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RESEARCH PAPER

Methane Based Continuous Culture of *Methylosinus trichosporium* for Production of Poly-3-hydroxybutyrate Using Membrane Recycle System

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Abstract Poly-3-hydroxybutyrate (PHB) is a bio-based and biodegradable polymer produced by microbial fermentation from wide variety of feedstocks. Methanotrophic organism *Methylosinus trichosporium* has been reported for the production of PHB using alternate feedstock like methane which is economical and abundantly available. PHB was produced from methane by cultivating *M. trichosporium* in a bioreactor with continuous cell recycle system. Different gas sparging strategies were evaluated for impact on cell biomass and PHB production. The fermentation was conducted in different modes including batch and continuous culture with and without membrane recycle. Using membrane based recycle system the biomass increased from 1.5 to maximum of 7.31 g DCW/L in 27 days. Although PHB content was comparable in both batch mode and membrane based recycle system, a 4.9 fold increase in biomass production enhanced the PHB titer by 6.1 fold as compared to batch culture. Continuous cell recycle processes provide an opportunity to increase the competitiveness of gas fermentation based processes for methanotrophs based PHB production.

Keywords: methane, biogas, cell recycle system, methanotrophs, poly-3-hydroxybutyrate

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1. Introduction

As a potent greenhouse gas (GHG) methane (CH_4) has a crucial impact on global warming effect and climate change due to increased concentration in the environment. This GHG is mainly released into the atmosphere from different sources including landfills, agricultural activity, coal mining, certain industrial processes, wastewater treatment, oil and natural gas fields. However, there is lack of viable technological alternatives to valorize this carbon source without prior treatment of methane released in the environment. Development of cost-effective and environmentally-friendly technologies are required for the utilization of CH_4 and to achieve an effective climate change mitigation [1-4]. Methane is cheap and abundantly available with multiple natural reserves and anthropogenic sources. Methanotrophs have the capability to use methane as a carbon and energy source [5-7]. Methane based feedstock can be used as a carbon source to produce novel as well as value-added products. Methanotrophs present in the diverse environmental habitats helps to reduce methane emissions as well as have the potential to valorize methane into different product categories including single cell protein, biofuels, biopolymers, biochemicals, cosmetics and vitamins [8-11]. Production of biochemicals from methane can expand the suite of products generated from biorefineries, municipalities and agricultural operations. Use of methane and methanotrophic bacteria for the production of bio-plastics has been recently evaluated in bioreactors with promising results [8,12]. Polyhydroxyalkanoates (PHAs) are biodegradable plastic and can be an appropriate environmentally friendly solution for existing petrochemical based plastics which are non-renewable in nature [13,14]. Among all PHAs, naturally accumulating

poly-3-hydroxybutyrate (PHB) is the widely produced and simplest biopolymer product from different biobased feedstocks and has properties similar to polypropylene [15-18]. Anaerobic biodegradation of organic wastes generates biogas with methane as major component (30-70%) and is an established renewable energy source. Methanotrophs have been isolated from variety of habitats including waste treatment facilities, paddy field, soil and natural gas field [8,13]. Natural gas/biogas can be a cost-effective feedstock for PHB production and could economically replace other sugar or oil based agri feedstocks.

Relatively high prices of sugar or oil based raw materials is the major limiting factor for wide spread commercialization of PHB [19,20]. Production of PHB from gaseous substrate like methane has also been reported from multiple methanotrophic genera like *Methylosinus*, *Methylocystis*, and *Methylocella* in batch as well as continuous culture mode. In a study with mix methanotrophic culture the maximum PHB content up to 59.4% was reported using recycling of cells between growth and PHB production phase. Despite of multiple research reports, CH_4 bioconversion to PHB still needs to be optimized to develop a scalable and sustainable process for PHB production [1,21,22].

Conventional batch bioreactors are often limited by mass transfer of gaseous substrates like methane and oxygen. Improvement in performance of PHB production from methane will require significant improvement in reactor and process designs [12]. The overall production of cell biomass can be improved by effective cell recycle systems [23]. In the present study we report an improved method based on continuous culture of *Methylosinus trichosporium* for the production of PHB using cell recycle system.

