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Research Article

Evaluating feeding strategies for microbial oil production from glycerol by *Rhodotorula glutinis*

Oil production, from biodiesel by-product glycerol, through microbial fermentation provides a promising option as part of an integrated biorefinery process. However, bioprocessing improvements are required to make the process more efficient. In the present work, different glycerol feeding strategies were evaluated under fed-batch cultivation of the oleaginous yeast *Rhodotorula glutinis*. Results showed that the concept of targeting first a cell proliferation stage and then a lipid accumulation stage had beneficial effects on both biomass and oil yields. Continual feeding and pulsed feedings, delivering the same total amount of nutrients, resulted in similar values of cellular biomass (~25 g/L) and oil content (~40%). In contrast, continual supply of nutrients at higher rates (>0.8 g/L/h) led to an increase in both cell densities (30 g/L) and oil content (53%), attaining a high oil yield of 16.28 g/L. This suggests that a continual cultivation with two different rates for each stage constitutes an efficient approach to enhance microbial oil production.

Keywords: Biorefinery / Fed-batch fermentation / Oil content / Oleaginous yeast / Single-cell oil

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1 Introduction

Microbial oil has attracted much attention lately as an alternative oil feedstock due to its advantages of being independent from the food supply as well as being easier to produce than conventional plant oils [1]. Various oleaginous microorganisms, mainly yeasts, filamentous fungi, and microalgae are able to accumulate significant proportions of their weight in intracellular lipids when nitrogen or other nutrients such as sulfur and phosphorus are depleted in the presence of carbon excess [2]. Single-cell oil consists mainly of triacylglycerols with similar composition to plant oils and a portion of polyunsaturated fatty acids, which makes it a suitable feedstock for a wide variety of commercial applications, ranging from food additives and medicinal prod-

ucts [3–5] to biodiesel production [6]. Although microbial oil production has already been implemented industrially for food applications, biodiesel production from microbial oils is still under development. The current scenario relies on the development of cost-efficient processes for large-scale oil production either by identifying new robust species that can withstand harsh culture conditions with little nutrient requirements, or using low-cost carbon sources that can contribute to lower operation costs [7–10]. For example, yeasts are robust oleaginous microorganisms that can be cultured even on waste materials at high growth rates, resulting in high-cell density conditions under conventional cultivation modes [11, 12].

An inexpensive carbon source that can be used to produce oil is the biodiesel by-product, glycerol [13, 14]. Crude glycerol accounts for 10% of the produced biodiesel, but the impurities that it contains limit the further industrial use of this high-strength polluting waste. In this context, the use of glycerol for oil production not only supports the biorefinery concept for production of valuable products but also provides the potential to enhance the overall biodiesel production by up to 10% for the same oil supply. The first works from glycerol using oleaginous yeasts demonstrated that they utilized effectively this carbon source for cell proliferation and concomitant lipid accumulation under different fermentation modes and configurations. Meesters et al. [15, 16] obtained high cellular concentrations of *Cryptococcus curvatus* on pure glycerol using fed-batch cultivation, while a continuous fermentation of *Yarrowia lipolytica* on crude glycerol

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Abbreviations: CA, citric acid; CFB1, fed-batch experiment with continuous feeding of glycerol at a rate close to the glycerol uptake rate; CFB2, fed-batch experiment with continuous feeding of glycerol at rate twice as high as the glycerol uptake rate; CFB3, experiment with continuous feeding of glycerol at rate between that of CFB1 and CFB2; DCW, dry cell weight (g/L); DO, dissolved oxygen concentration (mg/L); OUR, oxygen uptake rate (mg/L/h); PFB, pulsed fed-batch cultivation; TN, total nitrogen

revealed the effect of dilution rates on the glycerol uptake and oil yield [17].

