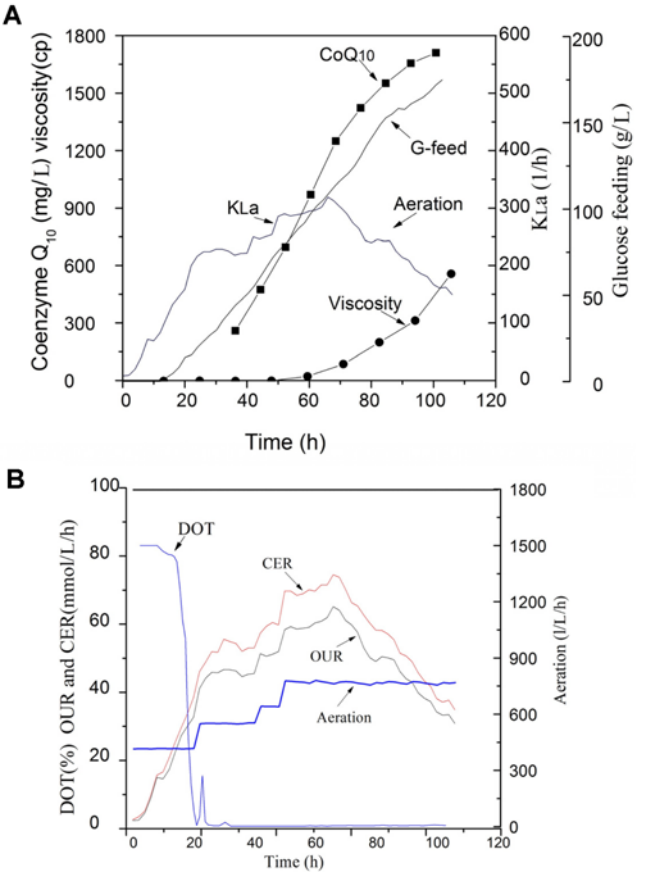

Fig. 1. Time course of CoQ_{10}, oxygen uptake rate (OUR), carbon dioxide evolution rate (CER), agitation speed (rpm), dissolved oxygen (DO), oxygen transfer coefficient (KLa), and glucose consumption (G-feed), and broth viscosity during the ordinary 50 L fermentation by *Rhodobacter sphaeroides*.

$$\frac{dC}{dt} = OTR - OUR = K_L a(C^* - C) - OUR = K_L a(C^* - C) - Qo_2 \times X \quad (2)$$

Where KLa (C*-C) and ($Qo_2 \times X$) represent OTR and OUR, respectively. KLa is the specific gas-liquid mass transfer coefficient for oxygen, C^* is the oxygen saturation concentration.

During CoQ_{10} fermentation process, DO had almost no change with the altering of oxygen supply levels; therefore, an acceptable pseudo steady state approximation could be simply considered dC/dt ≈ 0. Hence, it could draw the conclusion from the Eq. (2) that OUR ≈ OTR. Therefore, OUR can substitute OTR as an online monitor parameter to control the oxygen supply levels in the fermentation process.

It was validated that KLa in a bioreactor was mainly affected by agitation rate, aeration rate, viscosity coefficient, and so on of the broth [42]. The increased viscosity seriously inhibited the oxygen transfer rate in broth, which

significantly restricted OUR and CoQ_{10} biosynthesis in this period. Meanwhile, CoQ_{10} production rate greatly decreased with the increase of broth viscosity. Therefore, the control of oxygen supply levels on CoQ_{10} biosynthesis was crucial for the industrial CoQ_{10} fermentation.

3.2. Effect of oxygen supply levels on CoQ_{10} production

To better evaluate the effect of oxygen supply levels on CoQ_{10} production, different agitation speed were controlled at 340, 450, 550, and 640 rpm, respectively. The corresponding OUR levels were set at 30 ± 1.7 mmol/L/h (run-A), 40 ± 1.9 mmol/L/h (run-B), 45 ± 1.8 mmol/L/h (run-C), and 66 ± 2.3(run-D) mmol/L/h. As is well known, agitation rate always has a major effect on KLa of oxygen transfer in small-scale bioreactors [43]. The physiological characteristics of *R. sphaeroides* on CoQ_{10} biosynthesis were shown in Fig. 2. The results clearly revealed that cell growth, OUR, CoQ_{10} concentration, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone (demethoxyl-CoQ_{10}), and glucose consumption were significantly affected by oxygen supply levels.

