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A novel integrated biorefinery process for diesel fuel blendstock production using lipids from the methanotroph, *Methylomicrobium buryatense*

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ABSTRACT

In light of the availability of low-cost methane (CH_4) derived from natural gas and biogas along with increasing concerns of the greenhouse gas emissions, the production of alternative liquid biofuels directly from CH_4 is a promising approach to capturing wasted energy. A novel biorefinery concept integrating biological conversion of CH_4 to microbial lipids together with lipid extraction and generation of hydrocarbon fuels is demonstrated in this study for the first time. An aerobic methanotrophic bacterium, *Methylomicrobium buryatense* capable of using CH_4 as the sole carbon source was selected on the basis of genetic tractability, cultivation robustness, and ability to accumulate phospholipids in membranes. A maximum fatty acid content of 10% of dry cell weight was obtained in batch cultures grown in a continuous gas sparging fermentation system. Although phospholipids are not typically considered as a good feedstock for upgrading to hydrocarbon fuels, we set out to demonstrate that using a combination of novel lipid extraction methodology with advanced catalyst design, we could prove the feasibility of this approach. Up to 95% of the total fatty acids from membrane-bound phospholipids were recovered by a two-stage pretreatment method followed by hexane extraction of the aqueous hydrolysate. The upgrading of extracted lipids was then demonstrated in a hydrodeoxygeation process using palladium on silica as a catalyst. Lipid conversion in excess of 99% was achieved, with a full selectivity to hydrocarbons. The final hydrocarbon mixture is dominated by 88% pentadecane ($C_{15}H_{32}$) based on decarbonylation/decarboxylation and hydrogenation of C16 fatty acids, indicating that a biological gas-to-liquid fuel (Bio-GTL) process is technically feasible.

1. Introduction

Due to the development of shale gas production, the cost of natural gas has been reduced from $13/million BTU (British thermal unit) in 2008 to $3/million BTU in 2015 [1], which in turn has made methane (CH_4) a more attractive choice for biological conversion to fuels and chemicals when compared with more conventional but high priced feedstocks such as sugars. The CH_4 from landfills and digesters typically present as a mixture of 55–70% CH_4 and 45–30% CO_2 [2]. The 2014 Biogas Opportunities Roadmap report pointed out the potential to generate 376–490 trillion BTU of biogas from landfills, livestock and wastewater in the U.S. [3]. All biogas collected from biorefineries, landfills and anaerobic digesters for municipal and agricultural waste treatment is considered to be a renewable feedstock by the U.S. Environmental Protection Agency [4]. Therefore, liquid fuels derived from these renewable CH_4 sources can not only be economically competitive, but also effectively meet the lifecycle greenhouse gas (GHG) reduction requirement.

CH_4 has a high global warming potential (GWP) that is 72 times that of carbon dioxide (CO_2) within a 20-year period [5], which has driven research efforts into reducing the GHG effect caused by CH_4 release. To reduce CH_4 emissions, excess gas generated in energy extraction and anaerobic digestion is often flared [6,7] resulting in enormous energy waste, CO_2 emission and hazardous black carbon production by incomplete burning [8]. Thus, converting CH_4,

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especially which would otherwise be wasted, to liquid fuels, could potentially be an economical method to reduce GHG emissions.

The chemical conversion of CH_4 into liquid fuels (GTL), such as the Fischer-Tropsch (FT) process has gathered increased attention over the last few decades [9]. However, the FT process which requires the production of syngas from natural gas as a first step, is limited by its disadvantages of low yield, poor selectivity, huge capital investment and large energy inputs [10], although significant effort has being applied to make this process economically viable at small scale. All these drawbacks have limited the deployment of this FT process to a few instances. As an alternative, the biological conversion of CH_4 into liquid fuel (Bio-GTL process) offers great promise on these frontiers with either natural gas or biogas as feedstock, on the basis of potentially high carbon conversion efficiency (CCE), good selectivity, low capital expenditure, and low GHG emission [1,7,11]. The microbial lipids produced by microorganisms can serve as a potential substitute for the traditional fats and oils obtained from animals and plants (as well as oleaginous algae and yeast) to produce sustainable drop-in biofuels [12–14].

