Biochemical Engineering Journal 204 (2024) 109239

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Regular article

Fermentation strategies for PHB production in a novel membrane bioreactor: Investigating batch and fed-batch operations

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ARTICLE INFO

Keywords:
PHB production
Gas fermentation
Membrane bioreactor
Cupriavidus necator H16

ABSTRACT

Gas-transfer membranes have been successfully deployed as efficient aeration devices in wastewater treatment. There is an increasing interest in using such membrane technology in industrial biotechnology. This study proposes membrane bioreactors as a novel bioreactor setup for polyhydroxybutyrate (PHB) production using *Cupriavidus necator*, whereby gas-transfer membranes are used for aeration. A proof-of-concept membrane bioreactor was built by combining a 50 ml centrifuge tube with hollow fiber membrane bundles. Different numbers and length of polydimethylsiloxane (PDMS) hollow fiber membranes were used to create membrane bundles with varying specific surface areas for oxygen transfer. In batch mode, a maximum biomass concentration of 10.3 g/L, which corresponds to a yield of 0.67 g biomass/g substrate, was achieved with 250 m^2/m^3 as the specific surface area of the membranes and 40 rpm as the liquid recirculation rate. Two different fed-batch modes were investigated to induce PHB production by applying nitrogen source limitation via fill-and-draw and two-step feeding strategies. A PHB level of 22% was obtained with fill-and-draw feeding by supplying 0.25 g/L NH_4Cl after initial cultivation. Results indicate that membrane bioreactors are promising for *C. necator* cultivation, but further research is needed to enhance the PHB productivity.

1. Introduction

Polyhydroxybutyrate (PHB) is an intracellular carbon and energy storage material that is synthesized by many microorganisms, and it can be used to create biodegradable plastic. Under nutrient limitations such as nitrogen or phosphorus, microorganisms have a tendency to increase their production of PHB [1]. Among various microorganisms that can produce PHB, *Cupriavidus necator* (also referred as *Wautersia eutropha* and *Ralstonia eutropha*) stands out as the most extensively studied bacterial strain. This is due to its capacity to utilize a wider range of carbon sources, ability to accumulate larger quantities of PHB and its known genomic information [2]. *C. necator* can utilize various organic and inorganic carbon sources such as fructose, formic acid and carbon dioxide (CO_2). For cells to utilize CO_2 in their metabolism, hydrogen (H_2) and oxygen (O_2) gas are also required. The optimum gas composition ratio for cell growth is reported as 7:2:1 for H_2:O_2:CO_2 which is within the gas-explosion range [3]. To eliminate the explosion risk, one strategy is to keep the oxygen concentration below the lower explosion limit (6.9% v/v in the gas mixture) [4]. However, this would limit microbial growth and overall PHB productivity because of the limited availability of oxygen.

In general, gas fermentation encounters substrate limitation challenges due to the low solubility of gases in the liquid culture medium. Continuous stirred tank reactors (CSTR) are the most commonly used bioreactor types for gas fermentation [5]. However, CSTRs demand a lot of energy to reach high agitation speeds for increasing gas-to-liquid mass transfer [6]. Thus, there is ongoing research on designing bioreactors that would improve gas fermentation processes. In the literature, there are previous examples of different bioreactor designs to enhance gas transfer efficiencies for improving PHB production such as bubble column reactor [7], airlift bioreactor [8] and pressurised bioreactor [9]. Membranes were suggested as gas delivery devices for such applications due to their potential to help achieve high gas transfer efficiencies at low gas supply rates [10]. Previously, they have been successfully implemented as aeration devices for aerobic bioprocesses and oxidation reactions for wastewater treatment [11]. Membranes are also proposed as safe gas delivery devices for delivering explosive gas mixtures via diffusion for producing various chemicals [12]. Therefore, membrane

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https://doi.org/10.1016/j.bej.2024.109239
Received 21 November 2023; Received in revised form 25 January 2024; Accepted 26 January 2024
Available online 29 January 2024

bioreactors are proposed for PHB production processes where membranes are employed to enhance gas transfer.

Currently, commercialization of PHB faces challenges such as high production cost and performance loss from the requirement for large-scale operations to meet the market demand [13]. In industrial biotechnology, large-scale operation can be obtained by either adopting a scale-up or a scale-out approach. Scale-up involves the use of larger vessels to increase production capacity whereas scale-out systems increase production capacity by using multiple vessels in parallel [14]. Using the scale-out approach enables flexible process design and eliminates the product quality and process performance risks that occur with scale-up [15]. Furthermore, using the scale-out approach provides easier handling, better time management and reduces operational costs due to use of less media and smaller harvest volumes at downstream operations [16]. Therefore, a small-scale membrane bioreactor setup for PHB production is proposed, which has the potential to be employed in large-scale PHB production through the scale-out strategy.

In this work, a novel gas-transfer membrane bioreactor system was developed and characterized in terms of oxygen transfer for aeration, mixing and microbial growth using *Cupriavidus necator*. Various operating parameters were tested for batch operation and optimized for maximizing the biomass. Furthermore, fed-batch operation mode was investigated using the same setup and the effect of different feeding strategies on PHB production was studied.

2. Materials and methods

2.1. Membrane module fabrication

Dense polydimethylsiloxane (PDMS) hollow fiber membranes with 300 μm inner diameter (ID) and 500 μm outer diameter (OD) were used to fabricate the membrane bundles. Different numbers of membranes at different lengths were fabricated depending on the targeted specific surface area as shown in Table 1. Membrane bundles were potted into 6 mm OD (4 mm ID) nylon tubing using translucent epoxy resin (MG Chemicals) and excess length of tubing was cut with a sharp scalpel to expose the hollow fiber membrane lumens for gas transfer. Fig. 1 shows the membrane bundles and the cross-sectional view of one of the ends.

2.2. Membrane bioreactor design

The membrane bioreactors were built by modifying 50 ml centrifuge tubes (Merck) with silicone grommets to fit membrane modules and tubing for liquid recirculation. 6 mm OD Tygon tubing and 6 mm OD nylon tubing were used for connecting liquid and gas lines, respectively. The membrane module can be placed inside the modified tube in different configurations. Fig. 2 shows the modified centrifuge tube with a membrane module.

A control tube without membranes was filled with media and inoculated to determine whether any gas leakage occurred from any part of the modified tube. No growth was observed after 72 h of cultivation (optical density stayed at 0.12) which ensures that the design is robust where membranes are the only aeration source for microbial growth.

Table 1
Membrane module properties and calculated specific surface areas for gas transfer.

Module #	Number of membranes	Length (cm)	Specific surface area (m^2/m^3)
Module 1	10	10	30
Module 2	10	20	60
Module 3	20	20	125
Module 4	28	28.5	250

2.3. Bacterial strain, medium preparation and flask cultivation

Stock cultures of *Cupriavidus necator* H16 were maintained in 25% (v/v) glycerol at −80 °C. The strain was activated using nutrient rich Luria-Bertani (LB) agar plates in a 30 °C incubator. Precultures were prepared by transferring a single colony to 50 ml LB media in a 250 ml Erlenmeyer flask and cultivated overnight in a shaking incubator at 200 rpm and 30 °C. Minimal salts media (MSM) was used for the membrane bioreactor experiments which was prepared using (per litre) 9 g $Na_3PO_4.12H_2O$, 1.5 g KH_2PO_4 and 1 g NH_4Cl, 1 ml $MgSO_4.7H_2O$ (1 M stock solution; added after autoclaving) and 1 ml trace elements (per litre: 4 g $ZnSO_4.7H_2O$; 1 g MnCl .$4H_2O$; 0.2 g $Na_2B_4O_7.10H_2O$; 0.3 g $NiCl_2.6H_2O$; 1 g $Na_2MoO_4.2H_2O$; 1 g CuCl .$2H_2O$; 7.6 g $FeSO_4.7H_2O$; added after autoclaving). After autoclaving at 121 °C for 15 min, the media was supplemented with the separately autoclaved fructose as carbon source to the desired final concentration.

