Development of a real-time bioprocess monitoring method for docosaheanoic acid production by *Schizochytrium* sp.

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Abstract

Oxygen uptake rate (OUR) and respiratory quotient (RQ) are key respiratory parameters for docosahexaenoic acid (DHA) production by *Schizochytrium* sp. HX-308 under dissolved oxygen limited conditions. To investigate the relationship of OUR and RQ with culture status, three independent cultures with different aeration rates were performed in a 50 L bioreactor. OUR was found to be positively correlated with the aeration rate, which reflected the oxygen supply level in each culture. The highest biomass, reaching 124.5 g/L, was achieved under the highest OUR. DHA content was found to be highly correlated with the RQ value, and the highest DHA content (44.85% in total fatty acids, w/w) was achieved in the highest RQ level, which implies that the polyketide synthase pathway was more active. OUR and RQ, which reflect the physiological state of microorganisms, are suggested as synergistic real-time bioprocess monitoring parameters for DHA fermentation.

Keywords: Docosahexaenoic Acid, Oxygen Uptake Rate, Respiratory Quotient, *Schizochytrium* sp.

1. Introduction

Polyunsaturated fatty acids (PUFAs) are critical components of plasma membranes and play significant roles in improved physiological functions of humans (Metz et al., 2001; Ji et al., 2014). Especially, Docosahexaenoic acid (DHA; 22:6, n-3), an important kind of PUFAs, has received worldwide attention for its health benefits to both infants and adults (Lauritzen et al., 2001; Nordoy et al., 2001; Ji et al., 2015), and the market demand of high quality DHA-rich oil is expanding (Chin et al., 2006). Currently, the large-scale industrial production is being set up via biotechnological methods utilizing marine algae, such as *Schizochytrium* sp. and *Thraustochytrium* sp. (Sijtsma and de Swaaf, 2004). And, it was known that two typical pathways of the synthesis of PUFAs and saturated fatty acid were polyketide synthases (PKS) and fatty acid synthases (FAS) pathways in *Schizochytrium* sp. (Metz et al., 2001). However, the improvement of the bioprocessing and search for reliable real-time bioprocess monitoring method are crucial for the production of high quality DHA-rich oil.

Diverse fermentation bioprocesses have been reported that aim towards the improvement of biomass and the percentage of DHA in total fatty acids (TFAs). Li et al. (2015) used glucose and glycerol as mixed carbon sources for *Aurantiochytrium limacinum* SR21, and DHA productivity was 15.24% higher than that obtained using glucose as single carbon source. Zhang et al. (2013) studied a two-stage AEMR/shake mixed culture mode utilizing *Schizochytrium* sp. in the fermentation process, and the culture time was reduced in the mixed mode. Ethier et al. (2011) employed a

continuous culture method to feed *A. limacinum* SR21, whereby the yield of biomass was 11.78 g/L and DHA concentration reached 1.74 g/L. Ren et al. (2010) adopted a stepwise aeration control strategy for *Schizochytrium* sp., whereby cells dry weight (CDW) and TFAs reached 71 g/L and 35.75 g/L, respectively. Qu et al. (2011) developed a two-stage oxygen supply control strategy based on the oxygen transfer coefficient (K_La) for *Schizochytrium* sp., and they achieved a DCW and DHA concentration of 92.72 g/L and 17.7 g/L, respectively. Chi et al. (2009) proposed a control strategy involving shifting dissolved oxygen levels for *Schizochytrium limacinum* SR21, whereby the production of biomass and DHA improved to 37.9 g/L and 6.56 g/L, respectively. Meesters et al. (1996) designed a two-phase fermentation strategy according to the features of nutrient limitation and the cell density reached 118 g/L. Additionally, there are many parameters that have been researched previously. Wu et al. (2005) studied the effects of culture conditions, pH, carbon sources and nitrogen sources on the production of DHA by *Schizochytrium* sp. S31. Chang et al. (2013) reported a DHA concentration, DHA productivity and conversion yield of 28.93 g/L, 301 mg/L/h and 0.44 ± 0.02 g/g, respectively, by *Schizochytrium* sp. S31 under constantly high oxygen transfer conditions.