With cell growth at the early phase before 30 h, OUR

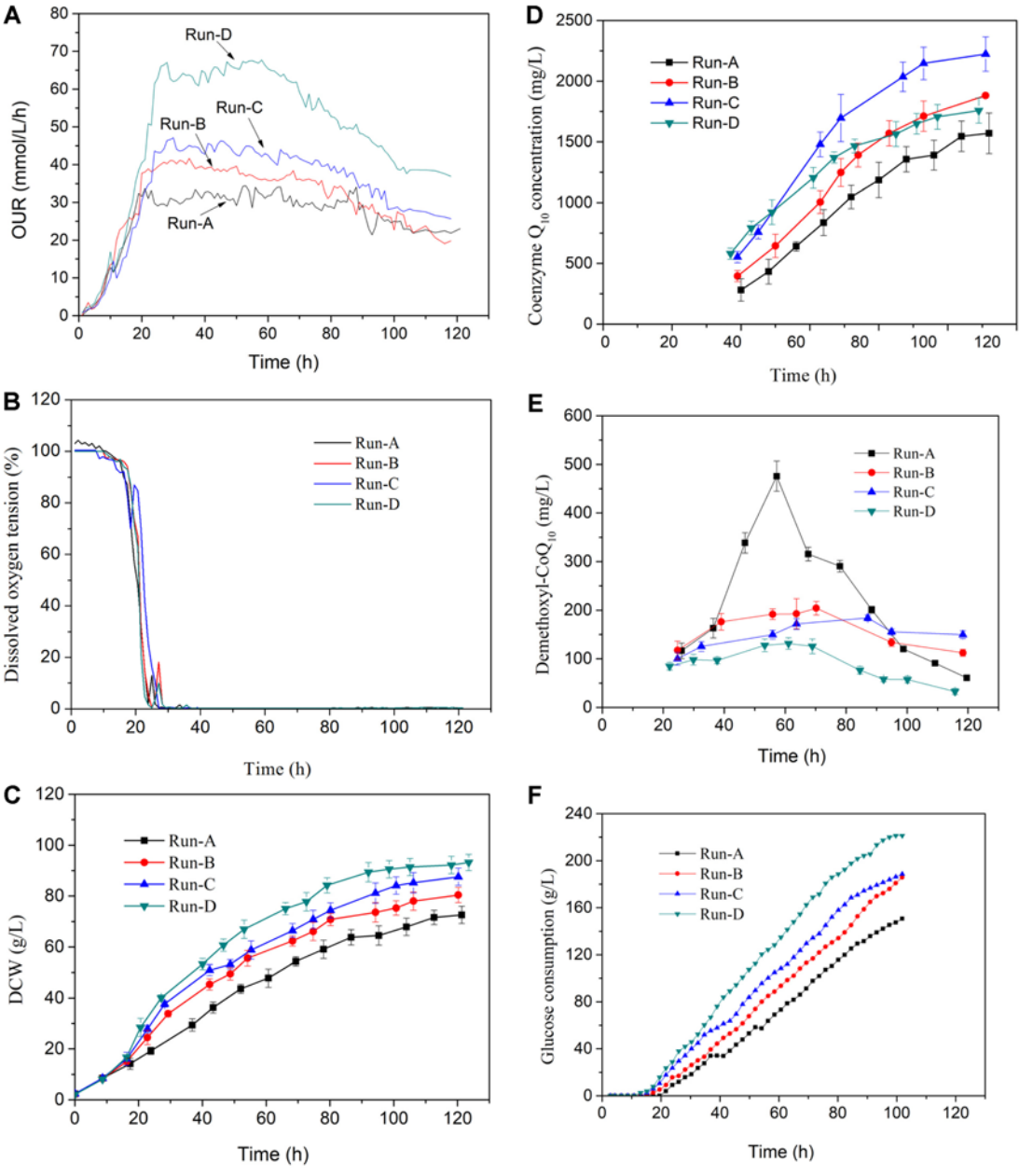

Fig. 2. Time course of physiological parameters of oxygen uptake rate (OUR) (A), dissolved oxygen tension (B), biomass (C), CoQ_{10} production (D), demethoxyl-CoQ_{10} production (E), and glucose consumption (F) in CoQ_{10} fermentation under different oxygen supply conditions at Run-A, 340 rpm (■), Run-B, 450 rpm (●), Run-C, 550 rpm (▲), Run-D, 640 rpm (▼).