Flask experiments of *Cupriavidus necator* H16 were performed by using 50 ml MSM in a 250 ml Erlenmeyer flask and precultures cultivated overnight in LB media. Overnight cultures were washed with MSM and transferred to flasks with MSM and fructose concentrations of 5, 10, 15 and 20 g/L to an initial optical density of 0.1.

2.4. Oxygen transfer rate measurement

The oxygen transfer rate (OTR) of the membrane bioreactors was measured using a dissolved oxygen (DO) probe (CellOx® 325, WTW). The liquid was first sparged with N_2 until the dissolved oxygen concentration dropped to zero. Air was then introduced through the membranes at 0.5 L/min and DO readings were recorded every minute until it reached the oxygen saturation point. Every experiment was run in duplicate. The OTR was calculated using the following equation [17]:

$$\frac{dC}{dt} = k_L a \quad (C^* - C_i) \tag{1}$$

Where C_i is the oxygen concentration in the liquid phase (mg/L) at any given time (s), C* is the saturated oxygen concentration (mg/L) and k_La is the volumetric mass transfer coefficient (1/s). After solving and replacing the initial conditions, Eq. 1 can be further simplified to the following:

$$\ln\frac{(C^* - C_0)}{(C^* - C_i)} = (k_L a)t \tag{2}$$

Where C_0 is the initial oxygen concentration in the liquid phase (mg/L).

2.5. Membrane bioreactor operation

2.5.1. Batch operation

Membrane bioreactors were operated in batch mode for 48 – 72 h, until the highest biomass concentration was reached. The temperature was maintained at 30 °C by placing the membrane bioreactor setup in a large incubator as shown in Fig. 3. Mixing was obtained by either recirculating the liquid using a peristaltic pump (Watson Marlow 323, RS) with a liquid flowrate of 105, 210, 315 and 520 ml/min or a shaker at 100 and 250 rpm. The initial fructose concentration for all experiments was 15 g/L. Air flowrate inside the membranes were kept at 0.5 L/min. Samples (0.8 – 1 ml) were withdrawn periodically to determine biomass, residual fructose and ammonium concentrations.

2.5.2. Fed-batch operation

Two different fed-batch modes were tested in this study. Both were initiated as batch mode with initial operating conditions the same as those of batch cultivation. The first fed-batch operation started after 33 h of batch cultivation. Biomass was collected via centrifuging at 9000 rpm for 5 min and pellets were resuspended in MSM with limiting nitrogen conditions. Two different limiting nitrogen concentrations

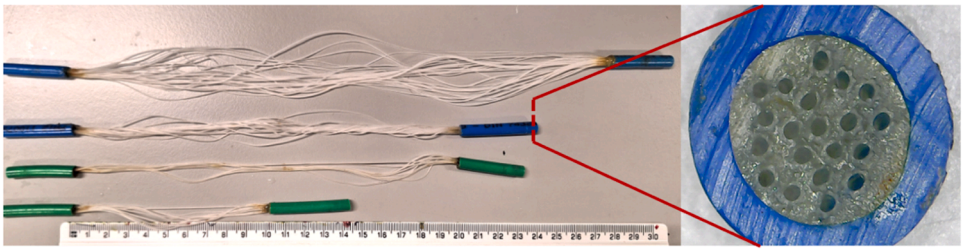

Fig. 1. Hollow fiber membrane modules used in the study and the cross-section of one of the module ends.

Fig. 2. Modified 50 ml centrifuge tube with silicone grommets to connect membrane bundles and tubing.

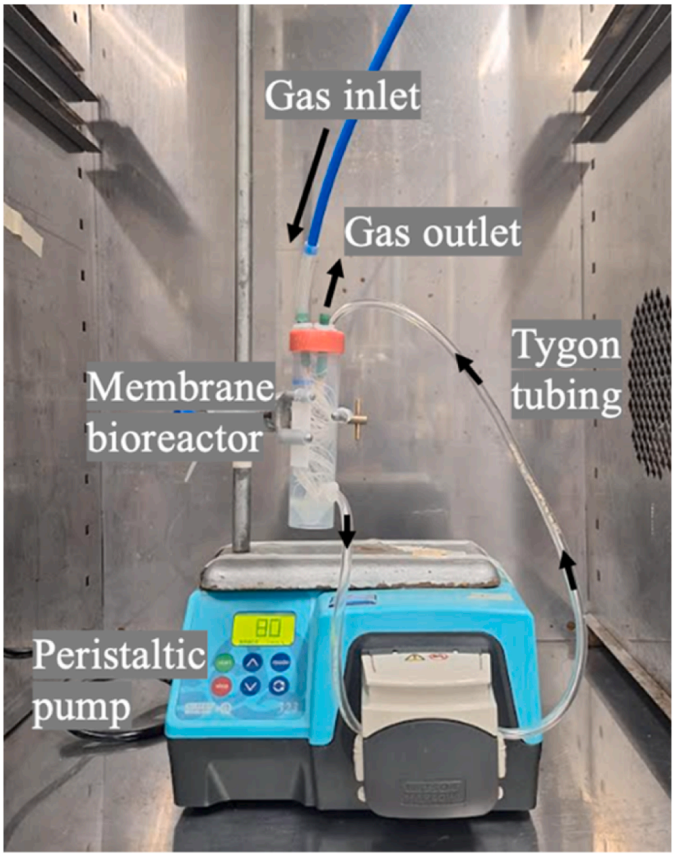

Fig. 3. Batch mode setup for the membrane bioreactor with pump in a large 30 °C incubator.

were made by preparing MSM with 0.25 and 0 g/L NH_4Cl. Resuspended biomass was cultivated for another 72 h and samples were withdrawn periodically.

The second fed-batch operation was based on a fill-and-draw strategy. After operating the membrane bioreactors in batch mode for 22 h, half of the fermentation broth (25 ml) was collected for sampling and fresh media with initial carbon concentration (15 g/L fructose) and limiting nitrogen concentrations of 0.25 g/L and 0 g/L NH_4Cl were added into the bioreactor every 12 h 5 times. A third condition where 0.25 g/L NH_4Cl and 30 g/L fructose was also added to study different carbon to nitrogen ratios on PHB production. After the last feeding, final samples were collected after 24 h.

2.6. Analytical methods

2.6.1. Biomass

Optical density of cells was detected for assessing cell growth at a wavelength of 600 nm using a spectrophotometer (Libra S11, Biochrom). Cell concentration was measured by collecting samples of

known volumes and harvesting bacterial cells by centrifugating the sample at 14000 x g for 3 min. The cell pellet was frozen at −80 °C and lyophilized (Labconco, Fisher Scientific) until dry. Dry pellets were weighed to obtain cell dry weight (CDW) and biomass concentration was calculated. The supernatant was retained for fructose and ammonium concentration analysis.