In addition to the carbon source, the culture conditions and the operation mode have significant effect on the oil yield. Hence, a proper fermentation design is necessary to achieve an effective use of the raw materials while maximizing the yields [18–21]. Such a design should take into account the conditions for lipogenesis. In particular, cell proliferation is the prevailing process when carbon and nitrogen are present in the medium. Upon the exhaustion of nitrogen, the excess carbon is still taken up by the cells and then converted into storage lipids rather than more cells [3]. A combination of excess carbon and low nitrogen, a high C/N ratio, would therefore appear to be a prerequisite for lipid synthesis. However, a certain amount of nitrogen must be supplied in order to provide substantial cell density to make the oil yield meaningful and worth producing at large scale. In view of that, cultivations consisting of two stages, where biomass is produced first and then lipid accumulation is induced, might be more appropriate. This is possible with fed-batch or continuous cultivation modes, in which the stages can be modified easily by altering the nutrient supply. Several cultivation modes have been developed using the two-stage strategy to improve oil productivity. In a recent study [22], a two-stage fed-batch cultivation of *Rhodotorula glutinis* on glycerol was found to be more effective and stimulated lipid and biomass production compared to one-stage fed-batch cultivation. Fontanille et al. [23] employed volatile fatty acids as carbon source for the second stage, enriched with ammonium sulfate while glucose and glycerol were used as growth promoting substrates during the first stage, employing a C/N ratio of 50 in both stages. In another approach, glycerol pulses were used after the end of an initial batch stage, when glycerol concentration fell below 3 g/L, and this offered higher productivities [21]. Such strategies are more advantageous because they are more adjusted to the needs of an oleaginous system. They achieve an unbiased cellular growth in the first stage, by avoiding growth inhibition from a high carbon source concentration in batch mode and can supply any kind of carbon source in the second stage where growth has been achieved and lipid accumulation takes place. Consequently fed-batch fermentations are increasingly attracting interest as efficient fermentation modes but a limited number of works refer to those in which continual feeding is applied with sugars or pure/crude glycerol as the carbon source [24,25]. While many studies focus on crude glycerol valorization through microbial oil production, pure glycerol has been mainly used in batch screening studies [14,26]. There is a lack of information on its use in more complex fed-batch systems, such as with different supply rates, where the stable composition of glycerol is essential to establishing clear effects.

In this work, different glycerol feeding schemes (pulsed and continual feeding at rates equal and higher than the glycerol uptake rate) were evaluated under fed-batch cultivation of the oleaginous yeast *R. glutinis*. The feeding strategy was based on a two-stage cultivation approach. After a batch period of 24 h, a further growth stage took place (to enhance cell production), followed by a lipid promoting stage with only glycerol supply (to enhance oil production). These feeding strategies enabled the evaluation of the effect of feeding rates on the glycerol uptake, cellular concentration, and lipid productivity, in order to build up a process for microbial oil production from glycerol.

2 Materials and methods

2.1 Microorganism and inoculum preparation

Rhodotorula glutinis CICC 31596, obtained from the Centre for Industrial Culture Collection (China), was maintained on YPGly (10 g/L Yeast extract, 10 g/L Peptone, 20 g/L Glycerol) Petri dishes at 4°C. A loopful of *R. glutinis*, after incubation for 4 days in a YPGly Petri dish at 30°C was used to inoculate the seed culture. For the seed culture preparation, 200 mL of medium containing 20 g/L glycerol (Sigma-Aldrich) and 10 g/L yeast extract (Sigma-Aldrich) at pH initially adjusted to 5.5 were added in a 500 mL shake-flask. The seed culture was incubated at 30°C for 24 h at 200 rpm in an orbital shaker (INFORS AG, Bottmingen, Switzerland).

2.2 Fed-batch bioreactor experiments

Fed-batch cultivations were conducted in a 2 L bioreactor (Electrolab, Tewkesbury, UK) with initial working volume of 1 L and an inoculation level of 10% v/v from the 24-h old seed culture. All fed-batch cultivations had the same starting conditions: 30 g/L glycerol and 2 g/L yeast extract (containing 10% w/w total nitrogen), 1 mL/L antifoam A (Sigma-Aldrich) and pH 5.5. The incubation temperature was 30°C, the agitation rate was 400 rpm and the air flow rate was 0.5 L/min throughout the cultivation. Dissolved oxygen was monitored using a polarographic electrode (Broadley-James, Bedford, UK) as Dissolved Oxygen Tension (DOT, percentage of air saturation).

2.2.1 Feeding scheme

A two-stage culture scheme was applied in the fed-batch cultivations. After an initial batch phase of 24 h, glycerol and yeast extract were fed to extend the Growth stage from 24 to 96 h, while from 96 to 144 h the Lipogenesis stage was promoted by feeding only glycerol. After that, the culture was left without nutrient input until the final harvesting of biomass. The first strategy (pulsed fed-batch, experiment PFB) consisted of three pulses of glycerol and yeast extract, every 24 h to maintain the glycerol and nitrogen concentration in the broth above 30 and 0.2 g/L, respectively. At 96 and 120 h two glycerol injections restored the glycerol concentration to 30 g/L. The average glycerol uptake rate was calculated for the growth stage 0.8 g/L/h and for the oil production stage 1.14 g/L/h. In the second strategy (fed-batch with continuous feeding, experiment CFB1), each feeding lasted for 24 h to supply by the end of the period the same amount of nutrients as the PFB did at the beginning (Fig. 1). Experiments PFB and CFB1 had the same stock media composition, which is shown in Table 1. The glycerol supply in CFB1 was 0.83 g/L/h from 24 to 48 h, 1.04 g/L/h from 48 to 96 h, and 1.25 g/L/h from 96 to 144 h. In the third strategy (experiment CFB2), the glycerol supply rate was twice as high as the average glycerol uptake rate of each stage in the PFB cultivation. Therefore, a feeding rate of 2 mL/h (1.6 g/L/h glycerol) for the growth stage and another feeding rate of 2.85 mL/h (2.28 g/L/h glycerol) for the lipogenesis stage were applied. The strategy for experiment CFB3 involved a