Table 2. Effects of different oxygen supply levels on CoQ_{10} fed-batch fermentation in 50-L bioreactors for 121 h (the agitation was adjusted at Run A 340 rpm, Run B 450 rpm, Run C 550 rpm, and Run D 640 rpm, respectively. Each value represents the mean of triplicate measurements)

OUR strategies	Run-A	Run-B	Run-C	Run-D
OUR (mmol/L/h)	30 ± 2.1	40 ± 2.3	45 ± 1.8	66 ± 2.3
CER (mmol/L/h)	36 ± 1.9	44 ± 2.1	55 ± 2.9	78 ± 1.9
Productivity	1,581 ± 67	1,879 ± 25	2,224 ± 42	1,754 ± 54
Qp (mg/(g DCW))	19.8 ± 1.1	23.3 ± 0.9	25.7 ± 1.2	20.4 ± 1.3
$Y_{x/s}$ (g/g)	0.47 ± 0.05	0.46 ± 0.05	0.45 ± 0.03	0.42 ± 0.07
$Y_{p/s}$ (mg/g)	14.48 ± 0.4	15.49 ± 0.9	17.63 ± 0.8	11.99 ± 1.0
Y_{CO2}/glucose (mol/mol)	3.23 ± 0.21	3.60 ± 0.18	4.18 ± 0.16	4.64 ± 0.20

OUR: oxygen uptake rate
CER: carbon dioxide elution rate
Qp: specific CoQ_{10} content.
Yx/s: yield of biomass to glucose consumption.
Yp/s: yield of CoQ_{10} to glucose consumption.
Y_{CO2}/glucose: yield coefficient of carbon dioxide to glucose.

increased simultaneously until oxygen limit conditions encountered at different agitation speeds (Fig. 2A, B). Higher cell growth rate was obtained under higher oxygen supply levels, the specific growth rate under OUR at 66 ± 2.3 mmol/L/h was 1.2-folds higher than that with OUR at 35 ± 1.7 mmol/L/h, indicating that oxygen consumption rate was the decisive factor on cell's specific growth rate. However, the statistical analysis showed that the yield of biomass to glucose decreased with the elevated oxygen supply conditions (Table 2). CoQ_{10} biosynthesis rate was increased with the elevated OUR levels, and the fast CoQ_{10} biosynthesis rate was obtained before 55 h. But the highest CoQ_{10} concentration of 2,224 ± 42 mg/L was obtained when OUR was controlled at 45 ± 1.8 mmol/L/h, which was 40.6% and 26.8% higher than that with OUR levels controlled at 30 ± 1.7 and 66 ± 2.3 mmol/L/h, respectively (Fig. 2D). Furthermore, the statistical analysis also indicated that the increase of OUR from 30 ± 1.7 to 45 ± 1.8 mmol/L/h greatly accelerated the specific CoQ_{10} content (Qp, mg/(g DCW)) (Table 2). While OUR was elevated to the highest level of 66 ± 2.3 mmol/L/h, CoQ_{10} biosynthesis was remarkably inhibited and Qp decreased to 20.4 ± 1.3 mg/(g DCW), which was 21% lower than that of 45 ± 1.8 mmol/L/h OUR condition (25.7 ± 1.2 mg/(g DCW)). The highest respiration of microorganism always leads to the unconscionable and economic pathway on CoQ_{10} biosynthesis [1]. During the industrial CoQ_{10} fermentation, the power energy and glucose consumption were always the main cost in the economical production. The experiment data showed that the glucose consumption rate was greatly aggravated with the increased oxygen uptake rate (Fig. 2F). While the lowest yield of CoQ_{10} to glucose consumption ($Y_{p/s}$) was only 11.99 ± 1.0 mg/g glucose at the highest OUR conditions, which was 35.3% lower than that under OUR controlled at 45 ± 1.8 mmol/L/h (17.63 mg/g). The excessive oxygen supply levels might accelerate the respiratory metabolism, and more glucose was oxidized to carbon dioxide at the highest OUR conditions. These were also reflected from the yield coefficient of carbon dioxide to glucose ($Y_{CO_2/glucose}$), the $Y_{CO_2/glucose}$ was increased greatly with OUR levels elevation (Table 2). In a word, the excessive oxygen uptake rate caused a higher respiration and a lower CoQ_{10} productivity, resulting in the excessive utilization of the carbon source by direct oxidation.