2.6.2. HPLC analysis

Fructose concentration was determined using a high-performance liquid chromatography (HPLC) system equipped with RID-10A refractive index detector (Shimatzu) at 50 °C. Appropriate dilutions of the substrates or culture supernatant were prepared and 20 µl of the sample was injected into the column after the culture supernatant was filtered through Mini-UniPrep syringeless filter devices (Agilent). The fructose was analysed by Aminex-87 H column (Bio-Rad) at 40 °C. The samples in the column were eluted with 0.014 N H_2SO_4 at a flow rate of 0.55 ml/min and pressure of 4.3 MPa. Quantification was achieved by reference to a fructose standard curve [18].

2.6.3. Ammonium determination

Ammonium concentration in the liquid phase was determined by analyzing the supernatant using Hach-Lange cuvette test kits (LCK303). Samples were diluted according to the concentration range of the test kit which was 2 – 47 mg/L.

2.6.4. PHB determination

The polymer content and the monomer composition of the polymer were determined by Gas Chromatography (GC) and confirmed by GC–Mass Spectroscopy (GC–MS). Approximately 5 – 10 mg of lyophilized cells were subjected to acidic methanolysis and the resultant 3-hydroxyalkanoic acid methyl esters were assayed using an Agilent 6890 N chromatography equipped with a HP-INNOWAX capillary column (30 m by 0.25 mm with 0.5 mm of film thickness) and a flame-ionization detector (FID). A temperature program of 120 °C for 4 min (a first temperature ramp of 10 °C per minute up to 170 °C, a second temperature ramp of 40 °C per minute up to 230 °C) was applied for determining PHB concentration [19].

3. Results

3.1. Membrane bioreactor design

Membrane module could be placed within the centrifuge tube in various configurations. To determine the optimal setup, three different configurations were examined. Fig. 4 shows different configurations to connect membrane modules to the modified centrifuge tubes.

Although Fig. 4a is the closest to a conventional membrane bioreactor design, Fig. 4c is used in the following sections since higher surface area for gas transfer is achieved by attaching longer membranes. The tubing for liquid inlet was used to hook the membranes, preventing them from floating. Fig. 5 shows the appearance of a membrane module with 20 cm length fibers, inside the centrifuge tube under different configurations.

When the configuration in Fig. 5b is used, the membrane fibers lose effective surface area for gas transfer due to the placement of the membrane module in the centrifuge tube. This difference is negligible for membrane modules with shorter membranes.

3.2. Oxygen transfer rate measurements

The effect of liquid recirculation rate and air flowrate on oxygen transfer rate was investigated by comparing the volumetric mass transfer coefficients (k_La) using the membrane bioreactor configuration in Fig. 4c. Fig. 6 shows the experimental k_La values using the membrane module with 20 hollow fiber membranes at different liquid flowrates where Fig. 7 shows the change in k_La with different air flowrates inside the membrane fibers with a constant liquid recirculation flowrate of 210 ml/min.

Within the range of investigation, the air flowrate within the lumen of the fibers did not have a major impact on the oxygen mass transfer coefficient. However, liquid recirculation did have a significant positive effect on the mass transfer rate. 40, 80, 120 and 200 rpm was tested which corresponded to 105, 210, 315 and 520 ml/min liquid flowrate, respectively. As the liquid recirculation flowrate increases, oxygen transfer rate increases which could be explained by the liquid boundary layer at the liquid side [22]. As the liquid flowrate increases, the resistance to mass transfer by liquid boundary layer decreases. Thus, k_La increases as the liquid flowrate increases.

3.3. Growth of Cupriavidus necator H16

The growth of *C. necator* using different fructose concentrations was investigated to find the optimum conditions. Flask experiments were employed for this purpose due to their operational simplicity. Fig. 8 shows growth of *C. necator* in flasks using various concentrations of fructose and Table 2 shows the final biomass obtained and corresponding biomass yields. Although the results are close, the highest biomass yield on substrate was obtained when 5 g/L fructose is used. Although a higher yield is achieved when 5 g/L fructose is used, 15 g/L is preferred to obtain a higher biomass concentration for PHB analysis.

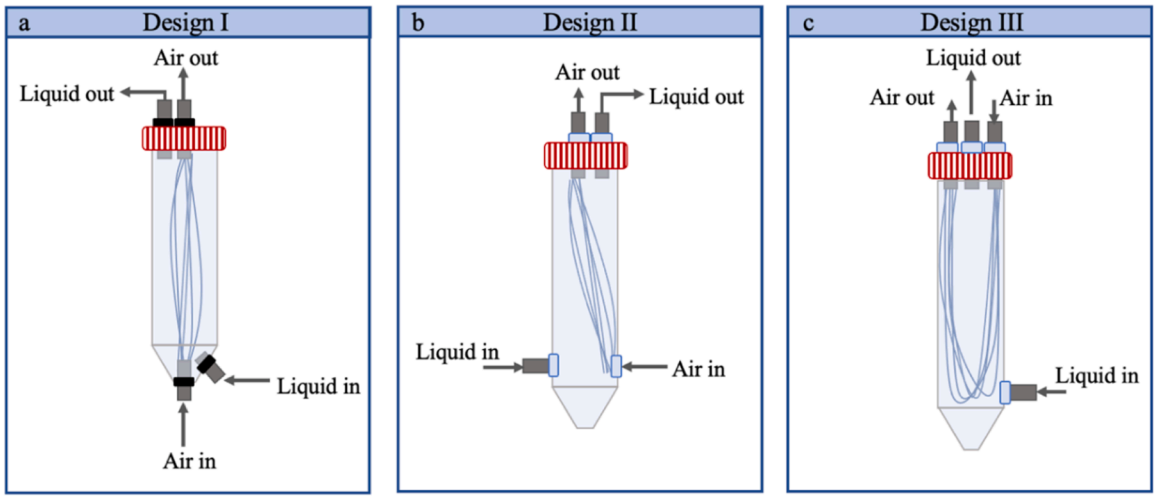

Fig. 4. Different configurations of attaching membrane modules into the centrifuge tubes.

Fig. 5. Different configurations of a membrane module with 20 cm long hollow fiber membranes in a modified centrifuge tube a) U-shaped configuration with both ends on the lid, b) I-shaped configuration with ends at opposite sides.

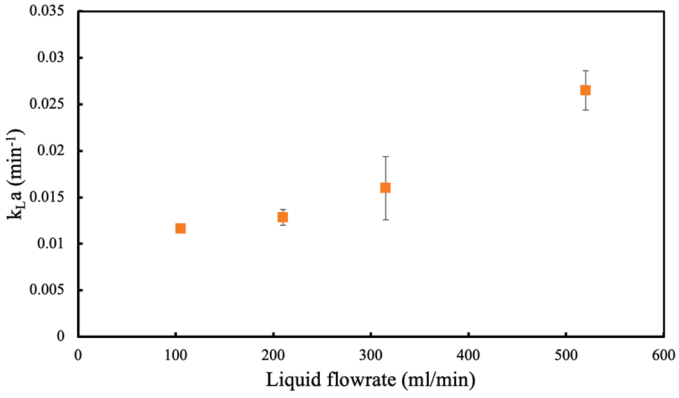

Fig. 6. Effect of liquid recirculation flowrate on volumetric mass transfer coefficient (k_La) in deionized water.

Thus, 15 g/L fructose was chosen as the fructose concentration for the subsequent experiments.

