Journal of the Taiwan Institute of Chemical Engineers 49 (2015) 67–71

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

journal homepage: www.elsevier.com/locate/jtice

The effects of feeding criteria on the growth of oleaginous yeast—*Rhodotorula glutinis* in a pilot-scale airlift bioreactor

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ARTICLE INFO

Article history:
Received 18 August 2014
Revised 9 November 2014
Accepted 14 November 2014
Available online 12 December 2014

Keywords:
Microbial oils
Oleaginous
Airlift bioreactors
Fed-batch culture
Glycerol
Integrated processing

ABSTRACT

Rhodotorula glutinis is an oleaginous microorganism, which is being able to accumulate great amounts of total lipid. To achieve high cell density cultivation, the fed-batch operation was adopted. The effects of three feeding strategies on the growth of *R. glutinis* in a pilot scale of 50 L airlift bioreactor were examined. Results indicate that operation in a 50 L airlift bioreactor had superior performance on the growth of *R. glutinis* than that of the 15 L agitation tank. The fed-batch operation could provide sufficient carbon source—crude glycerol while avoiding inhibition resulting from the accumulation of high crude glycerol. Therefore, fed-batch operation appears to promote a higher cell density as compared to that of batch operation. Among the feeding criteria, the exponential feeding criteria could ease the observed growth-lag phase by avoiding peaks in crude glycerol concentration. As such, it led to the highest obtained cell growth rate. Nevertheless, the conventional pulse feeding criteria could achieve the highest total lipid and β-carotene productivity. Consequently, if collecting the maximum total lipids for biodiesel production is the goal, the fed-batch operation by pulse feeding is suggested as a suitable criterion for the cultivation of *R. glutinis* with crude glycerol as the carbon source.

1. Introduction

A lipid content of microorganisms in excess of 20% (g/g) classifies them as oleaginous species [1]. Some yeast strains can accumulate intracellular lipids, comprising as high as 70% of their biomass dry weight. The majority of those lipids are triacylglycerol (TAG) containing long-chain fatty acids that are comparable to those of conventional vegetable oils, and can be further used as the feedstock for biodiesel production. The biological production of single cell oils (SCO) from oleaginous microorganisms had attracted much attention recently due to the advantageous characteristics of a short life cycle and varied carbon source choices; which in turn has raised research interest as a potential non-food feedstock for biodiesel production. Numerous oleaginous yeasts and microalgae have been reported to be capable of accumulating great amounts of lipids. More specifically, the characteristics of rapid growth and the ability to utilize a range of carbon sources mean that *Rhodotorula glutinis* is an attractive candidate for microbial oil production [2–5]. It was reported that the fatty acids from *R. glutinis* were mainly composed of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:2) [5,6]. The total of palmitic acid and oleic acid would be over 80% of total fatty acid, which made microbial oils derived from *R. glutinis* being suitable for biodiesel production.

Successful biotechnological processes for producing biodiesel feedstock should be supported by the utilization of cheap substrates to make the commercialization of SCO possible; and for this reason, crude glycerol is a particularly appealing material. About 10% (w/w) crude glycerol is the main byproduct produced in the biodiesel manufacture process [7]. Since the global production of biodiesel is increasing, the amount of crude glycerol production has also greatly increased, and consequently its market price has reduced leading to crude glycerol being a very promising potential substrate for the cultivation of oleaginous microorganisms [3,8,9]. In addition to the substrate's falling cost, the operating cost for the accumulation of microbial oils is also a major concern for the commercial viability of microbial oils. An airlift bioreactor has the advantages of simple operation and low energy consumption, and thus can be used for the growth of oleaginous microorganisms in a relatively inexpensive manner, facilitating the commercial production of microbial oils. The feasibility of using an airlift bioreactor instead of conventional agitation tank for the cultivation of *R. glutinis* has been reported previously [10].