In order to further investigate the effect of oxygen consumption rates on the physiological metabolism, the intracellular metabolites of the precursor demethoxyl-CoQ_{10}, intracellular amino acids and organic acids were determined with HPLC (Fig. 2E and Fig. 3). As is well known, demethoxyl-CoQ_{10} is a main precursor for CoQ_{10} biosynthesis, during the final steps catalyzed by oxido-reductase and methyltransferase [17]. Therefore, keeping enough oxygen uptake rate is significantly important for the oxidation step from demethoxyl-CoQ_{10} to CoQ_{10}. The demethoxyl-CoQ_{10} concentration were increased with the decreased oxygen supply conditions before 70 h (Fig. 2E), the highest demethoxyl-CoQ_{10} concentration reached 476 ± 31 mg/L under the lowest OUR level of 30 ± 1.7 mmol/L/h, which was 2 folds higher than that under a higher OUR level (45 ± 1.8 mmol/L/h). Furthermore, the demethoxyl-CoQ_{10} concentration decreased quickly under the lowest oxygen supply conditions after 70 h cultivation, which might be caused by suffering the extreme oxygen limitation. Additionally, the demethoxyl-CoQ_{10} concentration was maintained throughout the whole fermentation process under the agitation speed at 550 rpm in run-C, and a higher precursor concentration accelerated a higher biosynthesis rate of CoQ_{10} during the late fermentation phase.

The biosynthesis pathway of CoQ_{10} from glucose showed that phosphoenolpyruvic acid (PEP), chorismic acid (CA), p-hydroxybenzoic acid (PBA), and acetyl-CoA were the pivotal intermediate metabolites for CoQ_{10} biosynthesis [44].

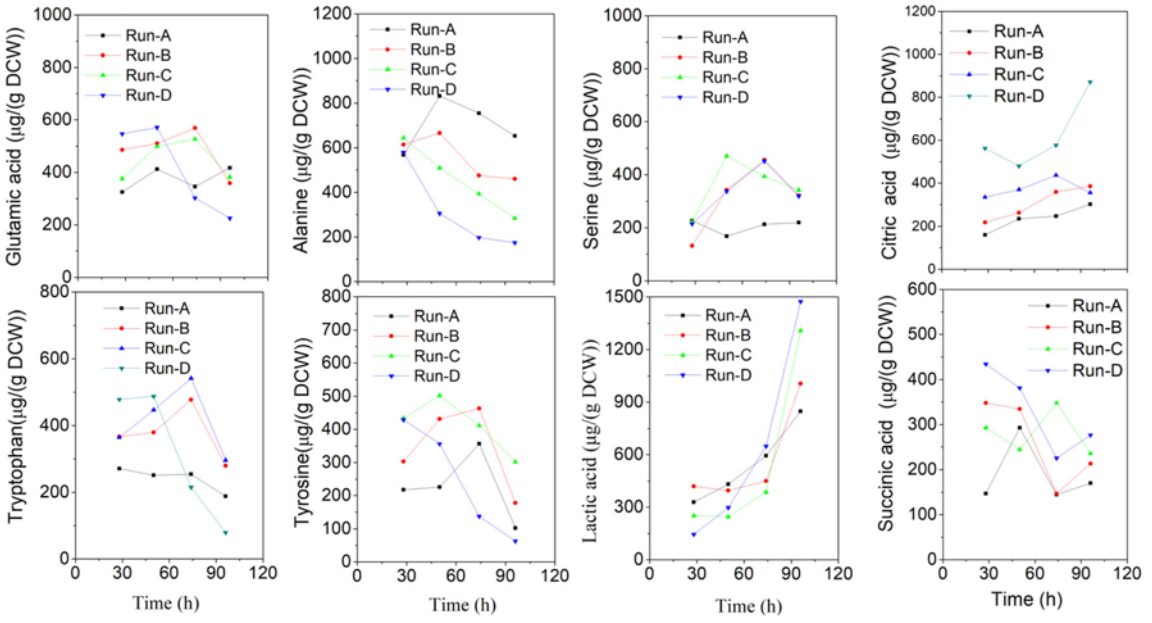

Fig. 3. Time course of intracellular metabolites in CoQ_{10} fermentation under different oxygen supply conditions at Run-A, 340 rpm (■), Run-B, 450 rpm (●), Run-C, 550 rpm (▲), Run-D, 640 rpm (▼).