2. Materials and Methods

2.1. Strain and culture conditions

Methylosinus trichosporium OB3b NCIMB 11131 (obtained from NCIMB, UK) has been used in the present study. The nitrate mineral salts (NMS) media containing buffering components for maintenance of neutral pH was used for *M. trichosporium* cultivation [9]. Culture of *M. trichosporium* was grown and maintained in 250 mL bottle containing 50 mL NMS media and headspace filled with methane oxygen mixture (50: 50 ratio). The NMS was composed of magnesium sulfate heptahydrate 1.0 g/L, calcium chloride hexahydrate 0.2 g/L, potassium nitrate 1.0 g/L, potassium dihydrogen phosphate 0.272 g/L, disodium hydrogen phosphate dodecahydrate 0.717 g/L. Solution with chemical constituents was sterilized in autoclave at 121°C for 20 min. It also contained 2.0 mL/L filter sterilized chelated iron solution which composed of Ferric (III) ammonium citrate 1.0 g/L, ethylenediamine tetraacetic acid, sodium salt 2.0 g/L and hydrochloric acid (concentrated) 3.0 mL/L. Trace element solution was added at concentration of 0.5 mL/L which contain ethylenediamine tetraacetic acid 500.0 mg/L, ferrous sulphate 200.0 mg/L, zinc sulfate heptahydrate 10.0 mg/L, manganese (II) chloride tetrahydrate 3.0 mg/L, boric acid 30.0 mg/L, cobalt (II) chloride hexahydrate 20.0 mg/L, calcium chloride dihydrate 1.0 mg/L, nickel chloride hexahydrate 2.0 mg/L and molybdic acid sodium salt dihydrate 3.0 mg/L respectively. Trace element solution was sterilized in autoclave at 121°C for 20 min prior to use. Seed culture for all experiments were prepared by inoculation of 2% (v/v) broth of *M. trichosporium* culture in screw cap bottles using previously described gassing protocol [10,24]. During incubation, headspace gas was replaced after every 12 h with fresh gas mixture. Screw cap bottles were incubated at 30°C temperature on 200 rpm shaking condition. The pH of the medium was observed to be in the range 6.8-7.0. The pH was not controlled in any mode of fermentation include batch and continuous experiment. Methane and biogas generated from rice straw biomass was used as a source of carbon for cell growth and PHB production. Composition of used biogas was ~52.6% v/v methane, ~38.6% v/v carbon dioxide, ~1% v/v oxygen, ~1% v/v hydrogen, ~3.5% v/v moisture, ~150 ppm v/v hydrogen sulfide and balance nitrogen.

2.2. Growth of *M. trichosporium* in screw cap bottles with methane and biogas

Comparison of growth on methane and biogas was conducted at 250 mL bottle with 50 mL NMS media. While using biogas as substrate, ratio of methane: oxygen (50:50) was maintained using mixture of 66% (v/v) biogas and 34% (v/v) oxygen. Headspace gas was replaced after every 12 h with fresh gas mixture and bottles were incubated till 120 h. The bottles were sampled every 24 h to determine optical density and purity of the culture. Final samples were analyzed for PHB content.

2.3. Batch culture for growth of *M. trichosporium* with methane in overlay mode

Batch culture experiment was carried out in 14 L bioreactor (Bioflo 415, New Brunswick Scientific) with 7 L of NMS medium. Mixture of methane and oxygen was supplied through sparger in the bioreactor using gas filling system (Fig. 1). Bioreactor set-up and gas saturation were performed as previously reported procedure [8]. In the present study, gas supply in the overlay mode was ensured by maintaining 14 psig pressure throughout the incubation period using auto control system. Agitation and temperature were maintained at 450 rpm and 30°C respectively. Samples were withdrawn every 24 h to check the optical density and PHB concentration.

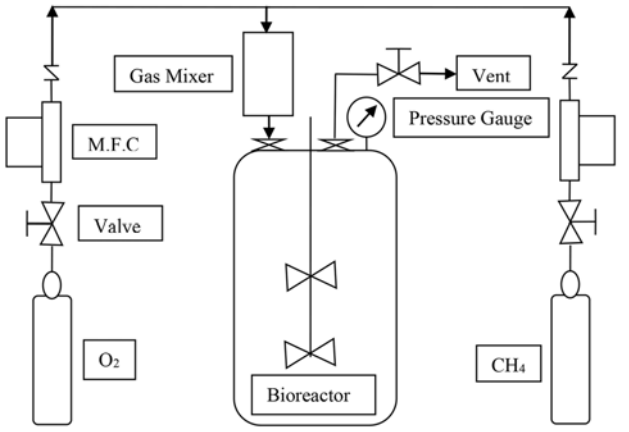

Fig. 1. Schematic illustration of batch culture set up at 14 L bioreactor.