Besides using the low operation cost of an airlift bioreactor for the cultivation of oleaginous microorganisms, the achievement of a

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http://dx.doi.org/10.1016/j.jtice.2014.11.019
1876-1070/

high cell density could further reduce the process cost. The fed-batch operation strategy is generally adopted for achieving a high cell density production process. The reason for using fed-batch operation is to ensure the substrate concentration does not exceed an inhibitory level. Various feeding strategies such as conventional pulse feeding, constant rate feeding and exponential feeding have been explored in the literature for different fermentation products including the conventional pulse feeding, constant rate feeding and exponential feeding [11–13]. Pulse feeding is mostly adopted in fed-batch operations; nevertheless, pulse feeding can lead to an unstable environment, which could potentially yield an unstable growing situation. Therefore, a fed-batch operation with constant feeding and exponential feeding probably better fits the requirement of cell growth by avoiding sudden nutrient accumulation [12].

In the fed-batch cultivation of *R. glutinis*, Pan et al. obtained a high cell density of 185 g/L with the aid of oxygen-enriched air [14]. Yamauchi et al. obtained a cell density of 153 g/L and a lipid content of 54% (w/w) by using fed-batch cultures of *Lipomyces starkeyi* for 140 h [15]. Further, Li et al. indicated that by using pilot-scale fed-batch cultivation of *Rhodosporidium toruloides* with discontinuous feeding in a 15-L stirred-tank fermenter resulted in dry biomass, lipid content and lipid productivity of 106.5 g/L, 67.5% (w/w) and 0.54 g/L h, respectively [16]. These three studies provide evidence that fed-batch operation could successfully enhance cell growth to a relatively high density.

Consequently, the present study attempted to compare three feeding strategies on the growth of *R. glutinis* in a pilot scale 50 L airlift bioreactor by using crude glycerol as the sole source of carbon and energy. The feeding strategies' effects on the total lipids and on β-carotene accumulation were also examined. Ultimately, a fed-batch operation process is proposed for the cultivation of oleaginous yeast—*R. glutinis* at a high cell density.

2. Materials and methods

2.1. Microorganism and medium

Freeze-dried *R. glutinis* BCRC 21418 was obtained from the Bioresource Collection and Research Center, Taiwan (BCRC). The seed medium composition and the cultivation methods followed the suggestions provided by the BCRC. The fermentation medium (per liter) comprised defined amounts of crude glycerol, 2 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of $CaCl_2$ and 0.1 g of NaCl [17]. The solution of 1.0 N NaOH or 1.0 N HCl was used to adjust the pH. The crude glycerol was provided by a local biodiesel manufacture company, with crude glycerol being the by-product of the conventional base catalyst transesterification process. The glycerol content of crude glycerol was 47 ± 8% (w/w) of glycerol content, which the content depended on the biodiesel production batch.

2.2. Fed-batch fermentation in 50 L airlift

Fermentation was carried out in a 50 L airlift bioreactor (working volume 40 L) with inner draft-tube and in a 15 L conventional standard agitation tank (with two sets of turbine impeller). The working volume in the 15 L agitation tank is 10 L. Specifications of the airlift bioreactor are as follows: an outer diameter of 26 cm, inner tube diameter of 18 cm, outer height of 115 cm and inner tube height of 53 cm. The 15 L conventional agitation tank was obtained from Biotop Company, Taiwan, with two sets of turbine impeller. All the experiments were performed at 24 °C and at pH 5.5 controlled by 1 N NaOH solution. The aeration rates would be 1 vvm in both operations of agitation and airlift bioreactor. To avoid the high shear force leading to the damage of cells, the agitation rate would be limited to less than 300 rpm in the operation of agitation tank.