The foundation for a Bio-GTL process lies in the ability of methanotrophic bacteria to metabolize methane. Methanotrophs, as a subset of a physiological group of bacteria known as methylotrophs, are characterized by their ability to utilize CH_4 as the sole carbon and energy source. Methanotrophs, discovered by Söhngen's group in 1906 [15], were first isolated and characterized in 1970s by Whittenbury and his coworkers [16], establishing the basis of the current classification of methanotrophic bacteria. Most methanotrophs are usually specified as aerobic microorganisms that can oxidize CH_4 to methanol and beyond for catabolism and anabolism [17,18], which in turn can serve as a production platform to convert CH_4 into myriad valuable products [19–22]. Recently, an aerobic Gamma-proteobacteria methanotroph, *Methylomicrobium buryatense*, was isolated from a soda lake in Russia. This strain assimilates carbon from methane via formaldehyde, employing the ribulose monophosphate (RuMP) cycle (Fig. S1) [23]. It grows rapidly at high pH in high salt medium which limits the ability of contaminating microorganisms to take over the culture [24]. It is naturally capable of producing 10% fatty acids on a dry cell weight basis. Genetic tools have been developed for this strain which could be used to increase the lipid content [25] or to turn the strain into a platform for a variety of bioproducts [26–28].

Membrane lipids represent a major fraction of the cell mass when cells are grown in the presence of copper ions because this growth condition causes cells to produce large amounts of particulate methane monooxygenase (pMMO), a membrane protein [29,30]. Because of the lack of phosphatidate phosphatase and acyl-CoA, diacylglycerol acyltransferase, *M. buryatense* is not able to convert phospholipids into triacylglyerates (TAGs) [31]. Since membrane lipids are the predominant source of fatty acids to be converted into fuels, *M. buryatense* produces mainly phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) [32]. Even though the lipid fraction relative to the cell mass can be highly relative to non-oleaginous organisms, the composition of the extractable lipid fraction from *M. buryatense* is very different from lipids accumulated by algae or oleaginous yeasts which can be predominantly TAGs. However, the high level of fatty acids in the membrane fraction coupled with the genetic tractability of the organism led us to test the feasibility of developing a methanotrophic route to hydrocarbon fuel production. The feasibility of this concept demands a successful extraction of phospholipids along with catalytic upgrading to hydrocarbons at high yield and with good phosphorus resistance to ensure long catalyst lifetimes, presenting significant technical challenges described below. The application of methanotrophic bacteria for the production of biofuel is a novel initiative; this report represents the first successful demonstration of the application of methanotrophic bacteria for the production of renewable diesel fuel blendstock.

During specific growth conditions (often through nitrogen depletion) algae and yeast usually accumulate large amounts of TAGs in lipid bodies. Lipid extraction from algae and yeast has been extensively investigated and developed for years [33,34], an efficient extraction of membrane phospholipid for biofuel production has not been explored. Based on techno-economic analysis (TEA) and life cycle assessment (LCA), it is clear that lipid extraction from dry biomass is energy-prohibitive. Therefore, it was necessary to develop a low-energy, scalable extraction process to directly recover lipids from wet biomass with minimal co-extraction of contaminants (for example inorganic catalyst poisons such as phosphorus, sulphur, and alkali metals) [35,36,33].

The extraction of phospholipids from wet biomass is mainly impeded by their amphiphilic nature and surfactant properties. The presence of polar moieties makes phospholipids partially water miscible. Analytical extraction of phospholipids from biomass typically utilizes the traditional Bligh and Dyer procedure (using a combination of chloroform and methanol) to extract an aqueous cell suspension [37]. Although effective, the co-solvent extraction system is not likely to be practical for industrial application due to the high energy requirement for water-miscible-solvent recovery and waste water treatment. In addition, phospholipids are prone to emulsions due to their surfactant properties [33]. The formation of an emulsion will reduce the lipid recovery, resulting in solvent waste or total failure in phase separation. Lastly, phospholipids are generally considered to be an inferior feedstock for biofuel because phosphorus (P) and nitrogen (N) might deactivate catalysts in deoxygenation [38]. Therefore a process built at least in part upon polar lipids will require a deoxygenation catalyst that will remain active in the presence of these heteroatoms as well as an extraction process that minimizes contamination of the lipid stream with other hydrophobic molecules such as pigments and membrane proteins.