The intracellular metabolite of amino acids and organic acids were determined and shown in Fig. 3, higher concentrations of tryptophan and tyrosine were greatly biosynthesized under higher OUR conditions at the former 70 h. As tryptophan and tyrosine were the main precursor for CA and PBA biosynthesis, they further accelerated CoQ_{10} production. However, the intracellular tryptophan and tyrosine concentrations decreased sharply with the decline of OUR value at every oxygen supply conditions after 70 h, which were consistent with the decreased CoQ_{10} production rate.

The decrease of OUR after 70 h greatly promoted the generation of intracellular lactic acid. At the end of the fermentation, the highest lactic acid concentration reached 1,489 μg/(g DCW) in the run-D with the highest oxygen supply condition, which was nearly 5 times higher than the lactic acid at 70 h. With the decrease of respiratory metabolism activity at the late fermentation phase, reduced nicotinamide adenine dinucleotide (NADH) would be accumulated and greatly inhibited the pathway from pyruvate to tricarboxylic acid cycle (TCA), and then seriously led to the flux from pyruvate to lactic acid.

Under the relative oxygen limitation, this phenomenon also appeared at the other oxygen supply conditions. The intracellular alanine concentration decreased with the elevate OUR levels, indicating that more phosphoenolpyruvate (PEP) was used for CoQ_{10} biosynthesis, rather than used for the further pyruvate and alanine generation. The serine and glutamate acid concentrations have almost no difference under the four oxygen supply levels. Higher intracellular concentrations of citrate acid and succinic acid at the late phase under higher oxygen supply condition of run-D really revealed a relatively more active flux in TCA cycle than that of the other three oxygen supply conditions. Higher OUR levels were in accordance with higher glucose consumption rates and yield coefficients of carbon dioxide to glucose ($Y_{CO_2/glucose}$) (Table 2). Therefore, controlling the oxygen uptake rate at appropriate levels is pivotal for higher relevant intermediates accumulation and CoQ_{10} biosynthesis.

3.3. Effects of ammonium sulfate supply on oxygen transfer rate and CoQ_{10} biosynthesis

In order to illustrate the reasons for high broth viscosity coefficient increased after 73 h, an identification method was used with extraction and hydrolysis analysis of the viscous byproduct in the broth. The results showed that exclusive glucose was detected after hydrolysis of the viscous materials, demonstrating that the generated viscous material was a polysaccharide. It was also proved to be soluble in the alkaline environment and insoluble under acidic conditions. The extracellular polysaccharide accumulation directly affected the oxygen transfer rate in broth, the power consumption on agitator and the effective utilization of glucose during CoQ_{10} biosynthesis. It's frequently reported that the polysaccharide biosynthesis was always affected by the high C/N ratio during the fermentation of microorganism, and the deficiency of nitrogen or the high carbon source consumption rate were always the reason to stimulate the polysaccharide production [45]. The accumulation of the internal keto acid and organic acid

concentrations generated from central carbon metabolic pathways would inhibit the glucose degradation and stimulate the polysaccharide biosynthesis [46,47]. In CoQ_{10} production by *R. sphaeroides*, a higher biomass and the enough ATP and NADH supply from glucose were all the significant factors affected CoQ_{10} biosynthesis rate.

Previous researches demonstrated that decreasing the sucrose concentration during the fed-batch fermentation was effective to inhibit polysaccharide production by *A. tumefaciens* KCCM 10413. But in industrial CoQ_{10} fed-batch fermentation by *R. sphaeroides*, glucose was used as the sole carbon source and maintained at a lower concentration. The unbalanced C/N ratio under limited nitrogen and higher glucose consumption rate would be the reason for stimulating the polysaccharide accumulation.