The stoichiometric equation for biomass growth when 15 g/L fructose is utilized is calculated as follows:

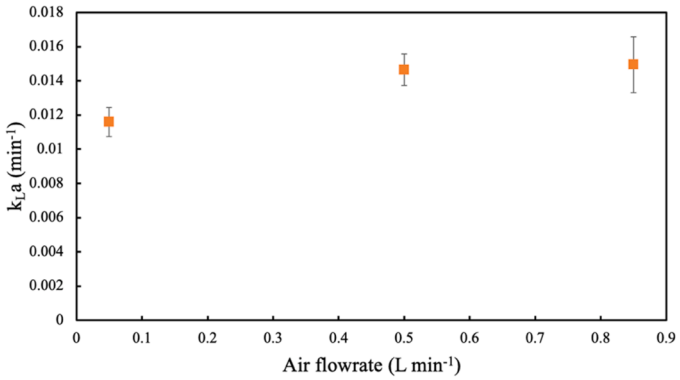

Fig. 7. Effect of air flowrate on volumetric mass transfer coefficient (k_La) in deionized water.

$$C_6H_{12}O_6 + 2.81O_2 + 0.57NH_3 \rightarrow 3CH_{1.74}O_{0.46}N_{0.19} + 3CO_2 + 4.24H_2O \quad (3)$$

where $CH_{1.74}O_{0.46}N_{0.19}$ is the biomass formula with a molecular weight of 25.35 g per mole [20]. Depending on this equation, to produce 1 mol of biomass, 0.94 moles of oxygen need to be utilized.

3.4. Batch operation

3.4.1. Mixing

To achieve uniform mixing in the laboratory-built membrane bioreactor, a shaker and a pump were compared. Their effectiveness was tested indirectly by comparing the maximum amount of biomass obtained after 30 h of batch cultivation. Membrane modules with 10, 20 and 28 hollow fiber membranes at the same length were used for aeration (see Table 1) and the results are shown in Fig. 9).

The highest biomass concentration was obtained when 28 hollow fiber membranes were used for aeration and a pump at 80 rpm was used for mixing. When comparing the shaker to the pump, it became evident that employing a shaker did not provide adequate mixing conditions. This had a direct impact on aeration and the resulting biomass. However, a relatively higher biomass was obtained when a shaker at 250 rpm was used with 28 membranes for aeration. This suggests that when the surface area for gas transfer is high, aeration becomes sufficient for microbial growth without the necessity for optimal mixing conditions.

3.4.2. Bacterial adhesion

The membrane bioreactor with the highest specific surface area for gas transfer was cultivated for 72 h to investigate the possibility of microbial cells adhering on the membrane surface. After 72 h, the membrane bundle was carefully removed from the centrifuge tube that contained the media and placed on a tray to dry for 48 h at 60 °C. When weighing the membrane module before and after cultivation, there was no detectible change in weight after 72 h, indicating that all biomass was in suspension. Nevertheless, the centrifuge tubes were vortexed before taking any samples in the following section to make sure that no microbial cells were attached on the membrane and all biomass was in suspension when calculating the total biomass concentration.

3.4.3. Batch mode optimization

To optimize the batch mode operation, different specific surface areas for aeration and liquid recirculation rates were tested. Fig. 10 shows biomass growth for four different membrane modules at 20, 40 and 80 rpm as the liquid recirculation rates.

The highest biomass was reached when the membrane module with 250 m^2/m^3 as the specific surface area for gas transfer was used for all three liquid recirculation rates. Although the transfer rate of oxygen increases as the liquid flowrate increases, the maximum biomass concentration obtained remains the same when oxygen is not limiting. This

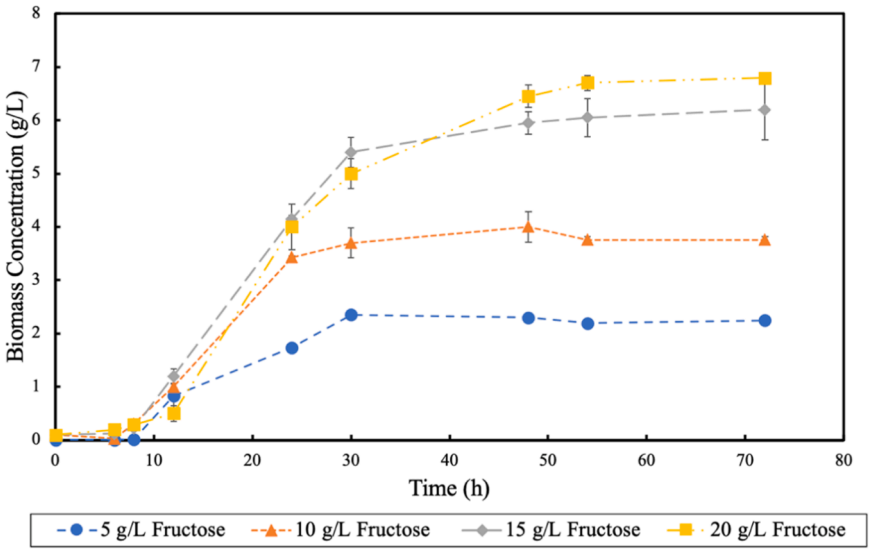

Fig. 8. Growth of Cupriavidus necator H16 on fructose at different concentrations in batch mode for 72 h using flasks.

Table 2
Final biomass and corresponding yield of biomass on substrate for C. necator H16 on different initial fructose concentrations.

Fructose (g/L)	Average Final Biomass (g/L)	Yield of biomass on substrate
5	2.20 ± 0.07	0.44 ± 0.01
10	3.96 ± 0.20	0.40 ± 0.02
15	6.24 ± 0.18	0.42 ± 0.02
20	6.86 ± 0.04	0.34 ± 0

can be better seen in Fig. 11 where the maximum amount of biomass concentration is shown for all batch experiments.

When membrane modules with lower specific surface areas are used, the final biomass concentration after 72 h increases as the liquid recirculation rate increases. This shows that oxygen is limiting for the smaller membrane modules and the oxygen transfer can be improved by increasing the liquid flowrate. Fig. 10 also shows that the liquid flowrate affects the rate and phases of cell growth. Although the final biomass concentration reached is similar for a given condition, the time when cells enter stationary phase differs with changing liquid flowrate. The optimum conditions with the highest biomass of 10.3 g/L were obtained when the membrane module with 250 m^2/m^3 specific surface area and 40 rpm is used. For a more detailed look, nutrient profiles are shown in Fig. 12 where fructose and ammonium concentrations are given. Although the nitrogen source depletes before fructose, cell growth continues until the fructose concentration depleted completely.

3.5. Fed-batch operation

The biomass and fructose profiles for the first fed-batch strategy is shown in Fig. 13. Initial %PHB before adding new media for both scenarios were 12%. When cells were resuspended in MSM with 0.25 and 0 g/L NH_4Cl (C/N = 100 and C/N = ∞), %PHB went up to 15% and 14%, respectively. Results show that for both fed-batch operation, cells showed initial growth but shortly after entered death phase. When MSM with 0 g/L NH_4Cl was used, less biomass was obtained in the end due to nitrogen scarcity.