Figure 1. (A) Glycerol feeding schemes presented as cumulative concentration during fed-batch cultivation of *R. glutinis* on glycerol-based media. (B) TN feeding schemes presented as cumulative concentration during fed-batch cultivation of *R. glutinis* on glycerol-based media.

constant medium feeding rate of 1.65 mL/min (1.32 g/L/h glycerol) from 24 h to 96 h, (glycerol and yeast extract) followed by glycerol only, at the same rate, from 96 to 144 h. The stock solutions in the CFB2 and CFB3 schemes contained 800 g/L glycerol in both growth and lipogenesis stages while the yeast extract was 41.5 g/L in CFB2 and 50.3 g/L in CFB3 to provide a constant, between all experiments, feeding rate of 0.083 g/L/h during the growth stage. Antifoam was supplied when needed to prevent foam formation. An LKB Perplex peristaltic pump was used to transfer the media into the bioreactor. Information regarding feeding rates and stock media compositions is summarized in Table 1.

2.3 Oxygen uptake rate

The Oxygen Uptake Rate (OUR, mg/L/h) was determined from the slope of the decreasing values of dissolved oxygen concentration (DO) after interrupting momentarily the air supply, according to the dynamic method [27]. Then, the specific OUR (qO_2 , mg/g/h) was calculated by dividing the OUR by the cell concentration (X , g/L) at each particular time point.

$$OUR = \frac{\Delta DO}{\Delta t} = \frac{DO_{t_2} - DO_{t_1}}{t_2 - t_1} \quad (1)$$

$$qO_2 \text{ (mg/g/h)} = \frac{OUR}{X} \quad (2)$$

2.4 Analytical methods

Cell growth was monitored as OD at 550 nm in a Shimadzu spectrophotometer (Shimadzu, UV mini-1240, Japan). For dry cell mass determination (DCW), 2 mL of cell suspension were centrifuged at $13\,000 \times g$ for 5 min and the residual cell pellet was dried overnight in predried aluminium weighing dishes at 60°C. Glycerol concentration was determined using HPLC, Total Nitrogen (TN) using a Total Organic Carbon analyzer coupled

Table 1. Stock media composition and feeding rates employed in the different feeding schemes

Feeding strategy	Feed stages	Stock media composition		Fed volume (mL)	Glycerol feeding rate (g/L/h)	Yeast extract feeding rate (g/L/h)
		Glycerol	N-source			
PFB	24 h: Gly + YE	333 g/L	33 g/L YE	60	Pulse-fed	Pulse-fed
	48 h: Gly + YE	417 g/L	38 g/L YE	60	Pulse-fed	Pulse-fed
	72 h: Gly + YE	417 g/L	38 g/L YE	60	Pulse-fed	Pulse-fed
	96 h: Gly	500 g/L	—	60	Pulse-fed	Pulse-fed
	120 h: Gly	500 g/L	—	60	Pulse-fed	Pulse-fed
CFB1	24–48 h: Gly + YE	333 g/L	33 g/L YE	60	0.83	0.083
	48–72 h: Gly + YE	417 g/L	38 g/L YE	60	1.04	0.095
	72–96 h: Gly + YE	417 g/L	38 g/L YE	60	1.04	0.095
	96–120 h: Gly	500 g/L	—	60	1.25	—
	120–144 h: Gly	500 g/L	—	60	1.25	—
CFB2	24–96 h: Gly + YE	800 g/L	41.5 g/L YE	144	1.6	0.083
	96–144 h: Gly	800 g/L	—	109	2.28	—
CFB3	24–96 h: Gly + YE	800 g/L	50.3 g/L YE	119	1.32	0.083
	96–144 h: Gly	800 g/L	—	80	1.32	—

Gly, glycerol; YE, yeast extract; (—), no yeast extract supply.

Figure 2. Time-course profiles of cell growth (■), oil concentration (●), CA (gray-filled ○), glycerol (▲), and OUR (◇) during different fed-batch fermentations of *R. glutinis* on glycerol. (A) Pulsed fed-batch cultivation PFB, (B) continuously fed-batch cultivation CFB1 (0.83 and 1.04 g/L/h from 24 to 96 h and 1.25 g/L/h from 96 to 144 h), (C) continuous fed-batch experiment CFB2 (1.6 g/L/h from 24 to 96 h and 2.28 g/L/h from 96 to 144 h), (D) continuous fed-batch experiment CFB3 (1.32 g/L/h from 24 to 144 h). The area I corresponds to the batch phase, area II to the extended growth stage, area III to the lipogenesis stage, and area IV to the last phase of the fermentation (harvesting stage). Values represent the average of triplicate assays with SD lower than 10%.

with a TN detection unit from cell-free fermentation samples. Total cellular lipids were determined gravimetrically using the Soxhlet extraction method in a Soxtec-HT6 System (FOSS, Warrington, UK) with a solvent mixture of chloroform:methanol at ratio 2:1 v/v. All analytical methods were carried out as previously reported [22]. All assays were analyzed in triplicate and the results presented here are the average values. In all cases, SD was <10%.

