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Continuous Cultivation of Photosynthetic Bacteria for Fatty Acids Production

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Abstract

In the present work, we introduced a novel approach for microbial fatty acids (FA) production. Photosynthetic bacteria, *Rhodobacter sphaeroides* KD131, were cultivated in a

continuous-flow, stirred-tank reactor (CFSTR) at various substrate (lactate) concentrations. At hydraulic retention time (HRT) 4 d, cell concentration continuously increased from 0.97 g dcw/L to 2.05 g dcw/L as lactate concentration increased from 30 mM to 60 mM. At 70 mM, however, cell concentration fluctuated with incomplete substrate degradation. By installing a membrane unit to CFSTR, a stable performance was observed under much higher substrate loading (lactate 100 mM and HRT 1.5 d). A maximum cell concentration of 16.2 g dcw/L, cell productivity of 1.9 g dcw/L/d, and FA productivity of 665 mg FA/L/d were attained, and these values were comparable with those achieved using microalgae. The FA content of *R. sphaeroides* was around 35% of dry cell weight, mainly composed of vaccenic acid (C18:1, omega-7).

Keywords: photosynthetic bacteria; fatty acids; membrane-coupled bioreactor; lactate; cell productivity

1. Introduction

Environmental concerns, energy shortage, and consequent increasing energy costs have emphasized the need to produce sustainable and renewable fuels (Steen et al., 2010). To this end, huge effort is now being focused on the production of lipids using microalgae (Chen et al., 2011). Under unfavourable culture conditions, they are able to store neutral lipids, 20-70% of their body in heterotrophic or autotrophic ways, mainly in the form of triacylglycerol (TAG). TAG is convenient storage compound for carbon and energy, possessing high calorific value, and can be used as industrial chemical and bioenergy feedstock (Alvarez et al., 2002).

On the other hand, although photosynthetic bacteria do not accumulate neutral lipids, they are able to synthesize fatty acids, principally for glycerol-based membrane lipids (Carlozzi et al., 2010). The oil content (20-40% of dry biomass weight) of photosynthetic bacteria is generally lower than that of microalgae, and therefore, the research on this subject has been scarce. However, they are simpler to cultivate than microalgae that they do not require stressful environments for lipids production. In addition, bacteria are much more genetically manipulatable than algae. The expression of specific genes could result in the overproduction of fatty acids and their secretion to the broth (Steen et al., 2010).

Until now, the use of purple non-sulfur phototrophic bacteria has been proposed mainly for the production of hydrogen and polyhydroxybutyrate (PHB), as well as for wastewater treatment (Khatipov et al., 1998; Kim et al., 2006; Wu et al., 2012). Electrons contained in organic materials can be released as hydrogen by nitrogenase with the help of light energy, and PHB is formed when PNS bacteria are faced with a suboptimal environment. Recently, the co-production of hydrogen and lipids under anaerobic conditions was suggested (Carlozzi et al., 2010). During the cultivation of *Rhodopseudomonas palustris* under anaerobic light conditions, lipid content of 22-39% of dry biomass weight was observed. However, this study was limited to batch operation: it is important to maximize the lipid productivity by continuous operation. In addition, the effective use and design of a continuous culture are known to lower the production cost (Chen et al., 2011).

During the production of photosynthetic bacteria in a continuous culture system, the ability to maintain a balanced concentration of chemical substances and cells is critical to obtain higher productivity than in a batch culture system. However, there are many difficulties in maintaining a continuous culture for long periods without mutation and microbial

contamination. Population changes due to mutation or microbial contamination in the pure culture of a microbial strain have frequently been reported in continuous culture systems (Toda, 2003).

The use of a membrane-coupled bioreactor has recently gained recognition as a solution to the above problems by decreasing contamination risks and cleaning frequencies, and by higher cell concentration compared to a standard stirred tank bioreactor (Glazyrina et al., 2010). In addition, it is possible to reuse the water and nutrients in the permeate from the membrane filter by retaining cells that are larger than the diameter of membrane pore. Recent works employing various microorganisms immobilized in membrane-based culture systems have shown that extremely high, sludge-like densities of ca. 10^{12} cells per ml were possible in such systems and that the productivity of membrane reactors continues at high levels for more than two weeks (Lee et al., 2008; Ríos et al., 2012).