In spite of the efforts that have been made previously, the mentioned real-time bioprocess monitoring parameters become unusable in their guiding roles when fermentation reaches the stabilizing phase (Chang et al., 2014). Therefore, real-time bioprocess monitoring method, which could effectively reflect physiological changes of cells in the culture, has been employed as tools for production scale-up and

optimization. These include the oxygen transfer rate (OTR), oxygen uptake rate (OUR), CO₂ evolution rate (CER) and respiratory quotient (RQ). The concentration of dissolved oxygen (DO) in the fermentation broth depends on the rate of oxygen transfer from the gas phase to the liquid, and the OUR by the microorganisms required for growth, maintenance and production (Garcia-Ochoa and Gomez, 2009). The OUR is approximately equal to the OTR under DO-limited conditions (Oliveira et al., 2004), and this situation also appears at the stationary phase of DHA production. Chang et al. (2014) showed that OUR is also influenced by the oxygen supply level, whereby the changes of OUR and CER are consistent with the alteration of cell activity, and the conversion yield of glycerol to biomass is highly correlated with the CER profiles, so OUR might be used as an on-line parameter to reflect the changes in oxygen supply level under DO-limited conditions. Moreover, RQ is one of the fundamental physiological parameters of cell culture (Shang et al., 2006), and it can be correlated with other parameters, e.g., OUR, CER and DO. RQ control strategy has been successfully applied to the optimization and scale-up in many industrial fermentation processes, such as the optimization of lactic acid or glutathione production (Wang et al., 2007). However, for the DHA production by *Schizochytrium* sp., the relationship of RQ to cell properties and oil composition is still unknown.

In the present work, three independent cultures using a fed-batch strategy, under three different aeration rates (0.5 vvm, 1.0 vvm, 1.5 vvm), were performed with *Schizochytrium* sp. HX-308 on glucose in a 50 L bioreactor with the aim to: first) investigate the relationship of OUR and RQ to culture status, second) propose

effective real-time bioprocess monitoring method which would be helpful for DHA production and last) analyze the variation of fatty acids from FAS and PKS pathways at different oxygen limitation levels. Additionally, the effects of different aeration rates on DHA production, OUR, RQ, oxygen limitation level, as well as the changes of fatty acid metabolism were surveyed.

2. Material and Methods

2.1. Microorganism

The strain *Schizochytrium* sp. HX-308 (CCTCC M209059) used in the present study was obtained from the China Center for Type Culture Collection (CCTCC) (Ji et al., 2015), and preserved in 20% (v/v) glycerol at -80 °C.

2.2. Culture conditions

The seed culture medium and the conditions were the same as those used in our previous study (Ren et al., 2014). The basic medium was composed of 50 g/L glucose and 0.4 g/L yeast extract in artificial seawater.

The culture preserved in the glycerin tube was inoculated into a 250 mL baffled shake flasks with 50 mL medium and cultivated for 24 h. After three generations, the culture (1%, v/v) was transferred to a 15 L seed bioreactor (Shanghai Guoqiang, China) with a liquid volume of 11 L and cultivated for 24 h. Then 5 L of seed culture was inoculated into a 50 L fermenter (Shanghai Guoqiang, China) containing 35 L of liquid volume with impeller speed set at 200 rpm and temperature at 30°C, and cultured for 120 h. The fermentation medium contained 70 g/L glucose and 8 g/L

yeast extract in artificial seawater.

During the fed-batch fermentation process, a feedback control strategy was implemented whereby a 800 g/L glucose feed solution was supplemented to keep the substrate concentration above 15 g/L. The pH was maintained at the state found in natural seawater. The agitation speed was set at 200 rpm, the cultivation temperature kept at 30°C and DO was monitored using an O₂ sensor electrode and expressed as percentage of O₂ saturation. Additionally, the bioreactor was equipped with an on-line gas analysis device, through which the oxygen and carbon dioxide concentration of the inlet and exhaust were determined. Consequently, the values of OUR, CER and RQ could be monitored on-line through real-time data collection software and used for analysis (Zhang et al., 2004).