3 Results and discussion

There are studies that highlight the importance of controlling the substrate feed in order to make the microbial oil production process more efficient [25, 28]. By following a two-stage fed-batch approach, different glycerol feeding styles (pulse and continuous) were evaluated in order to select an appropriate cultivation process for improved lipid production.

3.1 Kinetic profiles of *R. glutinis* using different feeding methods

At first a pulsed fed-batch experiment (PFB) was carried out with three glycerol and yeast extract feedings during the growth stage,

followed by two glycerol-only pulses in the lipogenesis stage. As can be seen from Fig. 2A, the cells grew quickly between 24 and 96 h, then the growth rate decreased when the lipogenesis stage began (phase III, Fig. 2A). The final cellular concentration was 23 g/L (IV, Fig. 2A). The oil concentration increased from 8.25 at 128 h to 9.38 g/L to 168 h. Since the changes in cellular density were insignificant during phases III and IV, the glycerol consumption was attributed to oil accumulation. All of the glycerol added was eventually consumed within 24 h of each injection. Nevertheless, in a pulsed fed-batch cultivation the substrate is supplied at once and the cells are left free to consume it at a rate probably influenced by the driving force (local concentration) of each component of the medium.

In order to evaluate the effect of supplying the same amount of glycerol at a lower rate, a fed-batch approach (CFB1) with continuous supply of the same stock medium as that used in experiment PFB was performed. According to Fig. 2B, the increase in cellular concentration was not quite as sharp between 24 and 72 h. However, it reached a concentration of 19.6 g/L at 96 h and continued to increase at the beginning of growth stage (III), reaching a final concentration of 24.23 g/L (Fig. 2B). The oil concentration increased from 7.42 g/L at 128 h to 9.55 g/L at 186 h. The only difference was the longer time that cells in CFB1 took to reach the stationary phase. As in PFB, all added

glycerol was consumed, despite a slight accumulation from 24 to 79 h (Fig. 2B), which was later eliminated during the lipogenesis stage.

Another experiment, CFB2, supplied glycerol continuously at rates twice as high as the glycerol uptake rate of each stage (growth and lipogenesis). In this way the cells would have available more glycerol than they apparently required. The cellular concentration increased smoothly and more sharply than in CFB1, even during lipogenesis stage (III) to 30.63 g/L at 168 h accompanied by the accumulation of lipids (Fig. 2C). The high glycerol supply rate during the lipid accumulation stage led to a glycerol peak of 98 g/L at 144 h, a value that could be inhibitory. This peak was a result of the accumulation of glycerol at a rate of 1.05 g/L/h, confirming that the supply rate was surplus to that required. However, the fact that this peak occurred during the lipid accumulation stage did not seem to have major detrimental impact on the final yields but resulted in a residual glycerol concentration of 68 g/L at the end of fermentation. Interestingly, the oil production was enhanced, reaching a concentration of 16.28 g/L by 168 h. In contrast to this study, in a continuous fed-batch cultivation of *Candida freyschussii* at a rate threefold higher than the glycerol uptake rate, the accumulation of glycerol occurred earlier and no residual glycerol was detected in the broth [24].

The last experiment, CFB3, examined further the effect of supplying glycerol at a constant rate throughout the cultivation, lower than CFB2 to avoid overfeeding of glycerol. The rate of 1.32 g/L/h glycerol was between the lower and upper rates applied in CFB1 and CFB2. The final cellular concentration reached 27.8 g/L at 168 h (Fig. 2D), lower than that achieved in CFB2 and higher than that of CFB1, accompanied by complete consumption of glycerol and production of 11.38 g/L oil. Similarly to CFB1, glycerol accumulated only during the growth stage (0.45 g/L/h), while during the lipid stage it remained almost steady (Fig. 2D).

Glycerol, or in general, substrate accumulation is a common phenomenon in continuous fed-batch cultures, when supply is higher than the uptake rate. However, subsequent cellular growth increases the uptake rate and eventually the substrate gets consumed.