2.3. The operation of fed-batch

According to the previous batch results, feeding was performed at the 48th hour to enhance cell growth. As aforementioned, three different feeding criteria were examined, namely pulse feeding, continuous feeding at a constant rate and exponential feeding. The feeding solution consisted of 3.5 L of solution with 2.4 kg of crude glycerol for all operations. Feeding occurred according to the following criteria:

(1) Pulse feeding was performed by feeding the 3.5 L solution within a short period (about 10 min).
(2) Constant feeding was performed by continuously feeding the 3.5 L solution at a rate of 0.3 L/h for 12 h.
(3) Modified exponential feeding was performed by adjusting the feeding rate according to the following equations [12]:

The mass balance for cells in fed-batch cultivation can be expressed as:

$$\frac{d(XV)}{dt} = \mu XV \tag{1}$$

where X is the cell concentration, V the culture volume, t the time, and μ the specific growth rate. If the fed-batch is operated under the conditions that the substrate feed rate is equal to its consumption rate, then the mass balance for limiting the substrate is expressed by;

$$FS_0 - \frac{\mu XV}{Y_{X/S}} = 0 \tag{2}$$

where F is the volumetric feed rate, S_0 the feeding concentration of the limiting substrate, and $Y_{X/S}$ the yield coefficient (assumed constant). In addition, the rate of increase in culture volume is

$$\frac{dV}{dt} = F \tag{3}$$

From Eqs. (1) and (2) with μ = constant, we have

$$FS_0 = \frac{\mu X_0 V_0 e^{\mu t}}{Y_{X/S}} \tag{4}$$

where X_0 and V_0 are the cell concentration and the culture volume at the beginning of the fed-batch operation, respectively. It should be noted that for the cells to grow at a predetermined μ, the substrate concentration S in the fermenter must be zero at the fed-batch phase. Nevertheless, such kind of steady-state condition would not be easily achievable.

In this study, the feeding concentration of S_0 was constant at 685.7 g/L, which represents 2400 g of crude glycerol dissolved in 3.5 L water. The volumetric rate F was calculated according to the values of μ, X_0 and $Y_{X/S}$ obtained at the end of the batch operation (Eq. (4)). Every 2 h, the volumetric feeding rate (F) was re-calculated to obtain a new feeding rate for the next 2 h operation. Accordingly, we can obtain the function as follows:

$$F \times 685.7 = \frac{0.0898 \times 25.4 \times 40 \times e^{0.0898 \times t}}{0.294} \tag{5}$$

2.4. Analytical methods

An infrared balance (Denver Instrument, IR 35) was adopted to rapidly measure the biomass concentration. Five milliliters of broth was centrifuged at 7000 rpm for 10 min. After removing the supernatant, about an equal volume of distilled water was added to eliminate impurities. This washing procedure was performed several times, and the final liquor was dried using the infrared balance at 150 °C to evaporate the water content.

The total lipid analysis was based on a modification of the procedure used by Bligh and Dyer [18]. The dry biomass was ground into a fine powder; then, 0.05 g of the powder was blended with 5 ml chloroform/methanol (2:1), and subsequently agitated for 20 min at

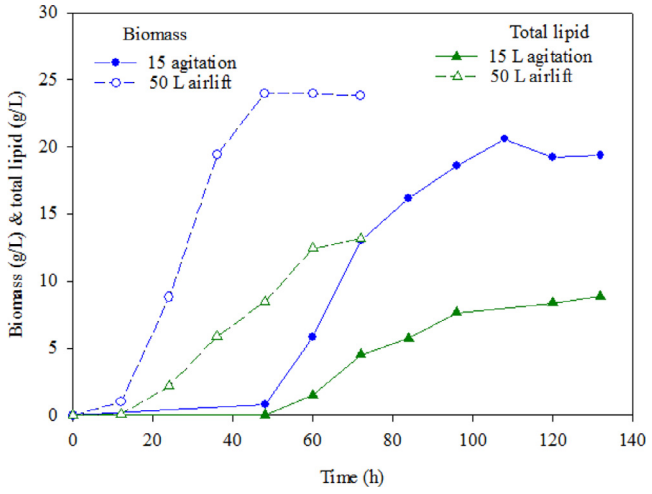

Fig. 1. The effect of different reactor systems on biomass and total lipids production at 60 g/L of crude glycerol at 24 °C and at pH 5.5.

room temperature in an orbital shaker. The solvent phase was recovered by centrifugation at 7000 rpm for 10 min. The same process was repeated twice, and the whole solvent was evaporated and dried under vacuum conditions.