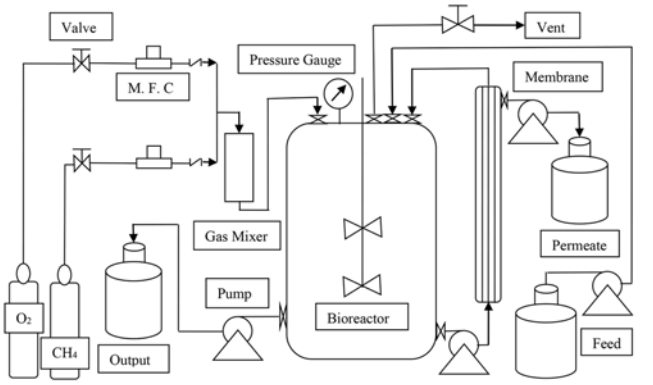

Fig. 2. Schematic illustration of cell recycle set up at 14 L bioreactor.

2.4. Batch culture for growth of *M. trichosporium* with continuous gas sparging mode

In continuous gas sparging mode, the setup of bioreactor, cultivation conditions were same as overlay mode without pressurizing the system. Mixture of methane and oxygen (50:50) was supplied continuously through the sparger at 0.025 and 0.05 vvm (Volume of gas sparged per unit volume of medium per minute) (Fig. 1).

2.5. Continuous culture of *M. trichosporium* with continuous gas sparging and cell recycle system

Continuous culture was carried out in a 14 L bioreactor (Bioflo 415, New Brunswick Scientific) with 7 L of working volume in NMS medium (Fig. 1). The temperature and agitation were maintained at 30°C and 450 rpm respectively. Mixture of methane and oxygen (50:50 ratio) was supplied through sparger at gas flow of 0.05 vvm. Sampling of the bioreactor was performed after every 24 h to determine optical density, pH, PHB concentration and purity of the culture.

In continuous culture with cell recycle the bioreactor was operated in three stages each of 9 days. In the initial stage culture growth was allowed in batch mode with 0.05 vvm gas sparging. In the second stage, cell recycle was initiated using membrane system and continuous fermentation was performed by supplying fresh NMS media as input to bioreactor (Fig. 2). Cell broth fed to the membrane system was separated as permeate and cells were recycled back to bioreactor. During second stage, flow rate was increased gradually from 1.05 L/day to 3.67 L/day to increase biomass productivity. In the last stage of the experiment, input to the bioreactor was increase to 4.90 L/day and output stream was split into permeate (with 3.90 L/day flow) and harvest streams (with 1 L/day flow). Harvested cell broth was collected and used for further processing.

Membrane recycle system was set up by connecting the bioreactor with bench top perfusion and tangential flow filtration system (KML 100 System, Spectrum Labs) with Kros Flo MBT disposable module bag tubing set (P/N-N06-P20U-05, Spectrum Labs) having 0.2 μm pore size and 1 m^2 surface area as shown in Fig. 2. A polyethersulfone membrane was used for tangential flow filtration.

2.6. Analytical methods

2.6.1. Absorbance, dry cell weight (DCW) and calculations

The samples were taken out at predetermined time intervals, and the optical density (OD) was measured by spectrophotometer at 600 nm (Model UV-2450, Shimadzu, Kyoto, Japan) [23]. For analysis of dry cell weight, 10 mL of cell suspension was centrifuged at 8,000 rpm for 10 min at room temperature (Centrifuge 5804 R, Eppendorf). Cells were washed once with distilled water, and dried in vacuum oven at 80°C for 24 h [18,23].

Total biomass and PHB produced were calculated based on mass of dry biomass in the harvested broth and its PHB accumulation. Total dry biomass (B_T) and total PHB (PHB_T) (g) produced was calculated using equation (1) and (2).

$$B_T = (B_{H1} + B_{H2} + \ldots. + B_{Hn}) + B_{RV} \quad (1)$$

$$PHB_T = (PHB_{H1} + PHB_{H2} + \ldots. + PHB_{Hn}) + PHB_{RV} \quad (2)$$

Biomass harvested for day (B_H) (g/day) was calculated by multiplying biomass concentration (DCW) (g/L) and harvested volume (H_v) (L/day) for the day (B_H = DCW × H_v). PHB produced per day (PHB_H) (g/day) was calculated by multiplying biomass harvested for day with PHB accumulation (PHB) (%) ($PHB_H = B_H \times PHB$). Similarly, biomass (B_{RV}) and PHB (PHB_{RV}) accumulated in the reactor volume (R_v) (L) at end of batch was calculated.