A hydrodeoxygenation (HDO) process follows the extraction of methanotrophic membrane lipids to upgrade these into hydrocarbon molecules, suitable for use in diesel fuel. Applications of metal-containing catalysts (e.g., Pd, Pt, Rh, Ru, Cu and Fe) for HDO have been reviewed with noted advantages and disadvantages [39] and recent advances in hydrotreating platforms have also been summarized based upon various biomass-derived feedstocks [40]. However, there are no reports describing the transformation of membrane lipids into liquid transportation fuels. As noted above, phosphorus-containing molecules found in membrane lipid fractions are potent catalyst poisons [39]. Therefore the optimization of the upgrading process conditions and the development of the catalyst system are needed to efficiently convert these methanotrophic lipids to fuel range hydrocarbons. In the case of renewable diesel fuel production, the fatty acids derived from methanotrophic membrane lipids contain high levels of oxygen compared to petroleum-derived products and must be deoxygenated. An isomerization process may also be needed following deoxygenation to convert the n-alkanes to branched alkanes for improved diesel fuel characteristics.

This paper presents a novel integrated biological gas-to-liquid fuel (Bio-GTL) biorefinery process that includes high cell bacterial density culture using CH_4 providing biomass with high fatty acid content, efficient lipid extraction, and full conversion of these lipid precursors to obtain a hydrocarbon fuel that is an infrastructure-compatible diesel fuel blendstock (Fig. 1).

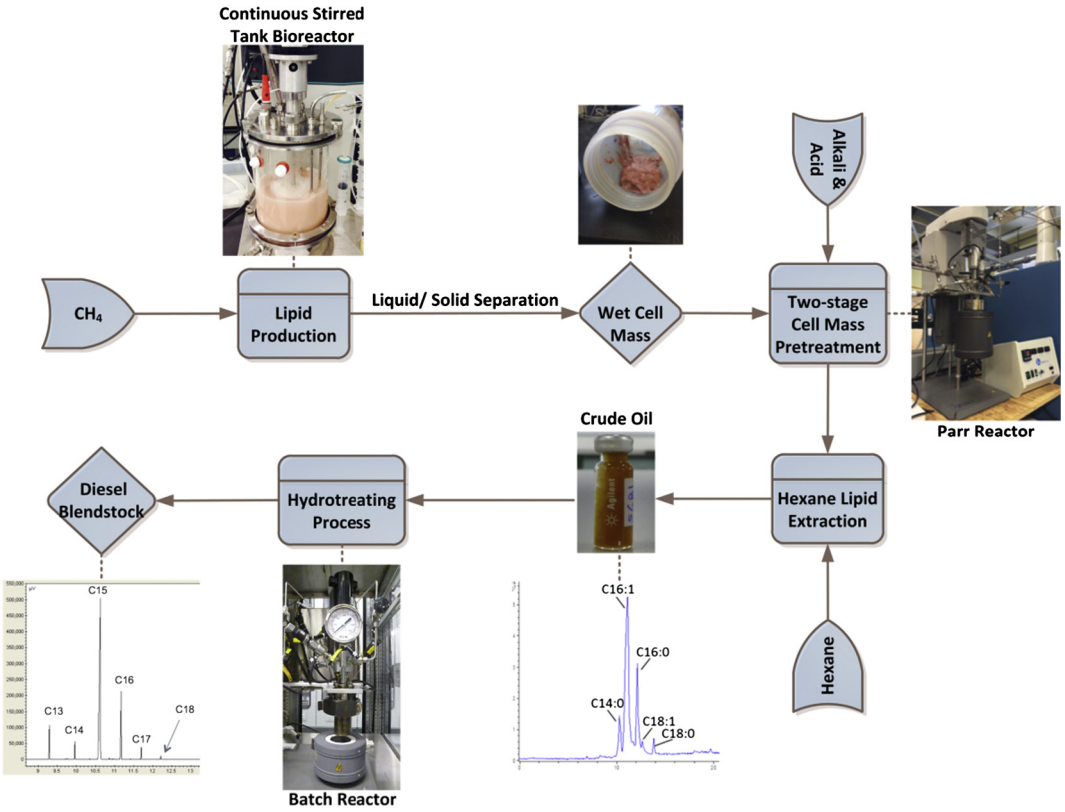

Fig. 1. Simplified scheme of the proposed biological conversion of CH_4 into diesel fuel blendstock process (Bio-GTL). A gas mixture of CH_4 and air is delivered to a 5 L continuously sparged stirred tank reactor for cell growth and lipid production. A centrifuge is used to harvest wet cell mass. A two-stage pretreatment using alkali and acid to disrupt the cell wall matrix is followed by a hexane lipid extraction. The crude oil mainly composed of free fatty acids is converted into the diesel fuel blendstock through a hydrodeoxygenation process in an autoclave reactor.