Therefore, the effects of different ammonium sulfate concentrations on the byproduct polysaccharide biosynthesis were implemented in shaking flasks. The results clearly showed that the highest polysaccharide concentration reached 16.2 g/L in the absence of ammonium sulfate (Fig. 4A), leading to the highest broth viscosity and serious inhibition on CoQ_{10} biosynthesis. While the polysaccharide concentration greatly decreased to 1.2 g/L when the ammonium sulfate concentration was increased to 6 g/L, and the broth viscosity was greatly lower than that without adding ammonium sulfate. The lowest CoQ_{10} production was only 29 mg/L without adding ammonium sulfate in the medium, which was significantly lower than that (542 mg/L) under 7 g/L ammonium sulfate addition (Fig. 4B). It seemed that feeding ammonium sulfate could prevent the extracellular polysaccharide biosynthesis and enhance CoQ_{10} biosynthesis.

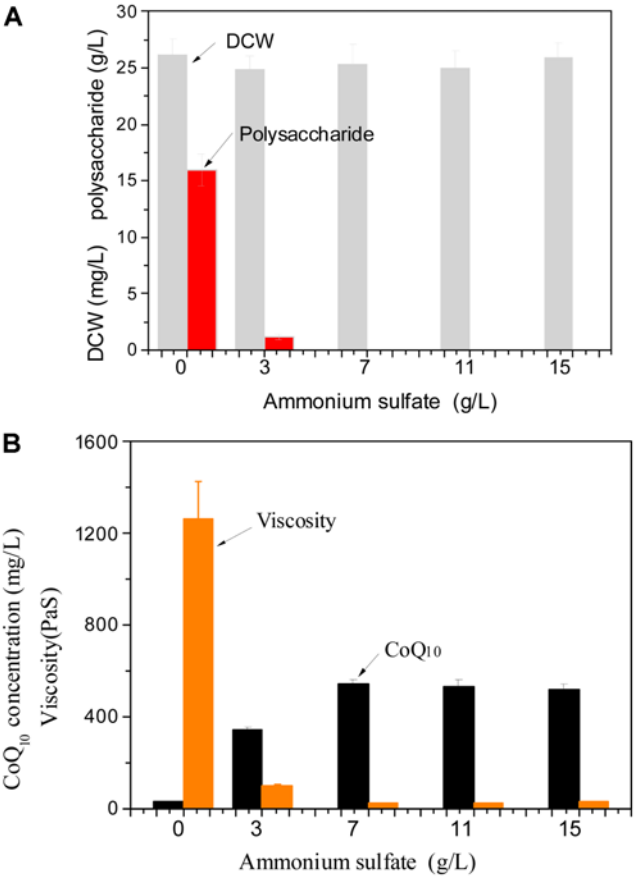

Fig. 4. Time effects of ammonium sulfate concentrations on the biomass, viscosity, and extracellular polysaccharide and CoQ_{10} concentration in the shaking flask fermentation.