The nitrogen (yeast extract) amount in all the continuous fed-batch experiments was controlled in such a way as to provide no more than the pulsed fed-batch fermentation over the same period of time (growth stage) and with the same supply rate of nitrogen. As can be seen in Fig. 3, nitrogen was immediately consumed after each injection. Similar levels of nitrogen were observed in all the continuous fed-batch fermentations as driven by the supply rate, confirming that nitrogen supply rate did not play a major role in the growth of *R. glutinis* but the biomass yield was rather a result of the supply and local concentration of carbon. Other studies on nitrogen have shown that the amount of nitrogen is linked to growth and increasing amounts affect beneficially the cellular concentration. Batch cultivations of *R. glutinis* at increasing C/N ratios, with increasing nitrogen concentrations favored growth, delayed the lipid accumulation phase but did not affect the glucose consumption profiles [29]. In another study of *R. glutinis* with constant medium supply, it was shown that sudden nitrogen limitation induced the lipid synthesis earlier [30].

Figure 3. Residual TN concentration during the PFB (●), CFB1 (○), CFB2 (◇), and CFB3 (■) fed-batch fermentations of *R. glutinis*. Values represent the average of triplicate assays with SD lower than 10%.

3.2 Effect of feeding style on the oxygen uptake rate

The OUR over time for each fed-batch cultivation is shown in Fig. 2. The sharp increase in OUR from 0 to 8 h coincided with the exponential growth phase and was followed by a drop at 24 h in all cases. At that time the nitrogen had been consumed, the growth rate ceased, and the oxygen requirements and uptake became less. With the onset of the fed-batch phase, the cells started consuming the new nutrients and proliferating. This led to an increase in OUR, which remained at high levels during the growth stage and most of the lipogenesis stage. In the case of PFB, the OUR was almost constant during the growth stage while the continuous fed-batch cultivations showed a clear increasing trend with the rising cell weight. OUR is linked to the metabolic activity as a result of growth that was a result of the available nutrients. High DO was used by Meesters et al. [15] as a sign of growth deceleration after exhaustion of nutrients in order to feed glycerol during fed-batch cultivation of *C. curvatus*. In contrast to the pulse fed-batch cultivation, the medium introduced gradually into the bioreactor in all the CFB experiments and that is the reason for the slower increase in OUR. The OUR decreased during the lipid stage while the DO was increasing, since the cells were not consuming much oxygen. However, the decrease in qO_2 (Fig. 4) meant less oxygen consumption per cell due to the rise in the number of cells present. The highest qO_2 values after the onset of the fed-batch phase was in PFB, while in the other experiments the qO_2 did not rise significantly. Moreover, in all fermentations except CFB2, the peak in qO_2 appeared before 32 h while despite the differences in feeding method, all cases reached the same level of $qO_2 \sim 5\text{--}6$ mg/g from 96 h, showing that the DCW above 19 g/L is the limiting factor for qO_2 .

3.3 Effect of cumulative glycerol on growth and lipid production

Feeding the same stock medium under two different modes (pulsed and continuous feeding) did not result in major differences in the final DCW and oil yields of PFB and CFB1 as can be seen in Fig. 5A. The final DCW in CFB1 was 24.23 g/L while that of PFB was 23 g/L, indicating that growth simply

Figure 4. Evolution of specific OUR (qO_2) during the PFB (●), CFB1 (○), CFB2 (◇), and CFB3 (■) fed-batch cultivations of *R. glutinis* on glycerol-based media. Area I corresponds to the batch phase, area II to the extended growth stage, area III to the lipogenesis stage, and area IV to the harvesting stage. Values represent the average of triplicate assays with SD lower than 10%.

Figure 5. (A) Comparison of the final DCW and oil content for the experiments PFB (pulsed feeding of glycerol, black bars) and CFB1 (continuous feeding of glycerol, gray bars). (B) Final DCW (◆), oil content (bars), and oil concentration (■) according to the total glycerol added in each continual fermentation. Values represent the average of triplicates with SD <10%.

reflected the overall stoichiometry. Similarly the oil content was around 40% in both cases. Different glycerol feeding rates, however, add different total amounts of glycerol causing differences in effect (Fig. 5B). Feeding glycerol at higher supply rates led to an upward trend in DCW, oil content, and oil concentration. Interestingly, the surplus of glycerol during the lipogenesis stage in CFB2 appeared to be extra beneficial for oil production, giving a 53% w/w oil content, rather than about 46% w/w that would be expected from a simple extrapolation of CFB1 based on total glycerol provided. A similar effect has been reported for a sequential fed-batch fermentation using *Y. lipolytica* on crude glycerol [31].