The glycerol concentration was measured by HPLC (Agilent series 1100, Agilent Technologies, Santa Clara, CA) with a refractive index detector, while the analysis was performed in a C-18 column (Vercopak N5ODS, 250 mm × 4.6 mm, Taiwan). The mobile phase was composed of 0.01 N H_2SO_4 with a flow rate of 0.4 ml/min [19].

The measurement of β-carotene was performed as following: 50 mg of cells, after freeze-drying, were mixed with a 2 ml solution consisting of acetonitrile, isopropyl alcohol and ethyl acetate (40:40:20, v/v/v), followed by ultrasonication (125 W, reaction time 2 min for the frequency of 20 s action and 20 s resting) to crush the cells for pigment extraction. The extract was centrifuged at 7000 rpm for 10 min, and the supernatant filtered through a 0.45-μm membrane filter and subjected to HPLC analyses, which were performed on a reversed-phase C18 analytical column (N5 ODS (C-18) 4.6 mm i.d. × 250 mm). The mobile phase was composed of acetonitrile, isopropanol and ethyl acetate (40:40:20, v/v/v) and had a flow rate of 0.7 ml/min. The column thermostat was set at 25 °C, while the detector was operated at a wavelength of 457 nm [20]. The standard was purchased from M.P. Biomedical Company, USA.

3. Results and discussion

3.1. Batch operation of 50 L airlift compared to 15 L agitation tank

In general, conventional agitation tanks are mostly adopted for the cultivation of aerobic microorganisms, such as *R. glutinis*. However, low operation cost has made the airlift bioreactor very attractive for the cultivation of oleaginous *R. glutinis*. A comparison of the batch operation in a 15 L agitation tank and a 50 L airlift bioreactor for biomass and lipid production is shown in Fig. 1. Biomass production in the airlift bioreactor was obviously higher than that of the agitation tank. Besides the higher biomass concentration obtained, the airlift bioreactor promoted a faster biomass growth rate as compared to that of the agitation tank, which were 0.5 and 0.191 g/L h, respectively (as seen in Table 1). However, the reasons for the higher biomass and the faster growth rate in the airlift bioreactor are not clear. In general, the operation of an airlift bioreactor is characterized by its low shear force, which might be one reason leading to the increase of cell growth. High shear force exerted in the agitation tank often resulted in the broth foaming occurred, especially for the growth of high cells density. The results obtained in this study are similar to previous findings conducted in a 5 L lab-scale airlift bioreactor, which revealed that the increase of aeration rate over 1.5 vvm could produce a higher cell growth rate than that of an agitation tank [10]. Besides the higher growth rate in the 50 L pilot scale airlift bioreactor, a higher total lipid and β-carotene content could also be obtained as compared to that of the agitation tank. All these findings suggest that an airlift bioreactor is a superior choice for the cultivation of *R. glutinis*.

Table 1
Comprehensive values of different rector systems for the batch operation at 24 °C and at pH 5.5.

	Agitation	Airlift
Maximum biomass (g/L)	20.6	24
Average growth rate (g/L h)	0.191	0.5
Maximum total lipid content (g/g)	0.456	0.555
Maximum β-carotene content (mg/g)	0.166	0.582

3.2. Comparison of batch and fed-batch operation in a 50 L pilot scale airlift bioreactor

As shown in the previous section, the 50 L airlift bioreactor had superior cell growth performance than the 15 L agitation tank. It suggested that the airlift type was a suitable bioreactor for the growth of *R. glutinis*. The comparison of batch and fed-batch operation in the 50 L airlift bioreactor is further examined in this section. The pulse feeding of crude glycerol was performed at the 48th hour after inoculation, which raised the glycerol concentration of the medium to the initial concentration of 60 g/L. The fed-batch operation by adding crude glycerol could greatly enhance the growth of *R. glutinis* to the maximum biomass concentration of 46.4 g/L, as compared to only 24.0 g/L achieved in the batch operation (Fig. 2). A comprehensive comparison of several kinetic parameters in both the batch and fed-batch operations is shown in Table 2, which also suggests that the