To investigate the relationship of OUR and RQ with culture status and the change in DHA production and lipid composition of cultures, three independent cultures were conducted at aeration rates of 0.5 vvm, 1.0 vvm, and 1.5 vvm. All experiments were carried out in duplicate.

2.3. Analytical methods

The measurements of cell dry weight (CDW), total lipid contents, fatty acid composition and fatty acid methyl esters (FAMES) preparation were done as published previously (Qu et al., 2013). The FAME samples were analyzed by GC system (GC-2010, Shimadzu, Japan). The GC was equipped with a capillary column (DB-23, 60 m×0.22 mm) and flame ionization detector (FID). Nitrogen was used as the carrier gas. The injector was maintained at 250°C with an inject volume of 1 µL.

The column was raised from 100°C to 200°C at 25°C/min and then increased to 230°C at 4°C/min, keeping this temperature for 9 min. The FID detector temperature was set at 280°C. Fatty acids were identified through comparison with related external standards (Sigma, USA). The quantities of individual FAMES and squalene were estimated from the peak areas on the chromatogram using nonadecanoic acid (C19:0) as the internal standard.

Glucose and glutamate were measured enzymatically using a bioanalyzer (SBA-40C, Institute of Biology, Shandong Academy of Sciences, China) in the same way as published earlier (Ren et al., 2014).

2.4. Determination of OUR , CER and RQ

OUR, CER and RQ were determined based on gas analysis and were calculated using the following balance Equations (1), (2) and (3), in the same way as published earlier (Wang et al., 2010). And the values of OUR, CER and RQ could be on-line display through computer aided analysis software.

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

$$CER = \frac{F_{in}}{V} \left[\frac{C_{inert in} \times C_{CO_2 out}}{1 - (C_{O_2 out} + C_{CO_2 out})} - C_{CO_2 in} \right] \times \frac{273}{273 + t_{in}} \times P_{in} \times \frac{1}{1 + h} \times 10^{-5} \quad (2)$$

$$RQ = \frac{CER}{OUR} \quad (3)$$

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 fractions of nitrogen, oxygen and carbon dioxide, P_{in} is the pressure intensity of input gas, t_{in} is the temperature of input gas, and h is the humidity of input gas.

3. Results and discussion

3.1. Effect of different aeration rates on the fermentation

Different aeration rates (0.5 vvm, 1.0 vvm, and 1.5 vvm) resulted in significant differences of substrate assimilation, nitrogen consumption, cell growth, lipid accumulation and DHA production (Fig. 1).

During the fed-batch fermentation process, the glucose solution (800 g/L) was supplemented to keep the substrate concentration above 15 g/L after the initial glucose in the broth was exhausted. The amount of glucose feed has been recorded in real-time to accurately reflect the consumption capability of the algae. As shown in Fig. 1A, the glucose consumption rate was prominently increased with the increase of aeration rate. The total glucose feed quantities for the three fed-batch cultures were 9.64 Kg (0.5 vvm), 11.36 Kg (1.0 vvm) and 12.32 Kg (1.5 vvm). The glucose feed quantity of the culture under high aeration rate (1.5 vvm) was markedly higher than the others, which indicated a higher glucose consumption rate and substrate assimilation capacity. It has been reported previously that the substrate utilization capacity was strong at high oxygen supply level and was feeble in cultures with low oxygen supply (Chang et al., 2014). These results also demonstrated that the substrate utilization capacity was heightened with the increase in aeration rate.

Nitrogen consumption and cell growth were also considerably affected by the different aeration rates. The time of nitrogen exhaustion was correspondingly later the lower the aeration rate, and was observed at 24 h, 40 h, 56 h, respectively (Fig. 1C). The lipid accumulation was preceded by the nitrogen limitation, so that the achieved lipid content of biomass was more than 16% at the high aeration rate (Fig. 1B). This

was significantly higher than the middle and low aeration rates (13.7% and 4%, respectively). This might be explained by the fact that the activity of AMP deaminase (EC 3.5.4.6) was enhanced under nitrogen limitation. Isocitrate dehydrogenase (EC 1.1.1.42) is inhibited at low AMP levels, which reduces the accumulation of citrate in mitochondria and controls the tricarboxylic acid cycle, permitting the flux of additional carbon to lipid accumulation (Ratledge, 2004). Therefore, it was obvious that the low aeration rate resulted in lower lipid concentration.