3.4 Influence of the glycerol feeding rate on biomass yield from glycerol

To evaluate the effect of feeding rate on *R. glutinis* growth after the initiation of feeding, the yield of biomass ($Y_{X/Gly}$, g/g) was calculated as the slope of the regression line of a plot of DCW against consumed glycerol (Fig. 6A–D). In Fig. 6A–D, growth and lipogenesis are distinguished by two different slopes. These slopes were less steep for the lipogenesis stage due to the near absence of cell growth. As can be seen in Fig. 6E, the biomass yield on glycerol decreased with increasing glycerol feeding rate. A different trend was seen during the lipogenesis stage (Fig. 6F) where increasing feeding rate led to higher $Y_{X/Gly}$ values, indicating that higher local glycerol concentration is preferred for oil production over cell proliferation. This behavior is consistent with that reported for a two-stage cultivation of *Y. lipolytica* on glucose [23], where high and low yields were found for the growth and lipogenesis stages, respectively.

3.5 By-product formation and glycerol feeding rate

Citric acid (CA) was detected in the broth mainly during the lipogenesis stage (Fig. 2). Although CA is a potentially valuable by-product, it diverts carbon away from oil production. Lipid production along with CA secretion has been described extensively for the yeast *Y. lipolytica* [13, 32, 33]. In most of the cases, CA is produced under conditions of nitrogen-limitation and carbon excess, during the last part of the fermentations [34]. Regarding *R. glutinis*, acidification of the broth has been reported but without further details of the acids or their relationship with oil or carbon source [20, 35, 36]. The CA titers and yields are summarized in Table 2. About 11 g/L of CA was produced in the PFB experiment, which is very close to the levels of oil production (9.4 g/L). However, only about half as much (5.3 g/L) was produced in CFB2, where the glycerol was fed continuously but slowly. This suggests that when large amounts of glycerol are available instantly, there is more chance that it will be converted to both oil and CA while the continuous supply channeled the carbon to oil production. In chemostat cultures using crude glycerol with *Y. lipolytica*, CA and oil production decreased with increasing dilution rate from 0.03 to 0.13 h⁻¹ [17]. Conversely, Rakicka et al. [31] observed a competitive relationship between lipid and CA production. Low values of biomass and oil were attributed to the high value of CA concentration. This might

Figure 6. Graphical estimation of the yield of biomass on glycerol ($Y_{X/Gly}$) for the growth stage (●) and the lipogenesis stage (○) during the fed-batch experiments (A) PFB, (B) CFB1, (C) CFB2, and (D) CFB3. (E) Biomass yield on glycerol ($Y_{X/Gly}$) as a function of the glycerol feeding rate applied during the growth stage. (F) $Y_{X/Gly}$ according to the glycerol feeding rate applied during the lipogenesis stage.

explain the low citrate production in our CFB2, where biomass and oil were higher. Concerning the CFB3 experiment, the CA titer increased after the lipid titer had reached a high but constant value. Although the glycerol supply rate was higher than in CFB1, it was consumed faster in CFB3 possibly due to this high acid production. It has been reported that in some cases oil concentration reaches a threshold value after which CA is mainly synthesized [37]. Moreover, increasing the glycerol feeding rate decreased the yield of CA from glycerol ($Y_{CA/Gly}$). In terms of

yield, Fig. 7 shows a clear trend toward lower production as feeding rate is increased. Similar trends are shown by the specific productivity, where again CA per cell was reduced by increasing flow rate while the pulsed supply of glycerol again led to more acid secretion. On the contrary, constant yields were observed in the studies of Andre et al. [13] with batch cultivations of *Y. lipolytica* at increasing crude glycerol concentrations and Rywinska et al. [38] using pulsed and continuous fed-batch cultivation of the same yeast.

Table 2. Experimental yields of *R. glutinis* CICC 31596 during the fed-batch cultivations performed in the present work

Feeding approach	DCW (g/L)	$Y_{X/Gly}$ growth stage	$Y_{X/Gly}$ lipid stage	Oil (g/L)	Oil content (% w/w)	$Y_{L/Gly}$ (g/g)	CA (g/L)	$Y_{CA/Gly}$ (g/g)
PFB	23.00 ± 0.03	0.225	0.043	9.38 ± 0.17	40.8 ± 0.01	0.059	10.96 ± 0.14	0.22
CFB1	24.23 ± 0.74	0.308	0.058	9.55 ± 0.04	39.4 ± 0.16	0.060	10.56 ± 0.09	0.21
CFB2	30.63 ± 1.44	0.229	0.091	16.28 ± 0.23	53.0 ± 0.00	0.087	5.46 ± 0.04	0.10
CFB3	28.00 ± 0.9	0.287	0.079	11.38 ± 0.11	41.5 ± 0.28	0.061	41.5 ± 0.06	0.03

Values represent the average of triplicates with SD < 10%.

X, biomass.

Figure 7. CA yield on glycerol ($Y_{CA/Gly}$, $\diamond$) and specific volumetric productivity of CA (blue-filled $\diamond$) according to the glycerol feeding rate applied during the lipogenesis stage. Data presented herein are the average values of triplicates with SD < 10%.