The second fed-batch strategy was performed using a fill-and-draw method where three different media with different fructose and ammonium concentrations were applied to induce PHB production by cells. Nitrogen source was limiting at all scenarios. Fig. 14 shows the biomass concentration profiles where Fig. 15 shows the %PHB at

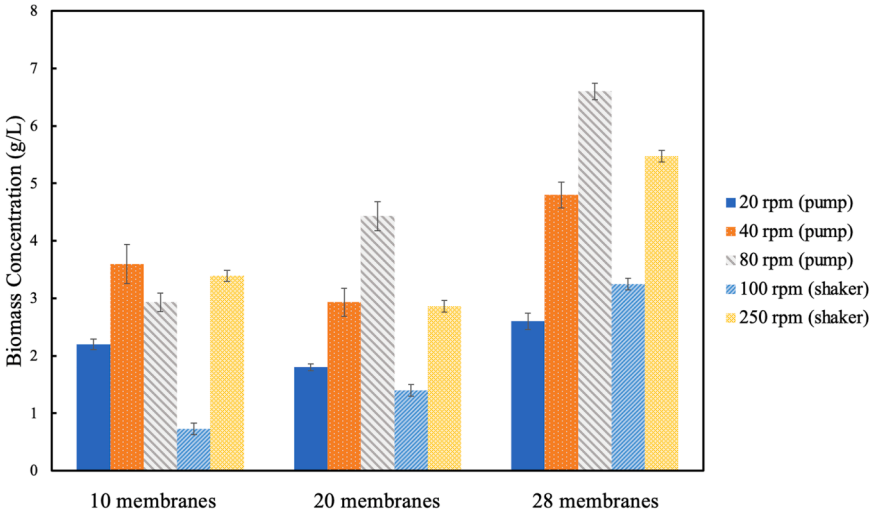

Fig. 9. Biomass concentrations at 30 h batch operation using membrane bioreactors with different mixing conditions and membrane modules with 60, 125 and 250 m^2/m^3 as specific surface areas.

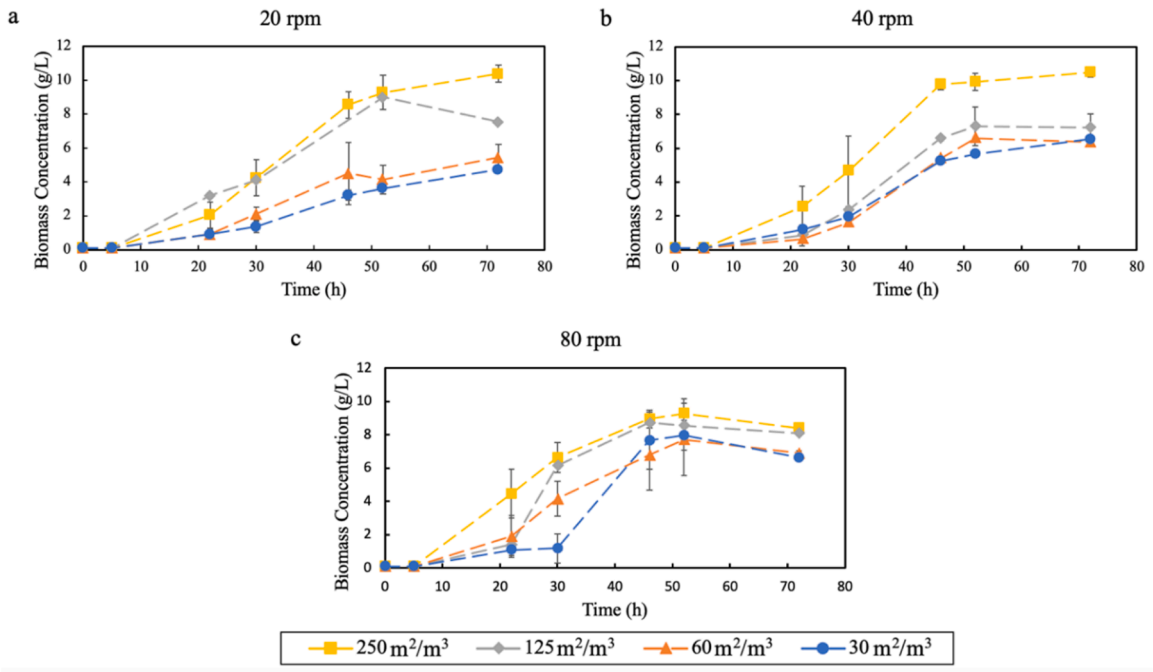

Fig. 10. Biomass growth using four different membrane modules with specific surface areas at liquid recirculation rates of a) 20 rpm, b) 40 rpm, c) 80 rpm.

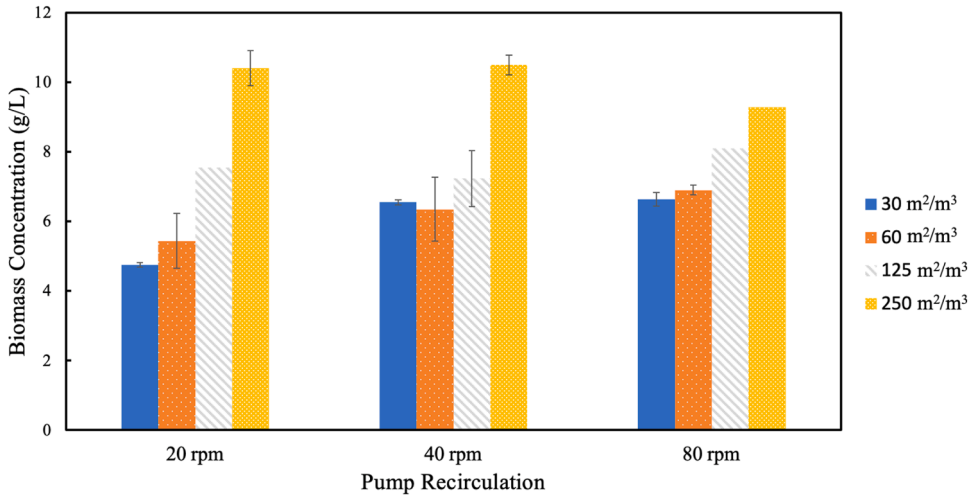

Fig. 11. Final biomass concentrations at the end of batch experiments for different membrane modules and liquid recirculation rates.

different time points where fructose concentration is shown as C and ammonium as N.

The biomass concentration for all fill-and-draw experiments initially went up but then decreased over time. This shows that the time between consecutive feedings were too short to reach the biomass concentration previously achieved. For the first two feedings, the highest PHB amount was observed when the nitrogen source was kept at 0 g/L. However, at 96 h the highest %PHB was measured when 0.25 g/L NH_4Cl and 15 g/L fructose was used. This shows that not all nitrogen limiting conditions increase the PHB production within the cell and there is an optimum ratio of C/N that can maximize the amount of PHB produced. It is seen that the proposed feeding strategy was not effective in terms of increasing %PHB within the specified experimental timeframe.

4. Discussion

In this study, a membrane bioreactor was designed and evaluated in terms of aeration efficiency and mixing through *Cupriavidus necator* H16 cultivation. 50 ml centrifuge tubes were used to create the membrane bioreactors due to their small volume, availability and ease of modification. This makes it possible to perform many experiments in parallel to investigate the effect of various operational conditions with replicates.

Aeration and mixing are two conditions that need to be optimized when designing a bioreactor. A conventional bioreactor would have impellers and a sparger for aeration. However, membranes are used for aeration in this study. Since the membranes used are dense, the mechanism of oxygen transfer is through diffusion [21]. Therefore, no bubbles were formed within the bioreactor which usually contributes to the mixing process. As for the mixing, a shaker was tested to simplify the setup. However, improved mixing was obtained by using a pump to circulate liquid media. Since the headspace within the tube is kept at minimum to fully submerse the membranes to maximize the area for gas transfer, shaking was inadequate for effective mixing.