Interestingly, at the end of fermentation, the highest lipid content (55.3 g/L) was achieved at middle aeration rate and the highest biomass (124.5 g/L) was achieved at high aeration rate (Fig. 1D). The conditions of high aeration benefit cell growth and low aeration benefits lipid accumulation. At the same time, excessive O₂ may cause potential damage to cells (Qu et al., 2011; Ren et al., 2010; Wang et al., 2010).

However, the lowest value for both was present at the low aeration rate. There appears to be a critical value of oxygen supply, so that lipid accumulation was limited and cell growth was restrained when the oxygen supply level fell under this value. Jakobsen et al. (2008) also presented an analogous conclusion for the *thraustochytrid*

Aurantiochytrium sp. Strain T66. Throughout the three fermentation processes, DHA percentage in TFAs was prominently different. As shown in Fig. 1E, the DHA content was low (36.1%) at the high aeration rate, and the highest DHA content (44.85%) was obtained at the middle oxygen supply level. The average efficiency of producing DHA was 105.3 mg/(L·h) at 0.5 vvm, 206.7 mg/(L·h) at 1.0 vvm and 146.8 mg/(L·h) at 1.5 vvm. The effect of high oxygen supply levels on cell growth was nevertheless clear

(Chi et al., 2009), and it led to a reduced ratio of DHA to fatty acids in the work of Bailey et al. (2003). This showed that high cell mass does not preclude a low DHA content. In contrast, all other feedback control studies in the literature reported that an increase in cell density often offsets a decrease in the DHA content (Huang et al., 2012). Therefore, more effective real-time bioprocess parameters were required to monitor the oxygen supply capacity and guide the simultaneous maximization of CDW, TFAs and DHA content.

3.2. Effect of different aeration rates on OUR and RQ

Currently, there are several real-time monitoring parameters which can be used to detect the status of oxygen supply, the level of biomass, and determine the optimum harvesting time. The real-time change of pH in these three cultures displayed a similar tendency, and was analogous to our earlier study (Ren et al., 2010), i.e. first increasing, then decreasing, later rising and finally maintaining a constant level (Fig.2B). This phenomenon mirrors the sequential metabolism of the variety of classes of nitrogen sources, the consumption of physiologically acid salts ($(\text{NH}_4)_2\text{SO}_4$ and similar), which could lead to acidification of the broth and finally a reduction of the pH, as well as physiologically alkaline salts (sodium glutamate and similar) which contribute to the alkalization of the broth (Wu et al., 2005). The DO displays a similar trend as is the case for pH. It shows a continuous decrease until reaching zero, after which it is maintained at a constant low level (Fig.2A). Thus, three different aeration levels were tested in bioreactors, and all three entered a dissolved oxygen limited state. DO and pH were not appropriate real-time bioprocess monitoring parameters to reflect

biomass, lipid and DHA content directly during the whole process of fermentation.

During the fermentation process, the DO reflected the dynamic balance between OTR and OUR (Oliveira et al., 2004). Based on the mass balance, an equation for the dissolved oxygen concentration changes in batch fermentation could be established via Eq. (4) (Bandaiphet & Prasertsan, 2006):

$$\frac{dC}{dt} = OTR - OUR = K_L a(C^* - C) - OUR = K_L a(C^* - C) - Q_{O_2} \times X \quad (4)$$

Where dC/dt is the oxygen accumulation rate in the liquid phase, whereas $K_L a(C^* - C)$ and $(Q_{O_2} \times X)$ represent the OTR and OUR, respectively. In the dissolved oxygen limited state, an acceptable pseudo-steady state approximation was considered to be simply $dC/dt \approx 0$, so we could draw the conclusion from the Eq. (4) that $OUR \approx OTR$, and that $K_L a$ has a synchronous tendency with OUR (Wang et al., 2010). Fig. 2C illustrates the profiles of the OUR. The OUR increased with the rapid decrease of DO in the exponential phase, and was subsequently maintained in a relatively stable state when DO almost did not change. These observations implied that the three cultures were all in an oxygen limited state, which generally occurred in all DHA fermentations utilizing *Schizochytrium* sp. HX-308. The OUR has a positive correlation with the aeration rate, and it increased with the increase of aeration rate, which approximately was 9.64 mol/(L·h) (0.5 vvm), 11.36 mol/(L·h) (1.0 vvm) and 12.32 mol/(L·h) (1.5 vvm), respectively. The OUR could be used as a real-time bioprocess monitoring parameter which reflects the change of oxygen supply level during the whole fermentation process. Additionally, higher OUR indicated that cells possess a higher metabolic activity, more substrate could be consumed and higher