3.6 Comparison of fed-batch modes

Among the fed-batch strategies studied here, the continual feeding of glycerol was seen to be the most beneficial mode, yielding the highest biomass and lipid production as confirmed in Table 2. However, the glycerol to oil conversion yields ($Y_{L/Gly}$) were very similar (6–9%) with a slightly increasing trend toward the higher glycerol supply rate, while the pulsed glycerol feed gave the lowest $Y_{L/Gly}$. Similar yields can be seen in fermentations included in Table 3. In the continuous feeding mode, the cells are supplied constantly with nutrients and there is less likelihood of them running out. Other studies have stated similar benefits of continual feeding compared to pulsed feeding. For example, Raimondi et al. [24] obtained their best results with continuous fed-batch fermentation while cultivating the yeast *C. freyschussii* on pure glycerol. Improved productivities were also obtained by Anschau et al. [19] during continuous cultivation of *Lipomyces starkeyi* on hemicellulose hydrolysate. The advantage of a continuous supply of substrate was also confirmed by Zhao et al. [25] who achieved 30% increase in DCW and 33% in oil concentration when using a continuous supply of glucose to maintain the broth concentration at 5 g/L throughout the cultivation of *Rhodospiridium. toruloides* Y4. *R. toruloides* DSM 4444 obtained high DCW (62.4 g/L) with 61% oil content in a fed-batch cultivation with continuous supply of glucose, targeting a constant concentration in the broth [39]. These values including the lipid productivity and glucose conversion yield were better than pulsed supply of glucose. Yen et al. [28]

compared pulsed, constant, and exponential feeding of crude glycerol on *R. glutinis*. In contrast to exponential feeding, pulsed and constant feedings were more efficient for growth and lipid production.

As well as continuous feeding being better than pulsed feeding, in a previous study we showed that two-stage feeding was better than single-stage [22]. The results reported here confirm these findings (all values in Table 2 are higher than those reported previously). Table 3 summarizes the various cultivation strategies for oleaginous yeasts on glycerol. For our PFB experiment, results for both biomass and oil were higher than those obtained by Kitcha et al. [20] (13.8 g/L biomass and oil 7.78 g/L) using a similar two stage fed-batch strategy with *R. glutinis*. Comparable oil content to the CFB1 and CFB3 experiments (~40%) but higher DCW (113 g/L) resulting in substantial lipid titer were acquired with the “red” yeast *R. toruloides* cultivated on sugars extracted from Jerusalem artichoke, with intermittent feeding [40]. The results for CFB2 were similar to those obtained for a two-stage cultivation of *C. curvatus* on crude glycerol (32.9 g/L cellular concentration and 52% oil content) [41]. An equivalent DCW of 30.5 g/L was reached by *C. freyschussii* cultivated on glycerol in pulsed fed-batch mode but the lower oil content (30%) led to lower oil titer than in the present study [24]. Higher values were, however, were obtained when auxiliary nutrients were added alongside glycerol. Two-stage fed-batch cultivation of *R. toruloides* on glycerol [42] resulted in similar values of oil content (40.3%) and oil concentration (8.1 g/L) to the PFB and CFB1 experiments of the present work, as can be seen in Table 3. In another two-stage approach using *R. glutinis* on sugars, oil content (47.2%) was comparable to the present work, though a higher cell concentration of 70.8 g/L led to a higher overall oil production [43]. Supplementation of lignocellulose derived sugars with crude glycerol improved the yields of *R. glutinis* compared to those with only sugars in a batch study [44]. Fontanille et al. [23] utilized volatile fatty acids as carbon source following a growth stage on glycerol using *Y. lipolytica*. They obtained similar results of 31 g/L biomass and 12.4 g/L oil concentration (oil content 40%), supporting the suggestion that two-stage cultivations are beneficial for oil production systems and indicating that during the second stage no inhibition occurs, which broadens the range of carbon sources and concentrations that can be used. Another aspect of continuous fed-batch cultivation was investigated by Cescut et al. [30], who investigated the effect of sudden and progressive nitrogen limitation using *R. glutinis*. The biomass and oil yields as well as the glucose uptake rate were higher in the case of sudden nitrogen limitation.