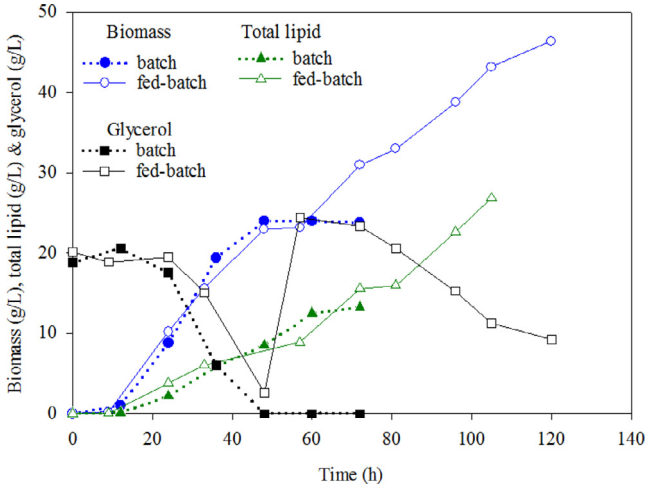

Fig. 2. The effect of batch and fed-batch operations in 50 L airlift bioreactor at 24 °C and at pH 5.5.

Table 2
Comprehensive values of batch and fed-batch operations in a 50 L airlift bioreactor at 24 °C and at pH 5.5.

	Batch	Fed-batch
Maximum biomass (g/L)	24.0	46.4
Average growth rate (g/L h)	0.5	0.387
Maximum total lipid content (g/g)	0.555	0.622
Maximum β-carotene content (mg/g)	1.163	1.417

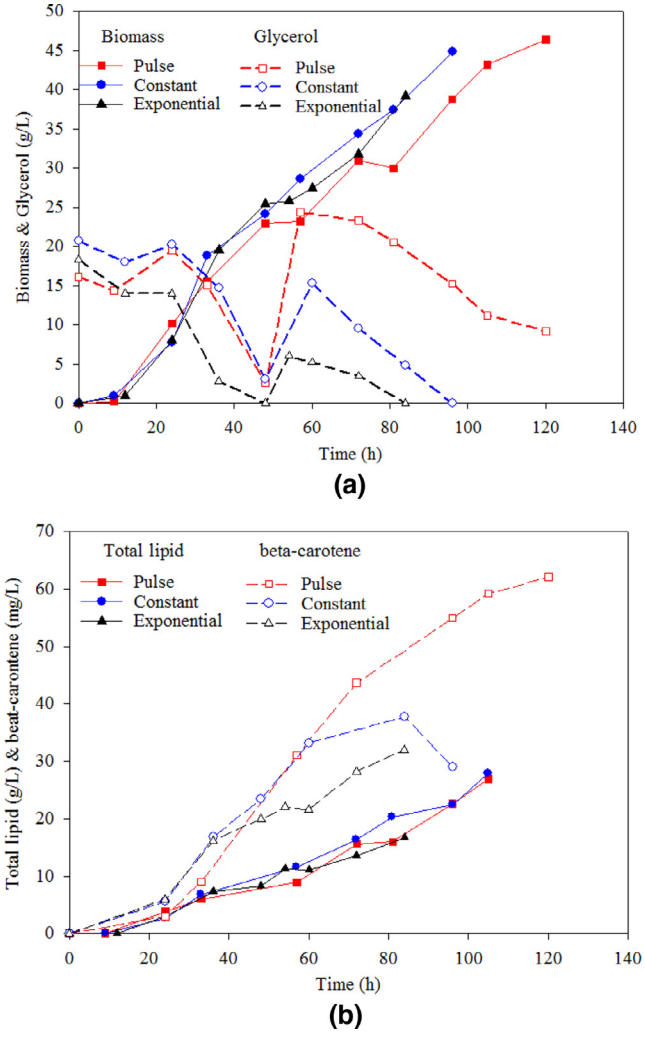

Fig. 3. (a) Effects of feeding criteria on the biomass and glycerol concentration in the 50 L airlift bioreactor. (b) Effects of feeding criteria on total lipid and β-carotene concentration in the 50 L airlift bioreactor.