biomass density could be obtained (Chang et al., 2014), which was consistent with this study where the relation of OUR to the microorganisms' growth state was obvious from the biomass achieved depending on the aeration rate with 85.6 g/L (0.5 vvm), 99 g/L (1.0 vvm) and 124.5 g/L (1.5 vvm). However, the highest content of DHA was achieved under the middle OUR condition at middle aeration rate. Thus, it was clearly shown that an appropriate oxygen supply level was important for DHA fermentation and this was dramatically reflected by the OUR.

In addition, RQ was also investigated in this study as a key cellular physiological parameter. As shown in Fig.2D, RQ increased rapidly with the decrease of DO, and maintained a dynamic equilibrium during final stages. The DHA content in TFAs for the three fermentations were 43.59% (0.5 vvm), 44.85% (1.0 vvm) and 36.10% (1.5 vvm), respectively, which was in good agreement with the RQ profiles. The more PUFAs are synthesized, the more CO₂ is released via decarboxylation reactions in PKS pathway, and less O₂ is consumed (Hauvermale et al., 2006; Ratledge, 2014), which leads to a high RQ value. Conversely, a lower RQ value (i.e., RQ $\approx$ 1.0) means that the tricarboxylic acid cycle is fully open and glucose is preferentially converted into H₂O and CO₂ (Xiong et al., 2010). The resulting conversion yield (5.7%) of glucose to DHA was quite low in the high aeration rate condition. Thus, RQ might be a viable real-time index to guide the fermentation of high DHA concentration cultures. Therefore, we suggest that a control strategy based on RQ could be applied effectively to DHA production. In order to inspect the relationship between lipid metabolism and RQ at different aeration rates, the lipid composition was studied as well.

3.3. Changes of fatty acid composition at different RQ values

GC analysis showed that 90% of TFAs extracted from *Schizochytrium* sp. were DHA (C22:6), docosapentenoic acid (DPA; C22:5, n-6), myristic acid (C14:0), tetradecanoic acid (C14:1), hexadecanoic acid (C16:0) and stearic acid (C18:0). Hereby, DHA and DPA syntheses relied on the PKS pathway, while C14:0, C14:1, C16:0 and C18:0 syntheses depended on the FAS pathway (Metz et al., 2001). The content of PUFAs (DHA and DPA) and saturated and monounsaturated fatty acids (C14:0, C14:1, C16:0 and C18:0) could reflect the relative activity of the PKS and FAS pathway, respectively. As shown in Table 1, the main fatty acid composition changed markedly when cells were cultured at different aeration rates, and the corresponding RQ values also displayed notable differences. The content of DHA was 43.59% (RQ $\approx$ 1.7), 44.85% (RQ $\approx$ 2.0) and 36.10% (RQ $\approx$ 1.0) at the end of fermentation, which is highly consisted with the RQ profiles. Moreover, the content of saturated and monounsaturated fatty acids, including C14:0, C14:1, C16:0 and C18:0, was reduced in accordance with the increase in RQ values. At the same time, the content of PUFAs had the opposite relation to the RQ values compared to the saturated fatty acids. Harwood and Guschina (2009) reported that the PKS pathway does not require aerobic desaturation and less oxygen is needed for it, so a possible reason for the increase in the DHA content and the reduction of the saturated and monounsaturated fatty acids in the cultures with high RQ values might be that the PKS pathway was more active relative to the others, and more carbon source was channeled toward the accumulation of unsaturated fatty acids.