2. Materials and methods

2.1. Cultivation of M. buryatense 5GB1 for lipid production

M. buryatense 5GB1 was obtained from Professor Mary Lidstrom's group, University of Washington (Seattle, WA, USA). Seed cultures were grown at pH 9.0 ± 0.2 using a NREL modified nitrate mineral salts (NMS) medium containing the following, per liter of distilled water: KNO_3 2 g, $MgSO_4{\cdot}7H_2O$ 1 g, $CaCl_2{\cdot}6H_2O$ 0.02 g, NaCl 7.5 g, phosphate solution (KH_2PO_4 5.44 g/L and Na_2HPO_4 10.73 g/L) 20 mL, carbonate solution (1 M $NaHCO_3$ 700 mL and 1 M Na_2CO_3 300 mL) 50 mL, and trace element solution 1 mL [32]. *M. buryatense* 5GB1 was maintained by a weekly subculture on NMS2 [24] plate containing 2% agar and 1% methanol. CH_4 of 99.97% purity (United States Welding Inc, Denver, USA) was used as a sole carbon source for all cultures. Due to safety considerations, a gas mixture with 20% CH_4 and 80% air (v/v) was used for this study to avoid the flammable zone [41]. A continuous gas delivery system was developed and utilized to supply the gas mixture for all CH_4 cultures. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA).

A loop of colonies taken from an agar plate was used for the seed culture of 300 mL NREL modified NMS medium in a spinner bottle at 30 °C and 200 rpm for 48 h until optical density (OD600) of the seed culture reached 6.0 ± 0.5. Production cultures with 10% inocula were grown with the same culture medium as the seed culture except using 8 g/L KNO_3, 40 mL phosphate solution and 4 mL of trace solution. The production cultures were performed in a 5-L Bioflo 3000 bioreactor (New Brunswick Scientific Co., New Brunswick, NJ, USA) with working volumes of 3L. pH was maintained at 9.0 ± 0.2 by the addition of 4 N NaOH. Dissolved O_2 was monitored with an Ingold polarographic probe and the agitation speed was manually controlled. The flow rate of the gas mixture was controlled by a mass flow controller (Vögtlin Instruments, Aesch, Switzerland) at 1 vvm (Volume per Volume per Minute). To prevent excessive foam formation, a 1% sterile antifoam solution (PPG-PEG-PPG, CAS Number 9003-11-6, Sigma-Aldrich. Co, USA) was automatically added with a rate of 25 μL/h during production cultures. The offgas sensors of CH_4 and O_2 (BlueSens, Reckling-hausen, Germany) were used to monitor the gas uptake rate (mmol/L/d). The large-scale cultivations were performed in triplicate in this study.

Cell growth was estimated by measuring OD at 600 nm. For biomass analyses, culture samples of 10–20 ml were transferred to pre-weighed centrifuge tubes and centrifuged at 4696g for 20 min. The pellets were dried for 24–48 h in a lyophilizer for dry cell weight (DCW) measurement and fatty acid methyl ester (FAME) analysis [42].

2.2. Extraction of membrane lipids

The biomass slurry (equivalent to 50 mg dry weight) and deionized water, alkali or acid were sequentially fed into a 10 mL microwave tube to a volume of 4 mL. The mixture was heated from 85 to 180 °C for 5 to 60 min in a microwave reactor (CEM, Matthews, NC). After the pretreatment, microwave tubes were allowed to cool down to room temperature. Then, an equal volume of hexane was added for lipid extraction. The extractions were carried out on a multistirrer plate (Velp Scientific, Bohemia, NY) for 1 h with 30 s vortex every 10 min. After the extraction, the slurry mixture was allowed to stand 1 h for phase separation. Centrifugation and freezing were applied to assist phase separation if an emulsion

layer formed between the solvent and aqueous phases. The solvent phase was then transferred to a pre-weighed glass tube. The solvent was evaporated at 40 °C in a TurboVap Concentration Workstation (Caliper Life Sciences, East Lyme, CT) and then dried in a vacuum oven at 40 °C overnight to obtain dry weight of the extracted oil. Subsequent fatty acid quantification on the evaporated extract was carried out as described in previous report [42]. Since fatty acids are the precursors for hydrocarbon fuels, fatty acid recovery and purity in crude extracted oil stream were used to evaluate the extraction efficiency. The following definitions were used to quantify extraction efficiency: completeness of extraction (Eq. (1)) and purity of the crude oil (Eq. (2)). The experiments were carried in duplicates.