2.6.2. Poly 3-hydroxybutyrate estimation

Analysis of PHB was performed using determination of monomer methyl-3-hydroxybutyrate using gas chromatography [20]. Sample of 20 mg dry cell pellet was suspended in a solution containing 2 mL chloroform and 2 mL of methanol with 2% of benzoic acid solution (40 mg/L) and 3% sulphuric acid. Sample was digested at 100°C for 5 h to depolymerize the polymer to its monomer and esterified with methanol in a capped glass vial. After cooling the sample at room temperature, 1 mL of deionized water was added and sample was thoroughly mixed by vortexing and kept for phase separation. Lower organic layer containing methyl ester of 3-hydroxybutrate was separated and pinch of sodium sulfate was added for removal of water traces in sample. Sample was then analyzed for quantification of methyl 3-hydroxybutrate using gas chromatography (GC). Standards of (R)-3-hydroxybutrate (with > 99% purity Sigma Aldrich, USA) were used for PHB quantification and methyl benzoate was considered as an internal standard. A gas chromatograph (GC7890A, Agilent Technologies, USA) equipped with an auto sampler (Agilent Technologies) and with a HP-5 or equivalent column (length 30 m, diameter 0.320 mm, film 0.25 μm) was used with flame ionized detector. The injection sample volume was 1 μl with flow rate 1.5 mL/min. Detector temperature was set at 300°C with run time of 21 min.

2.6.3. Methane monooxygenase (MMO) activity

Total MMO activity (both soluble MMO and particulate MMO) was estimated using previously reported propylene-oxidation assay [4]. Bacterial cells were harvested by centrifugation at 9,000×g for 10 min at 4°C. The cell pellets were washed twice with ice cold 20 mmol/L phosphate buffer (pH 7.0) and resuspended in the same buffer along with 5 mmol/L $MgCl_2$ and 20 mmol/L sodium formate. Produced epoxypropane was estimated on gas chromatograph and enzyme activity was represented as nmol of product formed per minute per milligram of DCW [25].

3. Results and Discussion

Several methanotrophic bacteria have been reported for the production of PHB using methane and biogas in the batch cultures [24,26,27]. Present study evaluated *M. trichosporium* as host in different modes of fermentation for improving cell growth and PHB production. In all experiments the culture density of seed culture of *M. trichosporium* was maintained in the range of 0.17 to 0.24 g DCW/L and inoculum of 10% v/v was used in each experiment. The pH was measured in all experiments and no change in pH was observed during the growth and PHB production.

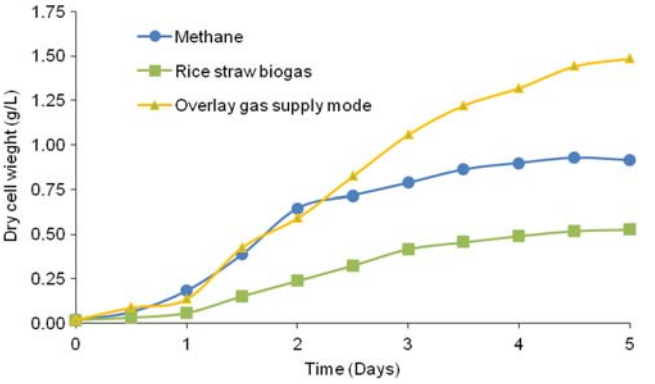

Fig. 3. Cell growth kinetics of *M. trichosporium* in screw cap bottle (circle and square) and bioreactor (triangle). Data is mean representation of three replicates at screw cap bottle and two replicates at 14 L bioreactor with < 10% standard deviation.

3.1. Growth of *M. trichosporium* in screw cap bottles with methane and biogas

As biogas can be a sustainable source of methane, effect of biogas on growth and PHB production ability of *M. trichosporium* was evaluated in 250 mL bottle in comparison to pure methane. Using methane as a substrate, maximum cell concentration was found to be 0.93 g/L along with 16.07% PHB accumulation. Whereas in the case of biogas, maximum cell concentration and PHB accumulation was found to be 0.53 g/L and 6.09% respectively (Fig. 3). As compared to biogas, methane resulted in 1.7 and 2.6 fold higher cell concentration and PHB production respectively. The biomass yield was found to be 51.27 and 42.34 mg-DCW/g-methane on pure methane and biogas respectively. The PHB yield was found to be 8.24 and 2.58 mg-DCW/g-methane on pure methane and biogas respectively. Yield was calculated based on total methane and biogas supplied to the fermentation. Methanotrophs growth on biogas could be inhibited due to hydrogen sulfide present in the biogas. Additionally, the presence of carbon dioxide and trace gases in biogas can also inhibit cell growth and PHB production [10]. In previous studies, similar effect of inhibition on use of biogas has been reported on cell concentration and product formation with *Methylotuvimicrobium alcaliphilum*, *Methylocella silvestris*, *Methylomonas methanica*, *Methylosinus trichosporium*, and *Methylosinus sporium* [10,28]. Another study investigating effect of sulfur compounds on methane oxidizing consortium revealed the competitive inhibition on methane oxidation [29]. Based on the results of screw cap bottle further experiments at bioreactor level were performed with pure methane.