Membrane layout within the bioreactor alters the uniformity of the

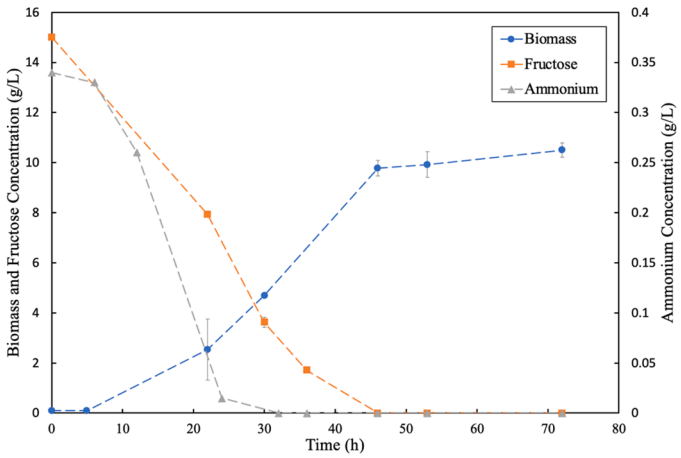

Fig. 12. Biomass, fructose and ammonium profiles for 250 m^2/m^3 membrane surface area and 40 rpm liquid recirculation rate.

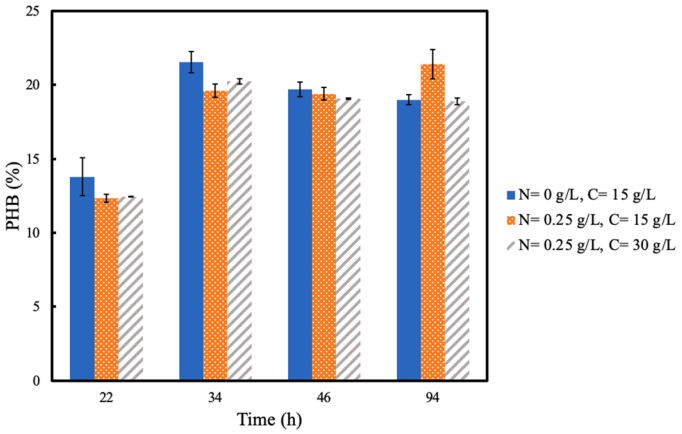

Fig. 15. PHB percentages for fill-and-draw experiments for three different media compositions.

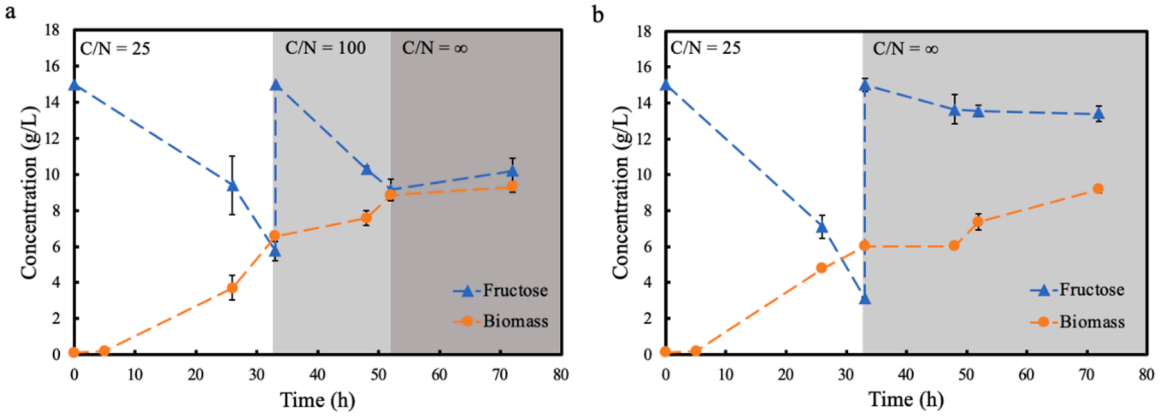

Fig. 13. Fructose and biomass concentrations for fed-batch operation where the biomass was resuspended in media with a) 0.25 g/L NH_4Cl and b) 0 g/L NH_4Cl.

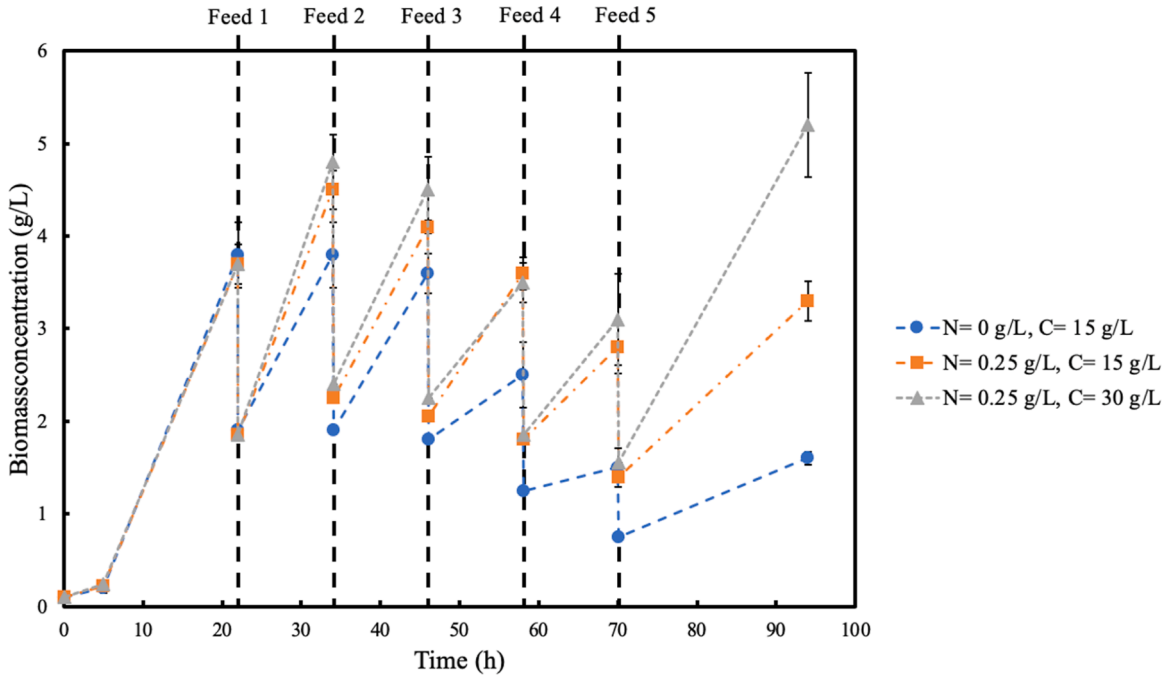

Fig. 14. Biomass concentration profile for fill-and-draw fed-batch strategy using three different media with different C/N ratios.

flow velocity field and the flow regime [22]. This has a significant effect on the gas-to-liquid mass transfer in membrane aerated systems [23]. Generally, membrane bioreactors are designed with membranes in bundles and bundles in rows. Depending on the spacing of the fibers and packing densities, different velocity fields are found within the reactor. These types of membrane distributions are studied extensively and computational fluid dynamic (CFD) simulations are applied to provide valuable information in such systems [22,24]. However, the obtained information is limited when designing a full-scale reactor. In this study, both co-current and counter-current flow regimes are present in the bioreactor due to the localization of the membranes within the tube. This creates a novel configuration with high packing density. Castrillo et al. studied a similar membrane configuration for denitrification, where they tested the effect of random membrane curvature and occupation of the whole cross section of the reactor [25]. They hypothesized that the random localization of the membranes would reduce the overall resistance and enhance the gas mass transfer. They concluded that the denitrification rates using this innovative membrane configuration was higher than conventional membrane designs and 4.4 times more energy efficient than systems aerated by diffusers.