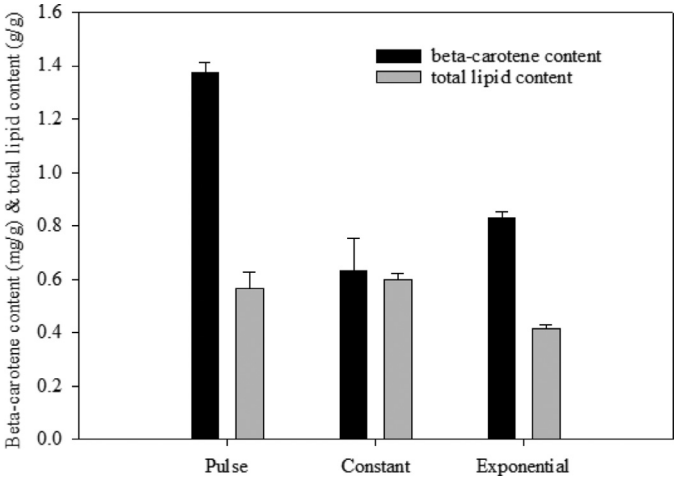

Fig. 4. Effects of feeding criteria on total lipid and β-carotene yield in the 50 L airlift bioreactor.

fed-batch operation could be a good fermentation criterion for increasing biomass concentration, and without serious negative impact on the total lipid and β-carotene content. However, a slight delay of cell growth after crude glycerol addition was observed from the biomass curve of Fig. 2. Crude glycerol was a complex material, which were consisting of many transesterification process derivatives and chemicals. Venkataramanan and his colleagues indicated that salt and methanol found in crude glycerol were found to have no negative effects on the growth and metabolism of the bacteria. However, the fatty acid with a higher degree of unsaturation, linoleic acid, was found to have strong inhibitory effect on the utilization of glycerol by the bacteria [21]. The cell growth delay might lead to a lower average cell growth rate measured in the fed-batch operation as compared to that of the batch operation, which were 0.387 and 0.5 g/L h respectively (as shown in Table 2). The pulse feeding performed in the fed-batch operation might result in the accumulation of crude glycerol, which led to the growth lag phase observed in the fed-batch operation. In the cultivation of oleaginous yeast—*Cryptococcus curvatus* using crude glycerol as the carbon source, the fed-batch operation could achieve a far higher obtained biomass than that of the batch operation. A one-stage fed-batch process would have a final obtained biomass density and lipid content of 31.2 g/L and 44.2%, respectively, as compared to less than 6 g/L of biomass in the batch operation [22]. In order to get a higher biomass concentration without reducing the cell growth rate, the effects of three feeding criteria on the growth are also examined.

3.3. *Effects of feeding criteria on the growth of* R. glutinis *in a 50 L pilot scale airlift bioreactor*