3.2. Batch culture for growth of *M. trichosporium* with gas supply in overlay mode

Pressurization of gas in the batch culture fermentation

provided advantage of increased mass transfer rate of gas to liquid phase. *M. trichosporium* cells were grown in batch mode with gas supply in the overlay mode for up to 5 days. After the incubation period methanotroph cell density increased up to 1.49 g DCW/L with maximum cell growth rate of 0.08 h^{-1} (Fig. 3). PHB accumulation in the harvested cells was found to be 29.90% w/w resulting in PHB titer of 0.441 g/L. Total MMO activity of *M. trichosporium* during the 5 days of incubation was observed to be in the range of 22.47 nmol/mg DCW/min to 35.78 nmol/mg DCW/min.

Previous studies have reported influence of pressure on the mass transfer rate and cell growth using different methanotrophs [7,8]. Study on *Methylomonas albus* reported improvement in methane consumption and growth rate by 50% and 10% respectively by increasing pressure up to 20 psig in the bioreactor [30]. Reports on other process modifications like immobilization, use of paraffin oil and membrane bioreactor using *M. trichosporium* have also been reported in literature [31-33]. In the present study we report comparative account of growth and PHB production using *M. trichosporium* in overlay as well as continuous gas sparging mode.

3.3. Batch culture for growth of *M. trichosporium* with continuous gas sparging mode

Continuous bubbling of methane-oxygen mixture (50:50 ratio) through sparger provides the higher surface area through bubbles and improves mass transfer from gas to liquid phase. Gas exiting from the bioreactor also include off gas like carbon dioxide and avoid any inhibition to the cells. In the present study, continuous gas sparging in batch culture was operated at two different flow rates of methane-oxygen mixture, 0.025 vvm and 0.050 vvm. At the gas flow rate of 0.025 vvm, *M. trichosporium* showed cell growth up to 0.72 g/L dry cells with PHB accumulation of 27.18%. Whereas in case of 0.050 vvm final cell concentration increased to 1.38 g/L with 28.20% PHB accumulation (Fig. 4). At 0.025 vvm cells entered stationary phase on 5th day while at 0.05 vvm the cell growth continued up to 12th day. Total MMO activity of *M. trichosporium* was found to be in the range of 24 to 34 nmol/mg DCW/min at both gas flow rates.

In the present study around 1.91 fold increase in cell density was achieved by increasing gas sparging of 0.025 to 0.05 vvm gas supply. The difference between final biomass produced for 0.025 vvm and 0.050 vvm gas flow rate was mainly due to increased gas flow rate resulting in higher mass transfer from gas to liquid phase. *M. trichosporium* is known to divert carbon towards PHB under nitrogen limiting conditions [20]. In the present study the total 0.135 g/L of nitrogen was provided in the form of nitrate was provided.

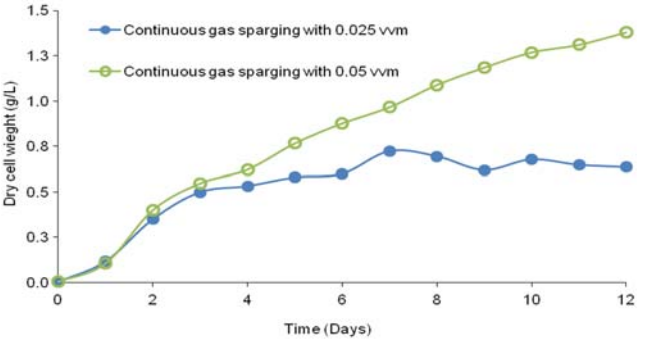

Fig. 4. Profiles of cell density with 0.025 and 0.05 vvm gas sparging during continuous gas system mode. Data is mean representation of two replicates with < 10% standard deviation.

The supplied nitrogen supported 0.72 g/L and 1.38 g/L dry cells during mix gas supply rate of 0.025vvm and 0.05 vvm respectively. Despite the change in carbon to nitrogen ratio, accumulation of PHB was found to be similar representing favorable stress condition for PHB production in both the cases. Higher PHB titer at 0.05 vvm gas supply was observed mainly due to increase in biomass production.