$$\text{Fatty acid recovery } (\%) = \frac{\text{Fatty acid in extracted oil (g)}}{\text{Fatty acid in biomass (g)}} \times 100\% \quad (1)$$

$$\text{Purity of extracted oil } (\%) = \frac{\text{Fatty acid in extracted oil (g)}}{\text{Extracted oil (g)}} \times 100\% \quad (2)$$

2.3. Scaled-up oil extraction

Fresh biomass slurry (16.6 g DCW), water and NaOH solution (20% w/v) were fed into a Parr reactor (Parr Instruments 4560 Mini Stirred Reactor) to obtain a slurry with biomass concentration of 10% and NaOH concentration of 1% (w/v). The biomass slurry was heated to 150 °C and held for 5 min. Then the reactor was cooled down in tap water and then H_2SO_4 was added to make up to an acid concentration of 2% (w/v) in the slurry. The biomass slurry was heated again to 150 °C and held for 5 min for acidic hydrolysis. After the treatment, the biomass slurry was transferred to a flask and extracted with equal volume of hexane overnight on a magnetic stir plate (Velp Scientific, Bohemia, NY). The mixture after the extraction was transferred to PTFE centrifuge bottles and centrifuged at 200*g* for 5 min to assist phase separation. The hexane phase was recovered by evaporation in a TurboVap Concentration Workstation at 40 °C and the remaining lipid fraction was dried in vacuum oven at 40 °C overnight.

2.4. HDO of extracted bacterial oil

A 5% Pd on silica catalyst was used throughout. This catalyst was selected based on earlier screening studies completed using model compounds (see Section 3.5 for more detail). The catalyst was prepared by incipient wetness impregnation using a solution of palladium nitrate and silica support (PQ Corporation, BET (Brunauer–Emmett–Teller) specific area = 490 m^2/g), followed by calcination at 450 °C. Elemental analysis by an inductively coupled plasma optical emission spectrometry (ICP-OES) confirmed a Pd loading of 4.7% by weight. H_2 reduction followed by CO chemisorption of the reduced catalyst gave a Pd surface area of 3.3 m^2/g.

Experiments were conducted in a small scale (45 mL) batch reactor made of solid superalloy (Alloy C-276). Catalyst (100 mg) was weighed into the reactor vessel. Solvent (decane, 25 mL) and extracted bacterial oil (0.5 mL) were added to the vessel, which was then secured to the reactor system in an extracted cabinet. Three purges were carried out with nitrogen. The reactor was then pressurized with hydrogen (4 MPa) and heated up to 360 °C for 6 h under stirring (800 rpm). Liquid samples were collected at the beginning and the end of the run for gas chromatography (GC) analysis.

2.5. Liquid chromatography-mass spectra (LC-MS) analysis of lipid samples

Intact lipid was extracted from lyophilized biomass using a Dionex ASE® 200 accelerated solvent extractor (ASE) system (Dionex, CA, USA). The dry sample was loaded into an 11 mL stainless steel vessel at a level of 0.2 g and was extracted with a mixture of chloroform/methanol (2:1, v/v) at a pressure of 10.3 MPa and a temperature of 50 °C. The solvent in extracted oil was evaporated in a TurboVap Concentration Workstation at 40 °C and the lipid fraction was dried in a vacuum oven at 40 °C overnight.