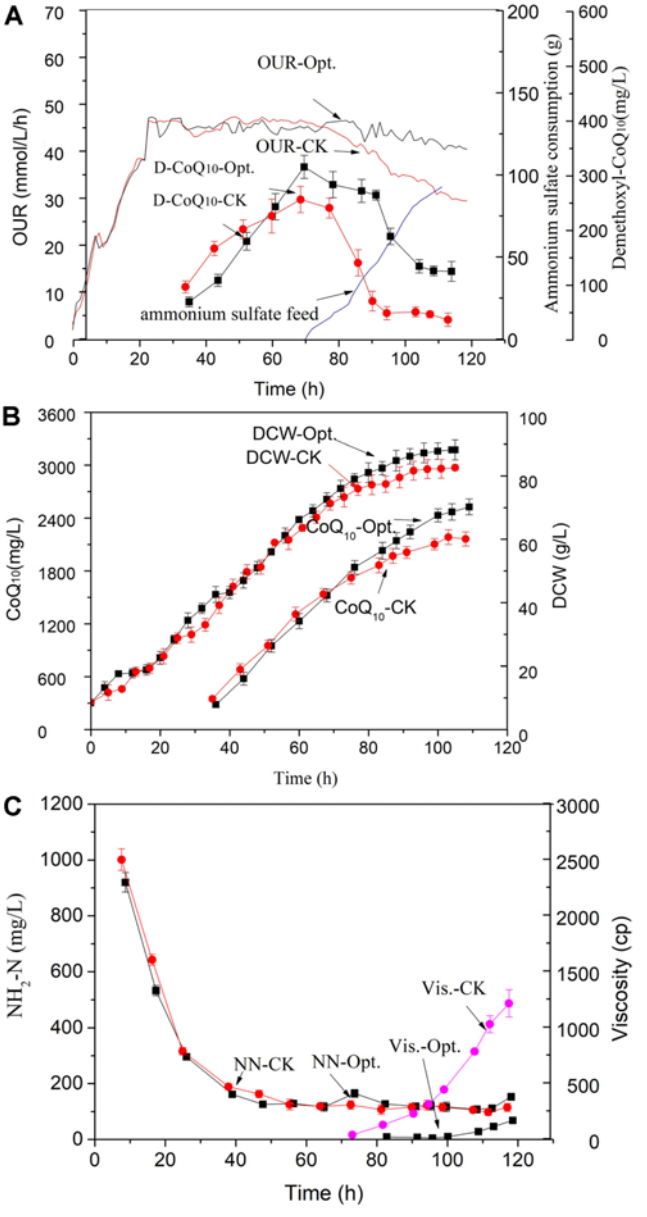

Fig. 5. OUR-Stat directed ammonium sulfate feeding strategy at the late stage of CoQ_{10} fermentation in a 50-L fermenter. (A) Time course of oxygen uptake rate (OUR), demethoxyl-CoQ_{10} and ammonium sulfate feeding. (B) Time course of biomass by dry cell weight (DCW) and CoQ_{10} concentration. (C) Time course of NH_2-N and broth viscosity. Optimal OUR-Stat strategy (Opt.: ■) and control (CK: ●).

3.4. OUR-stat control by balancing the oxygen transfer rate through the ammonium feeding strategy in a 50-L fermenter

Based on the aforementationed results, a new OUR-stat directed strategy with consecutive feeding ammonium sulfate was successfully established in the bioreactor. The optimal OUR was controlled at 45 ± 2.6 mmol/L/h online by adjusting the ammonium sulfate solution feeding rate at the agitation speed of 550 rpm and aeration rate of 1.0 vvm. As suggested in Fig. 5, with ammonium sulfate feeding rate at 130 ± 12 mg/L/h, OUR level could be maintained at nearly the similar level as the former phase during the late phase (Fig. 5A). The lowest broth viscosity no more than 230 cp revealed that the extracellular polysaccharide biosynthesis was dramatically suppressed (Fig. 5C). The improved OUR prevented the decline biosynthesis of precursor demethoxyl-CoQ_{10} (Fig. 5A), and a higher CoQ_{10} production rate was achieved under ammonium sulfate addition strategy. The maximal CoQ_{10} production reached 2,584 ± 82 mg/L with the optimal ammonium sulfate feed strategy, which was 15.4% higher than that of the control (2,239 ± 53 mg/L).

CoQ_{10} is an essential lipid-soluble component of the membrane-bound electron transport chains, and the accumulation of CoQ_{10} is mostly distributed in the membrane [44]. The increased amount of membrane was most important for containing a higher CoQ_{10} concentration. Therefore, keeping appropriate specific growth rate is of great importance for enhancing CoQ_{10} production [48]. The kinetics of NH_2-N concentrations were similar under the two controlling strategies, indicating that the added ammonium sulfate was completely consumed by cell growth (Fig. 5B). The microscope images exhibited that a higher cell volume size was achieved by the strategy of OUR-stat directed ammonium sulfate feeding than that of the control. The expansion of cell membrane improved the capacity for a higher CoQ_{10} production.