In overlay gas supply mode as well as continuous gas sparging at 0.05 vvm the final cell concentration as well as PHB was found to be in the same range. The biomass productivity in the case of overlay mode was found to be 2.15 fold higher than continuous gas sparging mode. Higher cell density in overlay mode shows potential to further increase the cell density by improving gas solubility at higher gas pressure.

3.4. Continuous culture of *M. trichosporium* with continuous gas sparging and cell recycle system

To produce growth associated product like PHB, it is important to achieve maximum cell densities prior to implementation of nitrogen stress conditions. Growth of culture in batch mode resulted in limited biomass and PHB production. To overcome these limitations, *M. trichosporium* cells were concentrated using membrane system to achieve higher cell density and cells containing PHB were harvested continuously. Due to limitations in operating cell recycle system in an overlay mode, continuous gas sparging at 0.05 vvm was used during continuous fermentation. For production of higher cell biomass, the overall substrate requirement is also higher, which can be fed in a controlled manner without allowing increase in substrate concentration to inhibitory levels in the bioreactor in batch mode [23].

Continuous culture with cell recycle was performed in three different stages namely cell growth stage, concentration using membrane and cell harvesting stage. During the initial stage *M. trichosporium* cells were grown in batch mode for 9 days with 0.05 vvm gas flow rate (methane to

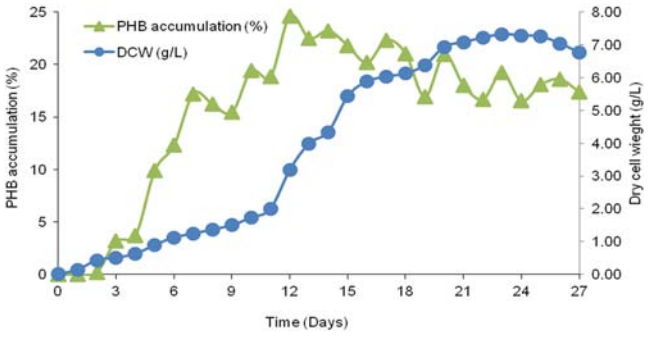

Fig. 5. Profiles of cell density and % PHB accumulation during continuous culture with cell recycle.

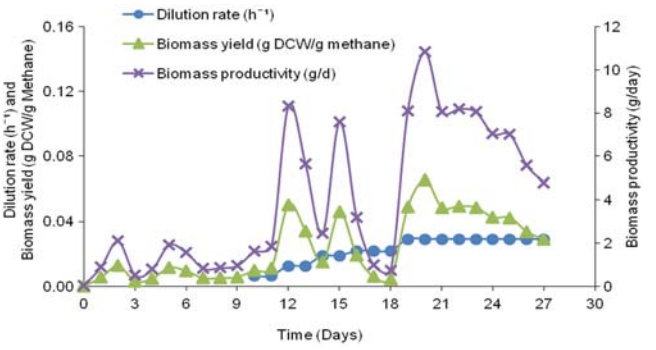

Fig. 6. Profiles of dilution rate, biomass yield and biomass productivity in continuous culture with cell recycle system.

oxygen in 50:50 ratio). At the end of the 9th day 1.5 g DCW/L cells were produced with 15.42% PHB content (Fig. 5). After 9th day, membrane cell recycle system was connected and operated as described in Table 1. During membrane recycle, flow rate of NMS media and permeate were increased from 0.0437 to 0.1529 L/h (Table 1). Supply of fresh nutrient and membrane based cell recycle resulted in an increased cell density up to 6.12 g DCW/L in the bioreactor. Simultaneously PHB accumulation in the cells was increased from 15 to 24.6% (Fig. 5).

After 19th day of fermentation, cell harvesting was initiated at 0.0416 L/h along with permeate flow rate of 0.162 L/h (Table 1). On 21st day steady state conditions were achieved and cell density of ~7.31 g DCW/L was observed in the harvest stream. Nitrogen limited condition was maintained during cell recycle stage and continuous cell harvesting stage by adjusting flow rates of NMS medium and harvested broth. Under steady state conditions (21st day onward), PHB accumulation in the range of 16.66% to 20.94 % was achieved (Fig. 5). During the controlled supply of nutrients, drop in biomass productivity and yield was observed at 14th and 18th day. To suffice the increased nitrogen requirement, feed flow rate was increased which resulted into improved biomass productivity on the consecutive day (Fig. 6).