Each of the three cultures with different aeration rates appear to reached a dissolved oxygen limited state at stationary phase. The OUR was selected as the parameter most likely to reflect the variation of oxygen supply level. The present study determined that the OUR is a reliable parameter in relation to biomass (Chang et al., 2014). In addition, RQ also showed good correlation with the DHA proportion, because it increased with the rise of the DHA proportion, and so could be an effective parameter to indicate the DHA content. Thus, a strategy based on OUR and RQ can be utilized to optimize the DHA proportion in fermentations.

4. Conclusion

The OUR had a positive correlation with aeration rates and reflected the reliable oxygen supply level under DO limited conditions in different fermentations by *Schizochytrium* sp. HX-308. The highest biomass at 124.5 g/L was achieved under the highest OUR at high aeration rate. The DHA content agreed with the RQ values, and the highest DHA content at 44.85% was achieved under the highest RQ at middle aeration rate and the PKS pathway was relatively active under said conditions. The OUR and RQ, which reflect the physiological changes of microorganisms, are suggested as synergistic real-time bioprocess monitoring for DHA fermentation.

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Table captions:

Table 1 The fatty acid composition (% , in total fatty acids, w/w) of *Schizochytrium* sp. HX-308 cultured at different aeration rates

Figure captions:

Fig.1. Time course of fermentation profiles in three independent fed-batch cultivations utilizing *Schizochytrium* sp. HX-308 with the aeration rates at 0.5 vvm (■), 1.0 vvm (●) and 1.5 vvm (▲), respectively: (A) glucose feed (g/L), (B) biomass (g/L), (C) monosodium glutamate consumption (g/L), (D) Total lipid (g/L), (E) DHA content (% in total fatty acids, w/w).

Fig.2. Time course of fermentation profiles in three independent fed-batch cultivations utilizing *Schizochytrium* sp. HX-308 with the aeration rate at 0.5 vvm (■), 1.0 vvm (●) and 1.5 vvm (▲), respectively: (A) dissolved oxygen, DO (%), (B) pH, (C) oxygen uptake rate, OUR (mol/(L·h)), (D) Respiratory quotient, RQ.

Figure 1

Figure 2

Table 1 The fatty acid composition (% , in total fatty acids, w/w) of *Schizochytrium* sp. HX-308 cultured at different aeration rates

Fatty acids (%,w/w)	0.5 vvm (RQ≈1.7)					1.0 vvm (RQ≈2.0)					1.5 vvm (RQ≈1.0)				
	40 h	64 h	88 h	100 h	120 h	40 h	64 h	88 h	100 h	120 h	40 h	64 h	88 h	100 h	120 h
C14:0	5.97	2.78	3.39	3.67	4.09	2.01	5.24	4.07	3.80	3.85	3.95	6.74	6.77	5.02	7.41
C14:1	0.89	0.74	0.70	0.67	0.58	1.99	0.66	0.49	0.52	0.60	1.25	0.91	0.91	1.59	1.27
C16:0	18.03	12.64	13.04	12.91	13.32	19.92	15.04	13.71	12.67	12.28	15.53	17.51	18.61	19.24	20.48
C18:0	0.97	1.24	1.05	0.92	0.78	1.36	0.96	0.83	0.77	0.64	0.63	0.72	0.78	0.75	0.68
Squalene	3.64	4.76	3.78	3.98	3.45	3.87	3.66	3.49	3.52	3.6	3.25	2.91	2.98	3.47	3.16
DPA	22.45	19.06	19.57	20.26	20.83	18.95	20.91	20.91	21.02	21.62	24.72	21.67	21.37	20.10	20.18
DHA	38.02	39.76	41.27	42.31	43.59	40.52	41.99	43.58	44.08	44.85	39.91	39.80	38.78	38.03	36.10
others	10.03	19.02	17.2	15.28	13.36	11.38	11.54	12.92	13.62	12.56	10.76	9.74	9.8	11.8	10.72

RQ, respiratory quotient; C14:0, myristic acid; C14:1, Tetradecenoic acid; C16:0, palmitic acid; C18:0, Stearic acid; DPA, docosapentaenoic acid, C22:5, n-6; DHA, docosahexaenoic acid, C22:6, n-3; others, which include unsaponifiables, pigments and trace amounts of fatty acids.