In this study, we suggested a novel approach for microbial fatty acids production, which is competitive with a traditional way of using microalgae. Photosynthetic bacteria, *Rhodobacter sphaeroides* KD131, were cultivated for fatty acids production using lactate as a substrate. Organic acids that mostly consisted of lactate can be easily obtained from fermentation products in agricultural and food waste (Kim et al., 2009). To maximize the cell productivity, a continuous-flow, stirred-tank reactor (CFSTR) was operated at various substrate concentrations and hydraulic retention times (HRT). A membrane unit was installed in the CFSTR for further increase of cell concentration and productivity. To our knowledge, this is the first attempt to apply the concept of a membrane-coupled bioreactor aiming at continuous cell harvesting. In addition, the fatty acids profile of the bacteria was assayed and the performance obtained in this study was compared with that using microalgae.

2. Methods

2.1 Inoculum preparation

The phototropic bacterium *R. sphaeroides* KD131 isolated from mudflats along the coast of Daebu Island in the West Sea of South Korea was cultivated for fatty acids production (Kim and Kim, 2012). The KD131 strain was pre-cultured in a modified Sistrom's broth (Kim et al., 2012) containing 4 mM $(NH_4)_2SO_4$, 0.3 mM L-aspartic acid, and 20 mM lactate at 30^{o}C for 24 hr under 54 W/m^2 irradiance using a halogen lamp (12V, 50W).

2.2 Reactor operation

A 1.2 L glass reactor (effective volume of 1.0 L, 200 mm high by 80 mm diameter) installed with a membrane unit was designed for the continuous cultivation of *R. sphaeroides* (Fig. 1). Three halogen lamps were properly located to adjust the light intensity at 54 W/m^2 on the reactor wall. The membranes are made of high density polyethylene (HDPE) with a normal pore size of 0.4 μm and an effective filtration area of 0.006 m^2. A certain amount of centrifuged microorganism was inoculated to reach an initial cell concentration of 0.5 g dcw/L. After purging with Ar gas for 1 hr, the reactor was operated for 48 hr by batch mode, and then switched to a continuous mode. It was agitated using a magnetic stirrer at 150 rpm. Feedstock contained 30 mM of lactate with nutrient medium as mentioned above. All experiments were conducted in a constant temperature room at 30±1^{o}C.

First, the reactor was operated by a CFSTR mode (w/o membrane unit operation). Lactate concentration was gradually increased from 30 mM to 70 mM at a fixed HRT 4 d. As the performance failure was observed at 70 mM, lactate concentration was decreased to 40 mM, and the reactor was operated as membrane-coupled bioreactor mode. Once a day, 50 mL of

cell broth, which was harvested for fatty acids production, was pulled out corresponding to a cell retention time (CRT) of 20 d, while HRT was kept at 4 d. In order to increase substrate loading, first, substrate concentration was gradually increased up to 120 mM. Afterwards, HRT was gradually shortened to 1 d at a fixed lactate concentration of 100 mM. The CRT/HRT ratio was kept at 5. Permeation of membranes was continuously maintained by using a peristaltic pump according to the HRT. One cycle of membrane filtration consisted of 45 min of filtration and 15 min of releasing. In order to reach the steady-state and to obtain average performance values, the reactor was operated for more than five times of HRT at each operating condition.

2.3 Analysis

Residual lactate was analyzed by a high performance liquid chromatograph (HPLC) (Finnigan Spectra SYSTEM LC, Thermo Electron Co.) with an ultraviolet (210 nm) detector (UV1000, Thermo Electron) and an 100 mm × 7.8 mm Fast Acid Analysis column (Bio-Rad Lab.) using 0.005 M H_2SO_4 as mobile phase. The liquid samples were pretreated with a 0.45 μm membrane filter before injection to both HPLCs. Cell concentration and fatty acids were measured according to the methods described in Kim et al. (2012) and Carlozzi et al. (2010), respectively.

3. Results and Discussion

3.1 CFSTR performance

High substrate concentration allows energy-efficient operation and results in concentrated biomass unless substrate inhibition occurs. In microbial oil production, in particular, a high

level of biomass concentration is essential for the ease of downstream processing such as harvesting, dewatering, and extracting (Chen et al., 2010).

Fig. 2 shows the daily performance of CFSTR in terms of cell concentration and lactate degradation at HRT 4 d. As lactate concentration increased from 30 mM to 60 mM, cell concentration continuously increased from 0.97 g dcw/L to 2.05 g dcw/L. The experimental period used to obtain average performance values are shown in Table 1. At 70 mM, however, cell concentration started to fluctuate with incomplete substrate degradation, suggesting substrate inhibition. At similar substrate strength, the inhibition has been observed in the continuous culture of heterotrophic microalgae and photosynthetic bacteria (Chen and Johns 1996; Xie et al., 2012). In the CFSTR operation of *R. sphaeroides* fed with lactate at HRT 4 d, the highest cell concentration of 2.05 g dcw/L and cell productivity of 0.54 g dcw/L/d were obtained at 60 mM. Cell productivity was calculated by dividing cell concentration to the corresponding CRT.