During 27 days of fermentation 110.50 g total dry biomass was produced along with 19.62 g of PHB. Biomass harvesting was performed for 9 days (19th day to 27th day) of the cell harvesting stage of continuous culture. Cells of *M. trichosporium* generated during the study were also analyzed for carbon (C), hydrogen (H), nitrogen (N), sulphur (S) content. In the CHNS analysis 44.96% of carbon, 6.91% of hydrogen, 6% nitrogen and 0.45% sulphur were observed. During 27 days of fermentation total of 3476.84 g of carbon (from methane), 1158.95 g of hydrogen (from methane), 10.09 g of nitrogen (from nitrate salt) and 8.97 g of sulphur (from media) was supplied. Among all macro compounds up to 1.43% carbon, 4.29% of hydrogen, 65.73% nitrogen and 5.55% sulphur were converted into biomass. Nitrogen limited condition throughout the fermentation resulted in consistent PHB accumulation during 10th to 27th day (Fig. 5). Low carbon utilization rate could be further improved by using different strategies like bioreactors of high L/D ratio; bubble column reactors; solubility enhancers of methane and oxygen; as well as recycling of unutilized gas. During the fermentation process of 27 days the average total MMO activity was found to be 19.07, 23.60 and 22.08 nmol/mg DCW/min in batch mode, membrane cell recycle mode and continuous culture with cell recycle mode respectively. In methanotrophic bacteria MMO is a key enzyme that catalyzes the first step in the oxidation of methane. As MMO activity increases, the methanotrophs can more efficiently convert methane into methanol, providing an increased supply of carbon for cell growth and PHB production. Both biomass production and PHB accumulation in the methanotrophs can be influenced by

Table 1. Parameters for continuous culture with cell recycle

Days	Feed flow rate (L/h)	Output flow rate (L/h)	Permeate flow rate (L/h)	Cell concentration in output (g DCW/L)	Dilution rate (h^{-1})
10	0.0437	NA	0.0437	1.99	0.0063
12	0.0875	NA	0.0875	3.99	0.0125
14	0.1312	NA	0.1312	5.42	0.0188
16	0.1529	NA	0.1529	6.12	0.0218
19	0.2041	0.0417	0.1625	7.31	0.0292

the activity of MMO enzyme. In the present study MMO activity remained constant with ± 15% variation to the average values except end of batch phase (6th to 8th day) and membrane recycle phase (17th and 18th day). At the end of batch phase and membrane recycle phase both cell growth rate and PHB production rate were decreased. MMO activity was restored upon increasing media feed flow rate. Variation in MMO activity could be the effect of limitations in supply nutrients and trace metals like copper which is cofactor for MMO enzyme and regulates expression of pMMO enzyme.

Batch culture was initiated with 0.945 g nitrogen which resulted in the nitrogen limited condition on 7th day after 8.67 g of dry biomass production. The nitrogen limited conditions triggered PHB accumulation above 17%. After 7th day, controlling nitrogen supply through feed rate (Table 1) supported continuous growth of *M. trichosporium* along with higher PHB accumulation. During 27 days of fermentation nitrogen limited condition with supply of 10.08 g of total nitrogen supported growth of 110.5 g total dry cells. The final steady state was established, at maximum dilution rate of 0.0292 h^{-1} (21st day onwards). The maximum biomass productivity and biomass yield reached up to 10.85 g/day and 65.5 mg DCW/g of methane respectively (Fig. 6). The maximum PHB productivity and PHB yield for continuous culture with cell recycle mode was found to be 2.27 g PHB/day and 13.7 mg PHB/day (Fig. 7). The average PHB yield was stabilized at 7.5 mg PHB/g of methane after achieving steady state condition (after 21st day). In the continuous culture with cell recycle mode overall biomass productivity and PHB productivity was found to be 4.09 g/day and 0.73 g PHB/day respectively (Table 2).

Previously PHB production from *M. trichosporium* using nitrogen free mineral salt media (NFMS) demonstrated up to 55.5% PHB accumulation resulting in to maximum PHB productivity of 12.5 mg/L/h in batch mode [34]. Similar effect of increase in PHB production under limiting nitrogen conditions was also observed by Zaldivar Carrillo et al. while optimizing nitrogen source and process conditions using response surface methodology [20]. In the present study, PHB accumulation in *M. trichosporium* cells was found to be 24.6% by using NMS media. The accumulation of PHB could be further increased by controlling the nitrogen concentration in the feed media. Another study with *M. trichosporium OB3b* fermentation on enhancing gas to liquid mass transfer rate using polyvinylidine fluoride hallow fiber membrane resulted up to 1.77 fold increase (from 1.06 to 1.88 O.D. 600 nm) in cell density [35]. In the present study up to 4.9 fold increase in cell density could be achieved using continuous culture with cell recycle of *M. trichosporium* at maximum cell concentration 7.31 g DCW/L as compare to maximum 1.49 g DCW/L in batch mode.