The statistical analysis results revealed that the highest specific CoQ_{10} content (27.90 mg/(g DCW)) were obtained using the effective OUR-Stat strategy, which were dramatically higher than that without OUR-Stat controlled fermentations. This was also the highest specific CoQ_{10} content and production among the previous reports regarding CoQ_{10} production (Table 3) [19,49-51]. Previous research demonstrated that the synergic effect of redox potential regulation and oxygen uptake improvement could enhance CoQ_{10} production in *R. sphaeroides*, and 600 mg/L of CoQ_{10} production was obtained in the fed-batch fermentation [20]. Zhang *et al.* [35] reported that phosphate limitation could induce a pleiotropic effect on the cell metabolism. And the improved CoQ_{10} biosynthesis efficiency of *R. sphaeroides* was possibly related to the disturbance of energy metabolism and redox potential, CoQ_{10} production reached 1,950 mg/L and a higher specific CoQ_{10} content reached 23.6 mg/(g DCW). Compared with the phosphate limitation strategy, an OUR-stat directed strategy with consecutive feeding ammonium sulfate could strengthen the specific CoQ_{10} content as high as 27.9 mg/(g DCW). The highest yield of CoQ_{10} and specific CoQ_{10} content demonstrated that the optimal OUR-Stat directed ammonium sulfate feeding strategy was efficient and economical for the industrial CoQ_{10} production.

As is well known, the agitation, aeration, pressure, temperature, and so on are usually the main methods for

Table 3. Comparison of CoQ_{10} production under the conditions with and without OUR-Stat directed controlling strategy in 50-L bioreactors and compared with the previous reports

Strain and Fed-batch	OUR (mmol/L/h)		CoQ_{10}	Qp	Reference
	Former phase	Later phase	mg/L	mg/(g DCW)	
R. sp[a] Control	65.3 ± 3.5	not control	1,683 ± 58	22.5 ± 0.9	This study
R. sp without OUR-Stat	45.9 ± 2.3	not control	2,239 ± 42	25.7 ± 1.2	This study
R. sp with OUR-Stat	45.0 ± 2.1	43.5 ± 2.3	2,584 ± 82	27.9 ± 1.7	This study
E. coli[b] GD-51	---	---	433	11.7	Dai *et al.* 2015 [15]
R. sphaeroides RspSRIDL	---	---	93.3	7.16	Lu *et al.* 2015 [19]
R. sphaeroides RspMQd	---	---	138.5	12.94	Lu *et al.* 2014 [24]
A. tumefaciens[c]	---	---	626.0	9.25	Ha *et al.* 2007 [49]
R. sphaeroides KY8598	---	---	770.0	8.7	Choi *et al.* 2005 [18]
R. sphaeroides 2.4.1			138.0	12.96	Lu *et al.* 2017 [19]
R. sphaeroides			600.0	8.3	Zhu *et al.* 2017 [20]
R. sphaeroides HY 01			1,950.0	23.6	Zhang *et al.* 2019 [35]

Each value represents the mean of triplicate measurements.
OUR: oxygen uptake rate
Qp: specific CoQ_{10} content.
a, *Rhodobacter sphaeroides*
b, *Escherichia coli*
c, *Agrobacterium tumefaciens*

regulating oxygen supply in the industrial fermentation. But these always encountered the highest power consumption, especially during a higher oxygen consumption fermentation. To maintain the stable OUR levels with OTR balance, continuously increasing the stirring speed or the aeration were needed to overcome the oxygen transfer limitation due to the increased viscosity. This will greatly increase the cost of power consumption in the industrial fermentation. However, the low-cost ammonium sulfate feeding not only stimulated the cell growth, but also inhibited the polysaccharide biosynthesis and viscosity increase. Especially, it significantly promoted CoQ_{10} biosynthesis without any cost consumption on elevating the agitation and aeration.

4. Conclusions

In this work, the multi-physiological parameter correlation analysis demonstrated that the oxygen transfer and consumption rate exhibited dramatic effects on the industrial CoQ_{10} production. As one of the crucial physiological parameters of microorganism, OUR is the reflection of microscopic intracellular metabolism and extracellular environment conditions in the fermentation process [50]. The experiment results demonstrated that the high C/N ratio with nitrogen limitation was the main reason for the accumulation of polysaccharide, leading to the decreased oxygen transfer rate and the inhibition of CoQ_{10} biosynthesis at the late fermentation phase. Ammonium sulfate addition could not only accelerate the cell growth, but also greatly prevent the polysaccharide generation. Therefore, an OUR-stat directed strategy with consecutive feeding ammonium sulfate could effectively maintain an OUR level and promote CoQ_{10} production.

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