It appears that cell yield, the conversion ratio of input substrate to cell growth, was constant at around 50%, although the substrate concentration changed. Cell yield was calculated by assuming the composition of $C_5H_7O_2N$, resulting in a chemical oxygen demand (COD) value of 1.42 g COD/g dcw (Kim et al., 2012). The reported cell yield of photosynthetic bacteria varied as 20-70% depending on the carbon sources, nutrient composition, and operational parameters such as pH and HRT (Kim et al., 2012; Yilmaz et al., 2010). 50% cell yield meant that half of the electrons contained in the substrate were used for cell growth while remaining electrons were converted to other metabolites such as soluble microbial products (SMPs). SMPs are defined as soluble organic compounds produced during substrate metabolism and biomass decay and they have been important research topics in biological wastewater

treatment for more than two decades (Laspidou and Rittmann, 2002). However, it was only recently recognized in the field of using photosynthetic bacteria that SMPs have a sizable impact on the electron distribution (Kim et al., 2012; Yilmaz et al., 2010). Further researches on SMP issue such as formation mechanisms, characteristics, and metabolic and genetic control strategies are needed to increase the cell yield.

3.2 Membrane-coupled bioreactor performance - Effect of substrate concentration

In the concept of a membrane-coupled bioreactor, a complete retention of biomass by membrane process makes it possible to maintain a high biomass concentration, and thus, a stable performance under high substrate loading can be expected (Judd, 2008).

In this study, first, lactate concentration gradually increased from 40 to 100 mM while the HRT and CRT were fixed at 4 d and 20 d, respectively. The cell concentration gradually increased and reached 14.38 g dcw/L at 100 mM (Fig. 3). Compared to the cell concentration in the CFSTR, this value was seven times higher, which could significantly reduce the cost in the downstreaming process (Table 2). To our knowledge, this is the highest cell concentration achieved to date in various application fields of photosynthetic bacteria. In photo-fermentative H_2 production, cell concentration was maintained at a low level (generally<3 g dcw/L) since light penetration is essential for the activation of nitrogenase, the central enzyme producing hydrogen (Pattanamanee et al., 2012). In wastewater treatment using photosynthetic bacteria, several attempts have been made to increase cell concentration, but the result was less than 5 g dcw/L (Kaewsuk et al., 2010).

The reactor performance significantly dropped immediately after increasing lactate concentration to 120 mM with incomplete lactate degradation. It seemed that *R. sphaeroides*

could not be adapted at this high substrate concentration and the cell concentration decreased by the pullout of biomass for CRT control. To recover the performance, lactate concentration decreased to 100 mM at further operation.

3.3 Membrane-coupled bioreactor performance – HRT and CRT control

Although high cell concentration was achieved by installing a membrane unit, cell productivity was only increased by 30% compared to the reactor performance without membrane, due to long CRT of 20 d (4 d for reactor without membrane). The way to increase cell productivity is to reduce CRT while increasing the substrate loading. As larger amount of biomass is removed from the reactor at short CRT, more substrate should be supplied to maintain the biomass concentration in the reactor. From the 193th day, HRT and CRT were gradually shortened to 1 d and 5 d, respectively, while keeping the CRT/HRT ratio at 5, meaning that the same amount of substrate was supplied to keep the biomass concentration unless the cell yield changed.

As HRT and CRT gradually shortened to 2 d and 10 d, respectively, both cell concentration and cell productivity increased up to 16.20 g dcw/L and 1.62 g dcw/L/d, respectively (Fig. 2, Table 2). As the CRT/HRT ratio was kept at the same substrate concentration, the increased cell concentration indicates the increased cell yield. In the application of membrane-coupled bioreactor, high cell yield has generally been observed under short CRT (Duan et al., 2009; Huang et al., 2001). At HRT 1.5 d and CRT 7.5 d, the cell concentration decreased to 14.29 g dcw/L with a small amount of lactate remaining. However, the overall mass of cultivated biomass was enhanced to 1.90 g dcw/L/d, which was 3.5 times higher than that in the CFSTR operation. Further decrease of HRT and CRT to 1 d and 5 d, respectively, caused the failure

of reactor operation. Moreover, the trans membrane pressure (TMP), which was maintained less than 20 kPa during the previous operation, increased up to 30 kPa. Because of membrane fouling, it was impossible to continue the reactor operation. The flux could be calculated based on the effective surface area of membrane module, 0.006 m^2. The calculated flux at HRT 4, 2, 1.5, and 1 d are 1.74, 3.47, 4.65, and 6.94 $L/m^2/h$, respectively. The significant increase of TMP was not observed during the operation periods under the HRT 4 to 1.5 days, but the significant membrane fouling was occurred at right after reactor operation under the HRT 1 d.