The effects of crude glycerol feeding on the cell growth with three feeding criteria were examined in this study, including conventional pulse feeding, constant feeding rate and exponential feeding. All fed-batch operations were initialized at the 48th hour. Conventional pulse feeding was performed by feeding 3.5 L of crude glycerol at one time, while constant feeding was executed by feeding 3.5 L of crude glycerol solution at a constant rate of 0.3 L/h until all the substrate was pumped in. Exponential feeding was performed according to the equation described in Section 2.3. The conventional crude glycerol was provided for all fed-batch operations. Results and the comprehensive comparison values are shown in Figs. 3 and 4 and in Table 3. Fig. 3 expresses the profiles of biomass and residual glycerol for the fed-batch operations with different feeding criteria. As seen in the figure, pulse feeding seems to have a slightly lower growth curve than that of other two fed-batch operations. It seems there is a lag phase observed in the fed-batch operation with pulse feeding after the feeding was performed at the 48th hour. It is clear that pulse feeding led to the highest glycerol content measured at the 60th hour after the feeding. To avoid the accumulation of high glycerol concentration as observed in the pulse-feeding, the same amount of glycerol solution was pumped into the fermenter within 12 h in the fed-batch operation with the constant feeding (as described in Section 2.3). Contrary to pulse feeding and constant feeding, the exponential feeding had the lowest glycerol profile measured at the fed-batch stage since the exponential feeding criteria only gradually increased the feeding rate. Therefore, a longer adaption period is required for cells to accommodate the high glycerol concentration. Crude glycerol derived from the biodiesel production process probably contains several potentially toxic compounds, which would be inhibitive to cell growth when using high concentration feeding [23]. Similar to exponential feeding by varying the feeding rate, a novel modified fed-batch cultivation method was developed for the cultivation of *Aurantiochytrium* sp., in which nitrogen and carbon sources were supplied at different time intervals [24]. This method increased biomass, palmitic acid, and DHA production 2- to 4-fold and utilized carbon and nitrogen sources more efficiently compared with the yields obtained using batch and non-modified fed-batch methods. Conclusively, the avoidance of high glycerol accumulation by the exponential feeding could prevent cell growth delay.

Table 3
Comprehensive values for the fed-batch operations with different feeding criteria in a 50 L airlft bioreactor at 24 °C and at pH 5.5.

	Pulse	Constant	Exponential
Maximum biomass (g/L)	46.4	44.8	39.2
Average growth rate (g/L h)	0.387	0.467	0.470
Maximum total lipid content (g/g)	0.622	0.621	0.433
Maximum total lipid production rate (g/L/day)	10.9	10.8	5.55
Maximum β-carotene concentration (mg/L)	62.1	51.8	41.6
Maximum β-carotene content (mg/g)	1.417	1.158	1.060
Maximum β-carotene production rate (mg/L/day)	19.73	18.23	10.26

It seems that the feeding criteria would slightly affect the cell growth due to the potential toxicity of high glycerol concentration accumulation. However, the effects of feeding criteria on the accumulation of total lipids were not obvious (Fig. 3). However, a high β-carotene concentration of 62.1 mg/L was observed in the operation with pulse feeding, which was almost three times that in the operations of constant and exponential feeding. Fig. 4 clearly indicates that pulse feeding results in more β-carotene yield. This implies that even high crude glycerol content could slightly retard the cell growth; however, it might be beneficial for the accumulation of β-carotene. The operation with exponential feeding resulted in the low glycerol measured for each time point, which had the low C/N ratio and led to the low total lipids content measured. Several kinetic parameters among all operations with different feeding criteria are shown in Table 3. The operation with exponential feeding had the highest cell growth rate, but had the lowest total lipids productivity and the lowest β-carotene productivity. Therefore, from the perspective of enhancing total lipids accumulation as the biodiesel feedstock, conventional pulse feeding might be the preferred feeding criteria for the cultivation of *R. glutinis* in a pilot scale 50 L airlift bioreactor using crude glycerol as the carbon source.

4. Conclusions

The airlift bioreactor had superior performance on the growth of *R. glutinis* as compared to the agitation tank. Furthermore, the operation of fed-batch by feeding crude glycerol could enhance cell growth as compared to simple batch cultivation. The exponential feeding criteria could avoid the high concentration of crude glycerol accumulated, and led to the enhancement of cell growth rate. Nevertheless, the conventional simple pulse feeding criteria achieved the highest total lipids and β-carotene productivity. Therefore, from the perspective of collecting the maximum total lipids for biodiesel production, the fed-batch operation by pulse feeding is suggested as a suitable criterion for the cultivation of *R. glutinis* with crude glycerol as the carbon source.

Acknowledgments

The authors gratefully acknowledge the financial support this study received from Taiwan's Ministry of Science and Technology (MOST) under grant numbers 103-2623-E-029-001-ET and 103-3113-E-006-006.

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