The effect of recirculation rate and gas flowrate inside the membranes on volumetric mass transfer coefficient can be explained by the liquid and gas boundary layers [26]. Within the range of investigation, liquid flowrate has a higher impact on k_La than gas flowrate. The resistance of the liquid boundary layer to the overall resistance is considerably higher than the resistance of the membrane when PDMS is used [27]. Thus, the thickness of the liquid boundary layer decreases when higher liquid flowrates are used, which reduces the resistance to gas transfer. Changing the gas flowrate does not have a significant effect on gas transfer since main resistance to transfer is at the liquid boundary layer. Although the gas pressure inside the membrane is an important parameter for gas transfer [28], its effects were not investigated in this study.

To optimize the operational conditions for microbial growth, a number of batch operation were performed. The scenario with the highest biomass is determined at 40 rpm and 250 m^2/m^3 as the specific membrane surface area. When using the same initial carbon source concentration, biomass yield on carbon is calculated as 0.67 g biomass/g fructose for the membrane bioreactor which is above the theoretical yield of 0.50 and flask experiment yield of 0.43 [18]. This shows that using membranes for aeration is sufficient for efficient microbial growth of *C. necator*.

Fed-batch operation is adopted by many studies that investigate PHB production since cells produce more PHB under limiting nitrogen or phosphorus conditions [29–33]. However, using any limiting substrate concentration inhibits growth. Thus, a strategy that is employed by many studies is to use a two-stage system where first stage is to maximize the biomass and second is to produce PHB. To induce PHB production within the cells, substrate limitation can be introduced by using fed-batch mode. In this study, two different fed-batch strategies were investigated. Overall, %PHB increased for all fed-batch strategies which shows the positive effect of nitrogen limitation on PHB production. However, the % increase for the first fed-batch strategy is minimal for both limiting NH_4Cl concentrations, ranging between 2–3%. On the other hand, 10% PHB increase was achieved when using the fill-and-draw feeding strategy. With the fill-and-draw strategy, nitrogen limitation condition is applied repeatedly as the new fresh medium is added to the bioreactor which explains the higher accumulation of PHB using this method. However, the final PHB percentages obtained are lower when compared to the literature [30]. This shows that there is still space for improvement to reach higher %PHB by applying a different feeding strategy.

In this study, membrane bioreactors are built which can be identified as modular. This means that a full-scale process can be obtained by using a set of these bioreactors in series or parallel. Thus, once a module is optimized for batch operation, scaling up can be effortlessly achieved by running multiple modules in series. This has advantages over designing a larger element such as simpler production, easier scalability and high maintainability. He et al. proposed a novel hollow fiber membrane contactor for gas-to-liquid mass transfer using many changeable and standard small contactors that consist of randomly packed membrane fibers [34]. They found that the small elements have comparable performance to the to the scaled-up contactor with small elements and stated that the performance of the novel contactor is slightly higher than the traditional contactor. They concluded that their design could be potentially promising for many industries. Therefore, the membrane bioreactor modules proposed in this study could potentially be used to produce PHB at large scale by simply employing multiple units.

5. Conclusion

In this work, a novel membrane bioreactor was designed and the characteristics such as oxygen transfer, mixing and microbial growth was investigated. *C. necator* was used as a model organism and PHB production was explored using the proposed bioreactor. Successful cultivation of *C. necator* was achieved with a high biomass of 10.3 g/L using the membrane module with the highest specific surface area. Results showed that mixing improves the oxygen transfer rate however oxygen uptake rate by cells stays the same when oxygen is not limiting. Fed-batch feeding strategies were tested to apply nitrogen source limitation to induce PHB and 22% PHB was achieved using fill-and-draw feeding. Results suggest that membrane bioreactors are promising for cultivating *C. necator*, but further research is required to improve PHB productivity to successfully employ membrane bioreactors in the future.

CRediT authorship contribution statement

Eoin Casey: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Eoin Syron:** Methodology, Conceptualization. **Sorcha Daly:** Writing – review & editing, Supervision. **Burcu Akkoyunlu:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors have received funding by Science Foundation Ireland (SFI) under Grant Number 16/RC/3889 for BiOrbic Bioeconomy SFI Research Centre, which is co-funded under the European Regional Development Fund and BiOrbic industry partners.

References

[1] A.F.M. de Mello, L.P.S. Vandenberghe, C.M.B. Machado, K.K. Valladares-Diestra, J. C. de Carvalho, C.R. Soccol, Polyhydroxybutyrate production by Cupriavidus necator in a corn biorefinery concept, Bioresour. Technol. (2023) 128537, https://doi.org/10.1016/j.biortech.2022.128537.

[2] S. Bellini, T. Tommasi, D. Fino, Poly(3-hydroxybutyrate) biosynthesis by Cupriavidus necator: a review on waste substrates utilization for a circular economy approach, Bioresour. Technol. Rep. vol. 17 (2022) 100985.

[3] L.G. Md. Salatul Islam Mozumder, Eveline Heleen DeWevera, I.P. Volcke, Poly(3-hydroxybutyrate) (PHB) production from CO_2: model development and process optimization, Biochem. Eng. J. vol. 98 (2015) 107–116.

[4] V. Lambauer, R. Kratzer, Lab-scale cultivation of Cupriavidus necator on explosive gas mixtures: carbon dioxide fixation into Polyhydroxybutyrate, Bioengineering vol. 9 (5) (2022), https://doi.org/10.3390/bioengineering9050204.