Using the stepwise increase of flux, the critical flux could be obtained. Critical flux is a criterion for the transition between concentration polarization and fouling. The critical flux is reached when irreversible fouling occurs locally on the membrane (Bacchin, 2004). The reactor was operated under an intermittent filtration regime (45 min filtration/15 min pause), and no irreversible fouling was observed under the flux of 1.74 - 4.65 $L/m^2/h$. On the other hand, a significant increase of TMP was observed under the flux of 6.94 $L/m^2/h$. Therefore, the critical flux for this membrane module and reactor system can be determined as 4.65 $L/m^2/h$.

3.4 Fatty acids profile and the performance comparison

The fatty acids profile of *R. sphaeroides* is shown in Fig. 4. During the membrane-coupled bioreactor operation, the microbial samples for fatty acid analysis were taken five times after reaching a steady-state at each substrate concentration, and the average values were obtained. The fatty acids content of *R. sphaeroides* investigated here was around 35% of the dry cell weight, and did not vary according to the substrate concentration. Although some microalgae

species can accumulate fatty acids over 50% of dry cell weight, it is obvious that *R. sphaeroides* are also suitable biomass resources for fatty acids generation. Ratledge and Wynn (2002) defined the microorganisms that can accumulate fatty acids at more than 20% of their body as oleaginous species. In addition, unlike phototrophic microalgae, dense cell cultivation was possible in the case of *R. sphaeroides* which was attributed their ability to capture every possible photon with high efficiency under light low intensity (Adams and Hunter, 2012). It is known that intracytoplasmic membranes (ICMs) containing structural components necessary for photosynthetic growth are developed when the light resource is limited.

The main fatty acid compositions of *R. sphaeroides* were C16-C18, which are appropriate for biodiesel production. In addition, the fatty acids from *R. sphaeroides* have special features in that they are mainly composed of unsaturated fatty acids whereas saturated forms are dominant in other microorganisms including microalgae, cyanobacteria, and *Escherichia-coli* (Chen et al., 2010; Lu, 2010; Steen et al., 2010). Saturated fatty acids have good oxidative stability, but poor fuel properties at low temperatures, which is a disadvantage in winter operation (Park et al., 2008). Also, unsaturated fatty acids are the raw materials for dietary supplements such as omega-3, omega-6, and omega-7 fatty acids. Vaccenic acid, comprising 60% of fatty acids of *R. sphaeroides*, is a main source of omega-7 fatty acids, which has a crucial role in maintaining the health of skin and mucous membranes.

From the continuous cultivation of *R. sphaeroides* using the membrane-coupled bioreactor, maximum cell concentration of 16.2 g dcw/L, cell productivity of 1.9 g dcw/L/d, and fatty acid productivity of 665 mg FA/L/d were attained, and these values were comparable with those achieved using microalgae (Table 3). Fatty acids productivity, representing the

combined effects of fatty acid content and cell productivity, is a suitable index to indicate the effectiveness of a process (Chen et al., 2011). In the case of fatty acids production by photoautotrophic microalgae, continuous and pilot-scale operation has frequently been reported. The fatty acids productivity was in a range of 30-180 mg FA/L/d, which was comparably lower than that achieved in this study. On the other hand, higher cell productivity and fatty acids productivity have been reported in the heterotrophic microalgae cultivation. However, most of them were obtained from the batch operation.

In microalgae cultivation, the membrane has only been applied for the harvesting of cultured microalgae as an extracting process even though it has potential to integrate the cultivation and harvesting process together. In this study, the membrane-coupled bioreactor for cultivation and harvesting of photosynthetic bacteria was successfully operated continuously. The productivity of cells and fatty acids was higher than photoautotrophic microalgae and it was comparable with the microalgae production under heterotrophic conditions. It is suggested that the novel method developed here that is cultivating photosynthetic bacteria using membrane-coupled bioreactor could be an alternative way of microbial fatty acids production.