Table 3. Comparison of cultivation modes employed for single oil production from glycerol by oleaginous yeasts

Yeast strain	Carbon source	Cultivation mode	Cultivation scale	DCW (g/L)	Oil content (% w/w)	Oil titer (g/L)	$Y_{L/Gly}$ (g/g)	Q_{oil} (g/L/h)	Reference
<i>C. curvatus</i> ATCC 20509	Pure glycerol	Fed-batch: two-stage	Bioreactor	118	25	29.5	0.11	0.59	[15]
<i>R. glutinis</i> CICC 31596	Pure glycerol	Fed-batch: two-stage	Bioreactor	16.8	34.6	5.07	0.03	0.03	[22]
<i>R. toruloides</i> Y4	Pure glycerol	Batch	Bioreactor	35.3	46.0	16.2	0.26	0.14	[14]
<i>Y. lipolytica</i> MUCL 28849	Pure glycerol ^{a)}	Fed-batch: two stage	Bioreactor	40.9	38.4	15.73	0.17	0.33	[23]
<i>R. toruloides</i> Y4	Pure glycerol	Fed-batch: two-stage	Shake-flask	21.1	40.3	8.5	0.22	0.07	[42]
<i>R. glutinis</i> TISTR 5159	Crude glycerol	Fed-batch	Bioreactor	10.5	60.7	6.1	0.06	0.12	[45]
<i>R. toruloides</i> Y-27012	Crude glycerol	Batch	Bioreactor	30.1	34	10.2	0.07	0.28	[46]
<i>Y. lipolytica</i> LGAM S(7)1	Crude glycerol	Continuous	Bioreactor	8.1	43	3.5	0.09	0.11	[17]
<i>C. freyschussii</i> ATCC 18737	Pure glycerol	Fed-batch (glycerol pulses)	Bioreactor	30.5	30	9.1	0.08	0.03	[24]
<i>C. freyschussii</i> ATCC 18737	Pure glycerol	Fed-batch (continuous feeding) ^{c)}	Bioreactor	82	34	28	0.07	1.8	[24]
<i>Y. lipolytica</i> JMY 4086	Crude glycerol ^{b)}	Fed-batch: two stage (continuous feeding) ^{c)}	Bioreactor	49.1	46	22.6	0.08	0.31	[31]
<i>R. glutinis</i> BCRC 21418	Crude glycerol	Fed-batch (continuous glycerol supply)	Bioreactor	44.8	62.1	27.82	-	0.45	[28]
<i>R. glutinis</i> BCRC 21418	Crude glycerol	Fed-batch (exponential glycerol supply)	Bioreactor	39.2	43.3	16.97	-	0.23	[28]
<i>R. glutinis</i> CICC 31596	Pure glycerol	Fed-batch: two stage (pulsed)	Bioreactor	23	40.8	9.38	0.059	0.06	This study
<i>R. glutinis</i> CICC 31596	Pure glycerol	Fed-batch: two stage (continuous feeding) ^{d)}	Bioreactor	24.23	39.4	9.55	0.06	0.06	This study
<i>R. glutinis</i> CICC 31596	Pure glycerol	Fed-batch: two stage (continuous feeding) ^{d)}	Bioreactor	28	41.5	11.38	0.061	0.07	This study
<i>R. glutinis</i> CICC 31596	Pure glycerol	Fed-batch: two stage (continuous feeding) ^{d)}	Bioreactor	30.63	53	16.28	0.087	0.10	This study

^{a)} Pure glycerol was used only during the growth stage.

^{b)} Crude glycerol was fed only during the lipid stage.

^{c)} Glycerol and nutrients were fed throughout the fermentation.

^{d)} Glycerol and yeast extract in the first stage, glycerol only feeding in the second stage.

4 Concluding remarks

This study used a two-stage fed-batch cultivation concept in order to examine the effect of the glycerol feeding rate on cellular growth and lipid production. Results showed that fed-batch cultivation with continuous feeding of glycerol is more efficient for both biomass and oil production than cultivation with pulsed feeding. Continuous feeding kept the cellular metabolism active, leading to high biomass and oil yields. However, provision of the same amount of nutrients in different ways (pulsed or continuous supply) did not significantly affect the final concentrations of biomass and lipids (and also CA) as these are defined more by the stoichiometry than the mode of operation. In addition, increasing the supply rate of glycerol had beneficial effects on the biomass production. Continuous glycerol supply at high rates resulted in enhanced cell densities and oil content, leading to higher overall productivities. Moreover, it was demonstrated that high glycerol levels were not inhibitory during the lipogenesis stage, resulting in less CA formation by channeling the available surplus of carbon source into oil production. In conclusion, a continuous feeding strategy with different nutrient supply

rates for each stage was an efficient cultivation mode for enhanced microbial oil production while reducing the by-product formation.

Practical application

The rise in biodiesel production has created a surplus of crude glycerol, which could potentially be used as a carbon source to produce oil of microbial origin. This could then be added to the feedstock for biodiesel production or used directly for other, higher value, applications. The results shown in this article suggest that a two-stage fed-batch cultivation (targeting growth first, then lipid accumulation) with continuous feeding of glycerol is more efficient for both biomass and oil production and leads to higher overall productivities. This study paves the way for establishing practical operating strategies to achieve efficient utilization of surplus glycerol in the production of microbial oil.

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