The lipid samples extracted from lyophilized and pretreated biomass were both analysed by LC-MS for characterization. Liquid chromatography was conducted on an Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA). Separations were conducted using two Agilent Zorbax Eclipse XDB-C8 columns in series (150 mm × 4.6 mm i.d., 5 μm; Foster City, CA) at 60 °C. Lipid samples were injected (injection volume, 5 μL) onto the column and analysed with a gradient of (A) isopropanol with 4 mM ammonium acetate, (B) methanol with 4 mM ammonium acetate, (C) acetonitrile and (D) water at a flow rate of 0.5 mL/min. The gradient was as follows: 0 min 85% A and 15% D which was held until 15 min; at 15.1 min mobile phase was switched to 10% A, 40% B, and 50% C; A increased to 50% while B decreased to 0% from 15.1 min to 55 min; at 55.1 min mobile phase was switched to 85% B and 15% D and was held until 60 min for a total run time of 60 min including equilibration.

Negative ion electrospray-mass spectrometry (ESI-MS) was performed on an LC/MSD Trap SL® ion trap mass spectrometer coupled to an electrospray ionization source with ChemStation® B. 01. 03 and MSD Trap Control 5.3 software (Agilent Technologies, Santa Clara, CA). The conditions used were: nebulizer gas 60 psi, auxillary gas 7 L/min, capillary voltage 3500 V, offset voltage −500 V, source temperature 350 °C, skimmer 60 V, end cap exit 157.8 V, direct current (DC)1 12 V, DC2 2.46 V, trap drive 78.9 V, radio frequency 200 Vpp, lens1 −5 V, lens2 −60 V with a scan range of m/z 100–1500. MS/MS was performed with a fragmentation amplitude of 0.3 V.

2.6. Hydrocarbon analysis

Off-line GC analyses were performed on a Varian CP-3800 equipped with an Agilent DB-5 column (cross-linked (5%-phenyl)-methylpolysiloxane, 30 m × 0.25 mm × 0.10 μm) using H_2 as carrier gas and a flame ionization detector. The starting temperature of the oven was set at 40 °C and kept for 5 min, after which the temperature was first raised to 240 °C at a ramp rate of 20 °C/min. Then the oven was heated to 320 °C at a ramp rate of 50 °C/min and held for 20 min. GC–MS analyses were performed on a ThermoScientific Trace 1300 GC-ISQ MS equipped with an Agilent DB-17MS column (cross-linked (50%-phenyl)-methylpolysiloxane 60 m × 0.250 mm × 0.225 μm), using H_2 as carrier gas. The same temperature program as for the GC was used. Ionization was done by electronic impact at 70 eV. Masses were scanned between 40amu and 700amu.

3. Results and discussion

3.1. Lipid production by M. buryatense 5GB1 using CH_4 as the sole carbon source

Even though much effort has gone in novel bioreactor design, continuous stirred tank bioreactors (CSTRs) are still the most widely used vessels for gas fermentation in industrial scale [43]. For this exercise, we employed a 5L CSTR with 3L working volume

with agitation rate of 500 rpm and gas mixture flow rate of 1 vvm for batch cultivation of 5GB1 to generate biomass for the demonstration of the production of microbial lipids as the precursors of diesel fuel production.

In our exploration of cultivation conditions we have observed that use of lower agitation rates can result in lower cell densities (9.9 g/L at 500 rpm and 17.4 g/L at 1000 rpm) but higher fatty acid content (8.5% at 500 rpm and 5.5% at 1000 rpm). Since the purpose of this work is to produce lipid for diesel production, the FAME content of the biomass was a more important parameter than total biomass concentration and so our biomass production run was conducted at 500 rpm agitation rate rather than the maximum 1000 rpm. As shown in Table 1, a dry cell weight (DCW) of 10.2 g/L along with a 9.5% FAME content was obtained at the end of the large-scale batch cultures, which provided a FAME titer of 0.96 g/L and FAME productivity of 0.0134 g/L/h. Under conditions of CH_4 limitation, carbon conversion efficiencies to cellular biomass as high as 61% (mol/mol) have been observed [37]. With 10% of the biomass as fatty acids, this equates to a yield of approximately 6.1% of the CH_4 carbon in the fuel intermediate. Thus it is clear that our process metrics are far too low for a commercial process, but we believe that this fermentation process offers a starting point to evaluate downstream processes of lipid extraction and upgrading. It is expected that a high cell density culture and lipid production can be achieved under a better gas transfer efficiency [25]. Although increasing the agitation speed or modifying the bioreactor's impellor of the CSTR can improve the gas-liquid interfacial area, high shear rates and massive energy input are obstacles for the application in industrial-scale. Therefore, many bioreactor designs to improve the gas transfer efficiency have been developed including bubble column bioreactor, loop and airlift bioreactor, trickle-bed bioreactor, monolithic biofilm bioreactor and membrane biofilm bioreactor, which can enhance both methanotroph growth and carbon conversion efficiency [1].