4. Conclusions

Photosynthetic bacteria, *R. sphaeroides*, were continuously cultivated for fatty acids production. By adopting a membrane-coupled bioreactor operation mode, a maximum cell concentration of 16.2 g dcw/L, cell productivity of 1.9 g dcw/L/d, and fatty acids productivity of 665 mg FA/L/d were attained, which were comparable with those achieved using microalgae. The fatty acids content of *R. sphaeroides* was around 35% of the dry cell weight

with mainly composed of unsaturated fatty acids, in particular, vaccenic acid (C18:1, omega-7). This work has high importance in the perspective of introducing a novel approach for microbial fatty acids production.

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Table 1 Average cell production performances at various substrate concentrations in the CFSTR

Substrate concentration (mM)	Data acquired (thday)	Cell concentration (g dcw/L)	Cell yield (%)	Cell productivity (g dcw/L/d)	Lactate degradation (%)
30	6-27	0.97±0.06	47.6±1.9	0.24±0.02	99.7
40	33-54	1.39±0.04	51.5±1.4	0.35±0.01	99.9
50	58-79	1.70±0.05	50.4±1.5	0.43±0.01	99.7
60	85-106	2.05±0.10	50.5±2.5	0.51±0.01	99.9

Table 2 Average cell production performances at various operation conditions in the membrane-coupled bioreactor

Substrate concentration (mM)	HRT, SRT (day)	Data acquired (thday)	Cell concentration (g dcw/L)	Cell productivity (g dcw/L/d)	Lactate degradation (%)
40	4, 20	19-41	5.77±0.17	0.29±0.01	99.9
60	4, 20	49-81	8.74±0.36	0.44±0.22	99.9
80	4, 20	91-122	11.27±0.37	0.57±0.01	99.9
100	4, 20	134-162	14.38±0.33	0.72±0.02	99.9
	3, 15	210-232	15.14±0.26	1.01±0.02	99.9
	2, 10	248-272	16.20±0.12	1.62±0.01	99.9
	1.5, 7.5	284-309	14.29±0.29	1.90±0.04	97.2

Table 3 Performance comparison of fatty acids productivity obtained in this study with microalgae

Cultivation conditions	Microbial species	Cell productivity (g dcw/L/d)	Fatty acids content (% of dcw)	Fatty acid productivity (mg FA/L/d)	Reactor and operation	Reference
Photo -heterotroph	*Rhodobacter sphaeroides*	1.9	35	665	Continuous (1 L) (HRT 1.5 d, CRT 7.5 d)	This study
Photo -autotroph	*Chlorella* sp.	0.53	34	178.8	Fed-batch (0.8 L)	Chiu et al., 2008
	Chlorella sp.	3.8	N/A[a]	N/A[a]	Fed-batch (400 L) Flat plate type	Doucha et al., 2005
	Chlorella vulgaris, *Scenedesmus* sp, etc.	0.17 - 0.28	16.1 - 21.1	30.4 - 53.9	Batch (0.25 L)	Rodolfi et al., 2009
	Nannochloropsis sp.	0.36	32.3	117	Continuous (110 L) (HRT 2.5 d)	Rodolfi et al., 2009
	Chlorella minutissima	0.137	36 - 44	60.2	Continuous (3 L) (HRT 3 d)	Tang et al., 2012
	Synechocystis aquatilis	0.932	18.58	177.1	Continuous (8 L) (HRT 8.5 d)	Kaiwan-arporn et al., 2012
Heterotroph	*Chlorella protothecoides*	1.7 - 7.4	43.0 - 57.8	732.7 - 3701.1	Batch (5 L)	Xiong et al., 2008
	Chlorella vulgaris	0.08 - 0.15	23.0 - 36.0	27.0 - 35.0	Batch (1 L)	Liang et al., 2009
	Galdieria sulphuraria	14.6 - 50	N/A[a]	N/A[a]	Continuous (3 L, HRT 2 d)	Graverholt and Eriksen, 2007

[a]N/A = Not attained

Fig. 1 Schematic of membrane-coupled bioreactor system for mass cultivation of photosynthetic bacteria

Fig. 2 Daily variation of cell concentration and lactate degradation in the CFSTR at various substrate concentrations

Fig. 3 Daily variation of cell concentration and lactate degradation in the membrane-coupled bioreactor at various operating conditions