[5] K. Asimakopoulos, H.N. Gavala, I.V. Skiadas, Reactor systems for syngas fermentation processes: a review, Chem. Eng. J. vol. 348 (2018) 732–744, https://doi.org/10.1016/j.cej.2018.05.003.
[6] M.D. Bredwell, P. Srivastava, R.M. Worden, Reactor design issues for synthesis-gas fermentations, Biotechnol. Prog. vol. 15 (5) (1999) 834–844, https://doi.org/10.1021/bp990108m.
[7] F. Ghoddosi, H. Golzar, F. Yazdian, K. Khosravi-Darani, E. Vasheghani-Farahani, Effect of carbon sources for PHB production in bubble column bioreactor: emphasis on improvement of methane uptake, (in English), J. Environ. Chem. Eng. vol. 7 (2) (2019). ARTN 10297810.1016/j.jece.2019.102978.
[8] F. Ahmadi, A.A. Zinatizadeh, A. Asadi, T. McKay, S. Azizi, Simultaneous carbon and nutrients removal and PHA production in a novel single air lift bioreactor treating an industrial wastewater, (in English), Environ. Technol. Innov. vol. 18 (2020). ARTN 10077610.1016/j.eti.2020.100776.
[9] F. Rahnama, E. Vasheghani-Farahani, F. Yazdian, S.A. Shojaosadati, PHB production by from natural gas in a bubble column and a vertical loop bioreactor, (in English), Biochem Eng. J. vol. 65 (2012) 51–56, https://doi.org/10.1016/j.bej.2012.03.014.
[10] J.J. Orgill, H.K. Atiyeh, M. Devarapalli, J.R. Phillips, R.S. Lewis, R.L. Huhnke, A comparison of mass transfer coefficients between trickle-bed, hollow fiber membrane and stirred tank reactors, Bioresour. Technol. vol. 133 (2013), https://doi.org/10.1016/j.biortech.2013.01.124.
[11] E. Syron, E. Casey, Membrane-aerated biofilms for high rate biotreatment: performance appraisal, engineering principles, scale-up, and development requirements, Environ. Sci. Technol. vol. 42 (6) (15 2008), https://doi.org/10.1021/es0719428.
[12] B. Valverde-Perez, W. Xing, A.A. Zachariae, M.M. Skadborg, A.F. Kjeldgaard, A. Palomo, B.F. Smets, Cultivation of methanotrophic bacteria in a novel bubble-free membrane bioreactor for microbial protein production, Bioresour. Technol. vol. 310 (2020) 123388, https://doi.org/10.1016/j.biortech.2020.123388.
[13] D. Kamravamanesh, D. Kiesenhofer, S. Fluch, M. Lackner, C. Herwig, Scale-up challenges and requirement of technology-transferfor cyanobacterial poly (3-hydroxybutyrate) production inindustrial scale, Int. J. biobased Plast. vol. 1 (2019) 60–71.
[14] H.T. Hornig-Do, How to Scale-Out and Scale-Up Cell Therapy Production. ⟨https://www.fishersci.ie/ie/en/scientific-products/publications/lab-reporter/2023/issue-1/how-to-scale-out-and-scale-up-cell-therapy-production.html⟩ (accessed 24 October 2023).
[15] B. International, Scale-Out Biomanufacturing - A Paradigm Change to Scale-Up. ⟨https://bioprocessintl.com/sponsored-content/scale-biomanufacturing-paradigm-change-scale/⟩ (accessed October 10, 2023).
[16] F. Mirasol, Single-use Bioreactors: To Scale Up or Sclae Out?. ⟨https://www.biopharminternational.com/view/single-use-bioreactors-scale-or-scale-out⟩ (accessed October 10, 2023).
[17] P.C. Munasinghe, S.K. Khanal, Syngas fermentation to biofuel: evaluation of carbon monoxide mass transfer and analytical modeling using a composite hollow fiber (CHF) membrane bioreactor (in English), Bioresour. Technol. vol. 122 (2012) 130–136, https://doi.org/10.1016/j.biortech.2012.03.053.
[18] A. Holohan, Production of Lactic Acid by Ralstonia Eutropha H16, a Gram-Negative Chemolithoautotroph, Using Tools of Synthetic Biology, MSc, Biomolecular and Biomedical Science, University College Dublin, 2021.
[19] F. Cerrone, et al., Use of a mannitol rich ensiled grass press juice (EGPJ) as a sole carbon source for polyhydroxyalkanoates (PHAs) production through high cell density cultivation, Bioresour. Technol. vol. 191 (2015) 45–52, https://doi.org/10.1016/j.biortech.2015.04.128.
[20] S. Grunwald, et al., Kinetic and stoichiometric characterization of organoautotrophic growth of Ralstonia eutropha on formic acid in fed-batch and continuous cultures, Microb. Biotechnol. vol. 8 (1) (2015), https://doi.org/10.1111/1751-7915.12149.
[21] F. Bazzarelli, L. Giorno, E. Piacentini, Porous membranes, Encycl. Membr. (2015).
[22] F. Kavousi, E. Syron, M. Semmens, E. Casey, Hydrodynamics and gas transfer performance of confined hollow fibre membrane modules with the aid of computational fluid dynamics, (in English), J. Membr. Sci. vol. 513 (2016) 117–128, https://doi.org/10.1016/j.memsci.2016.04.038.
[23] R. Nerenberg, The membrane-biofilm reactor (MBfR) as a counter-diffusional biofilm process, Curr. Opin. Biotechnol. vol. 38 (2016), https://doi.org/10.1016/j.copbio.2016.01.015.
[24] R. Plascencia-Jatomea, F.J. Almazán-Ruiz, J. Gómez, E.P. Rivero, O. Monroy, I. González, Hydrodynamic study of a novel membrane aerated biofilm reactor (MABR): tracer experiments and CFD simulation, Chem. Eng. Sci. vol. 138 (2015) 324–332.
[25] M. Castrillo, R. Diez-Montero, A.L. Esteban-Garcia, I. Tejero, Mass transfer enhancement and improved nitrification in MABR through specific membrane configuration, Water Res. vol. 152 (Apr 1) (2019) 1–11, https://doi.org/10.1016/j.watres.2019.01.001.
[26] M.P. Elisiario, H. De Wever, W. Van Hecke, H. Noorman, A.J.J. Straathof, Membrane bioreactors for syngas permeation and fermentation, Crit. Rev. Biotechnol. vol. 42 (6) (2022) 856–872, https://doi.org/10.1080/07388551.2021.1965952.
[27] C. Pellicer-Nàcher, C. Domingo-Félez, S. Lackner, B.F. Smets, Microbial activity catalyzes oxygen transfer in membrane-aerated nitritating biofilm reactors, J. Membr. Sci. vol. 446 (2013) 465–471.
[28] E. Casey, B. Glennon, G. Hamer, Biofilm development in a membrane-aerated biofilm reactor: effect of intra-membrane oxygen pressure on performance, Bioprocess Eng. vol. 23 (2000) 457–465, https://doi.org/10.1007/s004499900175.
[29] R.A. Scheel, T. Ho, Y. Kageyama, J. Masisak, S. McKenney, B.R. Lundgren, C. T. Nomura, Optimizing a fed-batch high-density fermentation process for medium chain-length poly(3-Hydroxyalkanoates) in Escherichia coli, Front. Bioeng. Biotechnol. vol. 9 (2021) 618259, https://doi.org/10.3389/fbioe.2021.618259.
[30] D. Nygaard, O. Yashchuk, D.G. Noseda, B. Araoz, E.B. Hermida, Improved fermentation strategies in a bioreactor for enhancing poly(3-hydroxybutyrate) (PHB) production by wild type Cupriavidus necator from fructose, Heliyon vol. 7 (1) (2021) e05979, https://doi.org/10.1016/j.heliyon.2021.e05979.
[31] T.T. Loan, D.T.Q. Trang, P.Q. Huy, P.X. Ninh, D. Van Thuoc, A fermentation process for the production of poly(3-hydroxybutyrate) using waste cooking oil or waste fish oil as inexpensive carbon substrate, Biotechnol. Rep. vol. 33 (2022) e00700, https://doi.org/10.1016/j.btre.2022.e00700.
[32] E. Vlaeminck, K. Quataert, E. Uitterhaegen, K. De Winter, W.K. Soetaert, Advanced PHB fermentation strategies with CO(2)-derived organic acids, J. Biotechnol. vol. 343 (2022) 102–109, https://doi.org/10.1016/j.jbiotec.2021.11.010.
[33] O. Psaki, I.I. Athanasoulia, A. Giannoulis, D. Briassoulis, A. Koutinas, D. Ladakis, Fermentation development using fruit waste derived mixed sugars for poly(3-hydroxybutyrate) production and property evaluation, Bioresour. Technol. vol. 382 (2023) 129077, https://doi.org/10.1016/j.biortech.2023.129077.
[34] K. He, S. Chen, C. Huang, L. Zhang, Fluid flow and mass transfer in an industrial-scale hollow fiber membrane contactor scaled up with small elements, Int. J. Heat. Mass Transf. vol. 127 (2018) 289–301.