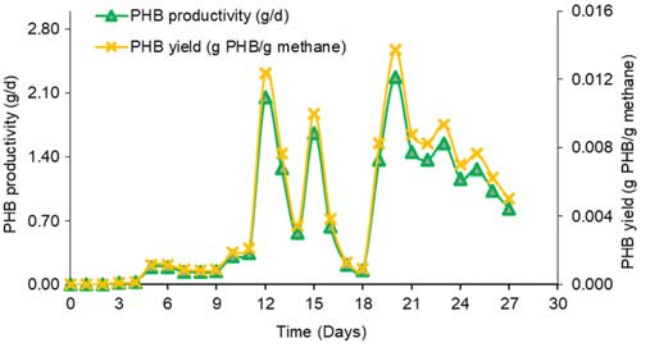

Fig. 7. Profiles of PHB yield and PHB productivity in continuous culture with cell recycle system.

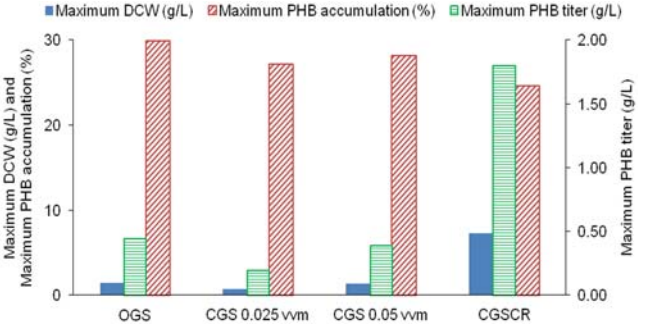

Fig. 8. Comparision of maximum dry cell weight (blue filled bars), PHB accumulation (red cross), PHB titer (green horizontal) in batch and continous cell recycle mode. On X axis: OGS (overlay gas supply), CGS (continuous gas sparging) with 0.025 vvm, CGS with 0.05 vvm and CGSCR (continuous gas sparging with cell recycle) respectively. Except CGSCR data is mean representation of two replicates with < 10% standard deviation.

Table 2. Different modes of fermentation with maximum PHB accumulation, biomass productivity and PHB productivity

Modes	Maximum PHB accumulation (%)	Biomass productivity (g/d)	PHB productivity (g/d)
Overlay gas supply	29.90	2.08	0.62
Continuous gas supply 0.025 vvm	27.18	0.72	0.20
Continuous gas supply 0.05 vvm	28.20	0.80	0.23
Continuous gas supply with cell recycle	24.6	4.09	0.73

To achieve the maximum throughput of products in a fixed reaction volume in the shortest possible time, the productivity must be maximized. As compared to batch process, continuous processes help in prolonging the exponential growth phase resulting in improvement in cell productivity (Fig. 8). It also helps in continuous removal of inhibitory substances through the harvested stream and has operational advantages like avoiding repeated cleaning, sterilization between subsequent batches and pre-culture preparation [23].

4. Conclusions

This is the first study on investigation of cell recycle system for enhancement of PHB production using methane rich gaseous feedstock. Our studies establish that continuous culture with cell recycle is suitable technique for cultivation of methanotrophs. It can also provide better control on cell growth rate by varying the dilution rate during cultivation. We demonstrated improvements in the continuous culture of *M. trichosporium* like increased growth rate and final cell density along with improved PHB production rate using cell recycle system. Being low cost and sustainable feedstock use of biogas can help in overall economy of PHB production. Our studies can help in advancement of industrial processes for PHB production as well as in the mitigation of green house gas emission in the environment. Operational flexibility, simple design and controlled flow process makes cell recycle system most suitable system for PHB production.

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Authors' Contributions

TRS: Conceived, design of research and experiments, performed experiment, samples analysis for PHB, data analysis, manuscript writing. PPK: Samples analysis for PHB, design of research, performed enzyme assay, data analysis, manuscript writing. ARG: Supervised and designed research, data analysis corrected and reviewed manuscript. All authors read and approved the manuscript.

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Ethical Statements

This article does not contain any studies with human participants or animals performed by any of the authors. The authors declare that they have no competing interests.

Availability of Data and Materials

Data will be provided on request.

Consent to Participate

Not applicable

Consent for Publication

Not applicable

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