Fig. 4 Fatty acids profile of *Rhodobacter sphaeroides*

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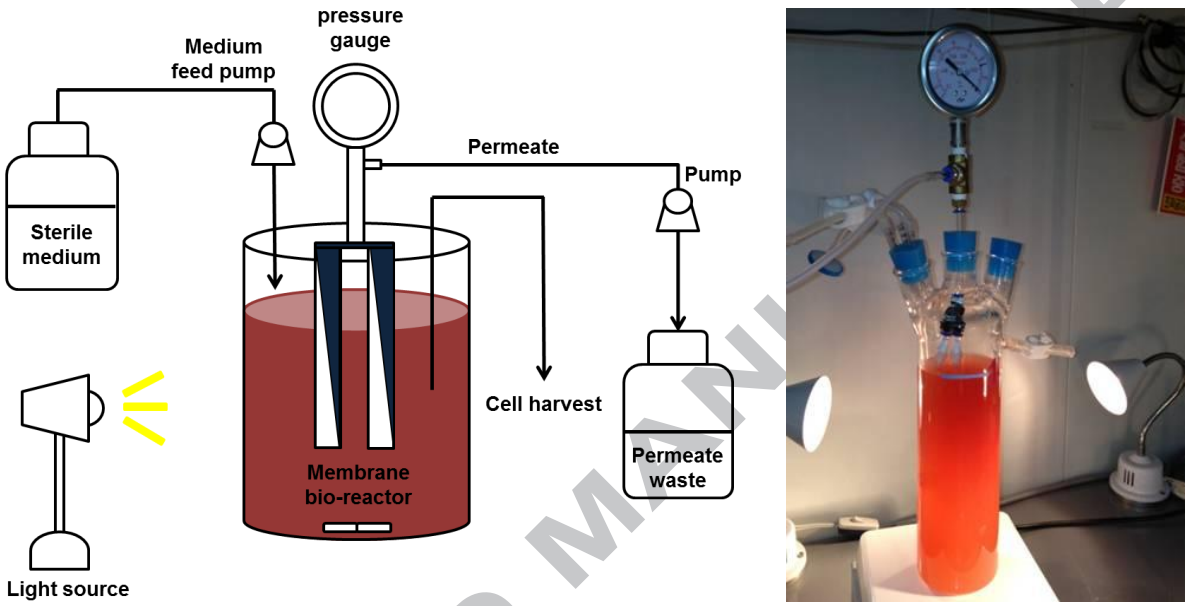

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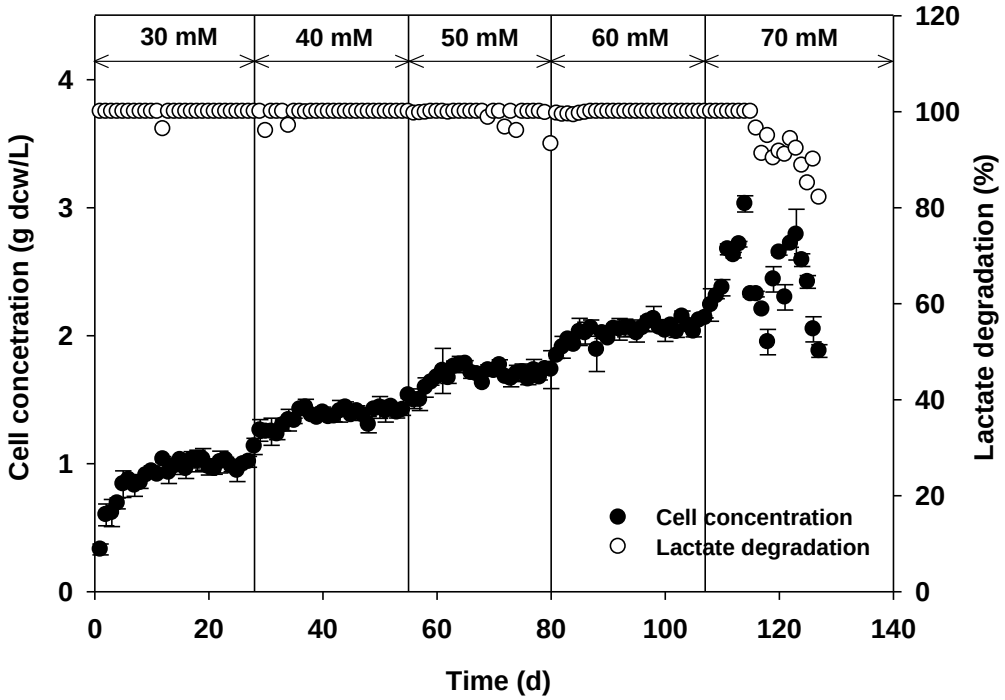


Fig. 3 Daily variation of cell concentration and lactate degradation in the membrane-coupled bioreactor at various operating conditions

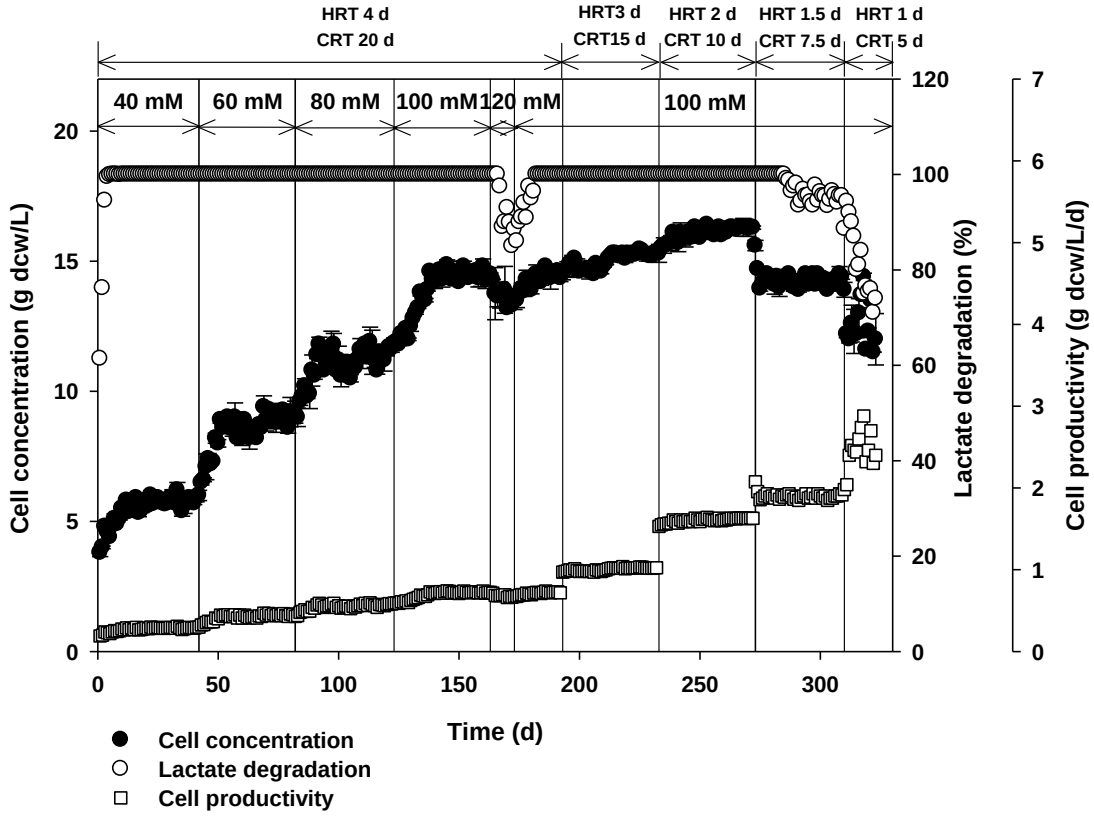
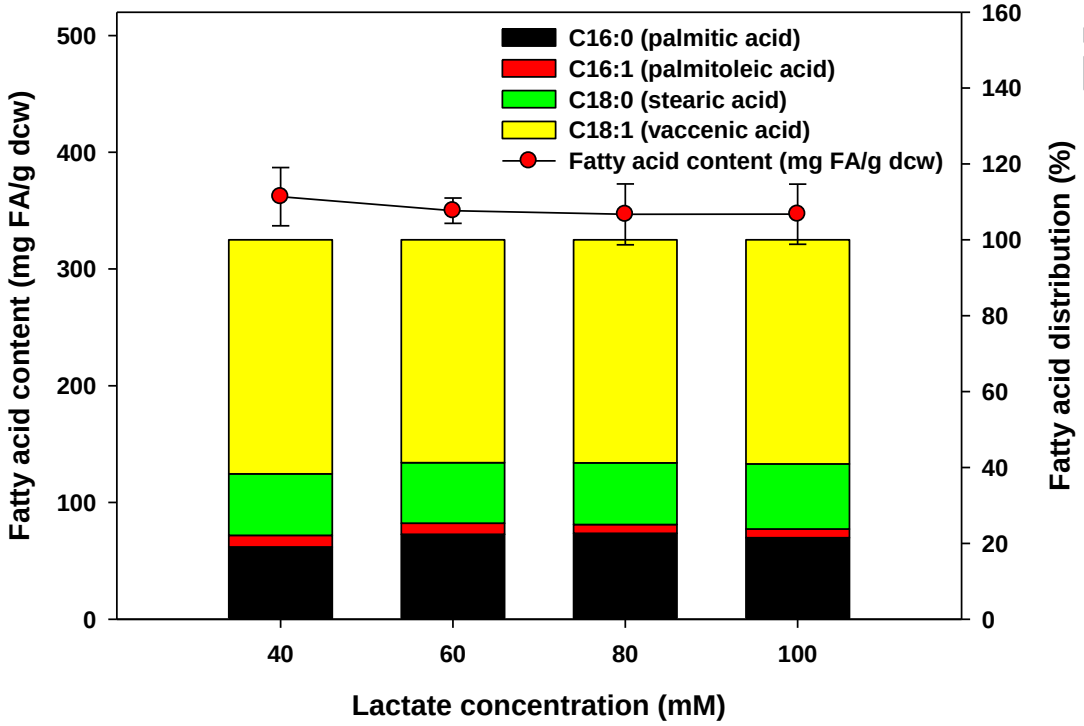
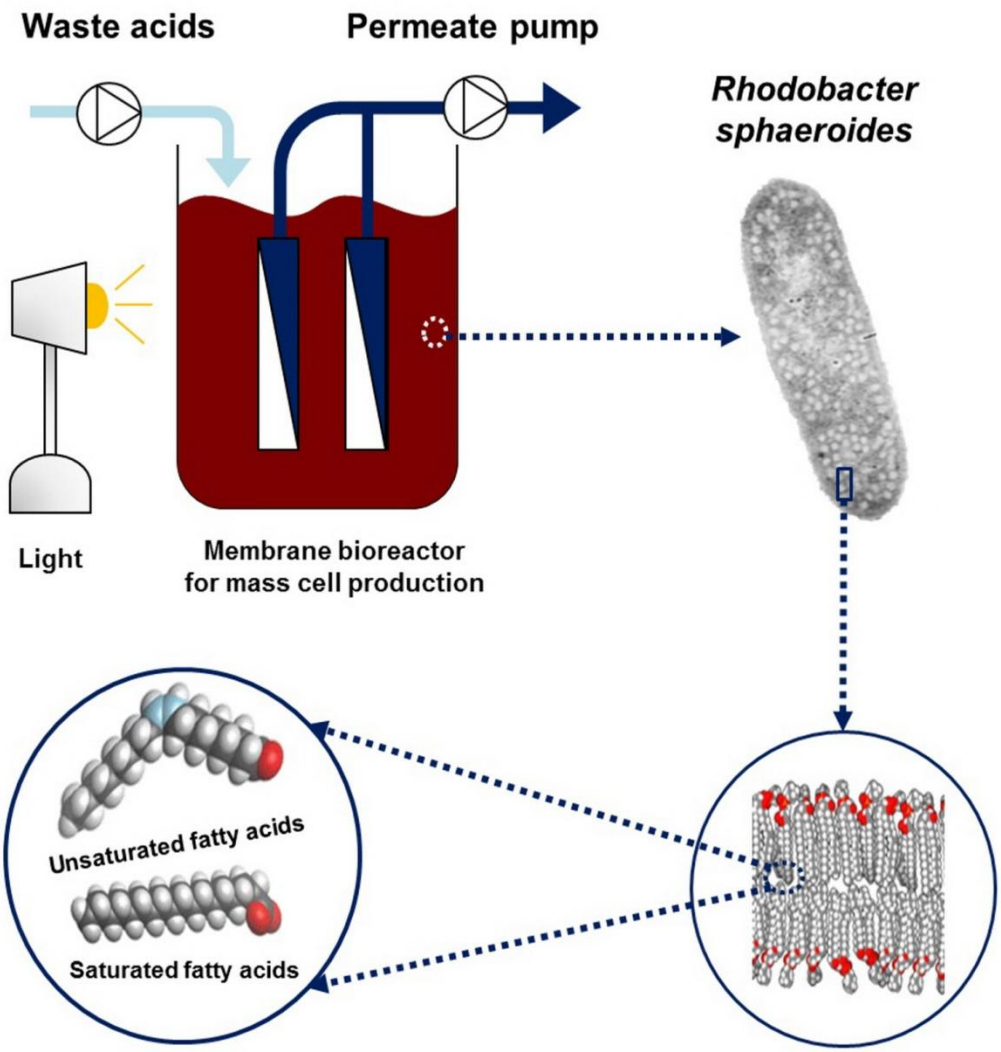


Fig. 4 Fatty acids profile of *Rhodobacter sphaeroides*





Highlights

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- Photosynthetic bacteria cultivation for fatty acids (FA) production from lactate
- Continuous-flow, stirred-tank reactor and membrane-coupled bioreactor
- Maximum cell productivity of 1.9 g dcw/L/d and FA productivity of 665 mg FA/L/d