As shown in Table 2, the fatty acids produced by *M. buryatense* 5GB1 were composed mainly of long chain fatty acids with myristic acid (C14:0), palmitic acid (C16:0) and mono-unsaturated C16 (C16:1). The composition of fatty acids remained notably constant throughout the cultivation process. Although C14 fatty acid significantly increased during cultivation, the overall content of C16 fatty acids (both saturated and unsaturated) made up over 90% of the total fatty acids. A total wet cell mass of 160 g harvested from the batch cultures was used for the large-scale lipid extraction and upgrading work.

3.2. Investigation of biomass pretreatment conditions for fatty acid recovery

Biomass pretreatment methods have been developed for deconstruction of lignocellulosic biomass as a means to disrupt the matrix, hydrolyzing hemicellulose to a varying degree and making the remainder of the carbohydrate accessible to enzymatic saccharification. More recently we have modified dilute acid pretreatment to rupture algal cell walls, liberating lipids for extraction [44,45,36]. Algal lipids, which mainly consist of triglycerides (when cultures are grown in a manner to produce high lipid content), are easily extracted with hexane to give yields as high as 95% from pretreated slurries [45]. However, lipids in methanotrophic biomass are mainly phospholipids, which are amphiphilic polar lipids. The solubility of polar lipids in organic solvent (hexane) is not as high as neutral lipids and they tend to behave as emulsifiers during extraction [33]. Thus, a successful pretreatment process should be able to facilitate extraction, and we evaluated alkaline, acidic and alkali-acid two-stage approaches prior to hexane extraction using fatty acid recovery, fatty acid purity and emulsion stability as metrics of success.

Table 1
The production of cell mass and lipid in bench-scale fermentation (replicates n = 3).

Time	DCW, g/L	FAME, g/L	FAME, % (w/w)
24 h	4.27 ± 0.99	0.43 ± 0.13	10.0 ± 0.9
48 h	8.37 ± 1.31	0.81 ± 0.16	9.7 ± 0.8
72 h	10.22 ± 1.16	0.96 ± 0.05	9.5 ± 0.8

The biomass slurry was almost totally dissolved after the alkaline pretreatment, generating a transparent solution with minor insoluble residue. This result suggests that alkali is capable of hydrolysing the biomass to liberate lipids for extraction [46]. However, there was a significant emulsion problem in the subsequent solvent extraction process, even though the slurry was neutralized. Freezing and centrifugation was able to break the otherwise stable emulsion to achieve sufficient phase separation, but this was unsatisfactory and the investigation of alkali pretreatment was discontinued. In contrast, emulsions were much less problematic when acid pretreatment was employed. However, when acid was introduced, the biomass coagulated, which could cause problems for a process carried out at a large scale. To take advantage of the biomass hydrolysis capacity of alkaline treatment and the emulsion reduction power of acidic treatment, a two-stage pretreatment, in which alkali hydrolysis is followed by acid hydrolysis, was investigated. Very little emulsion formation was observed after the two-stage pretreatment, allowing for an easy phase separation.

3.3. Optimization of pretreatment conditions for a high fatty acid recovery

Based on the initial data, the acid and two-stage pretreatment approaches showed promise in increasing the efficiency of lipid extraction and so we evaluated both, varying time, temperature and chemical concentrations. Both the recovery and the purity of the extracted oils were used as the metrics of process optimization.

As shown in Fig. 2A and B, lipid extraction efficiency generally increased with elevated temperature for both acid and two-stage pretreatments. It was observed that alkali was able to dissolve almost all of the biomass, which is consistent with alkali-induced protein hydrolysis [47], in this case applied to the glycoprotein cell matrix surrounding the 5GB1 cells. The application of alkali might help to disassemble biomass liberating embedded membrane lipids. At the best temperature of 150 °C, the highest fatty acid recovery of 82.2% and 100% was obtained for acid and two-stage pretreatment, respectively. Thus, a pretreatment temperature of 150 °C was chosen for the follow up experiments.

As shown in Fig. 3A, fatty acid recovery increased to 84.6% with prolonged acid pretreatment time until 30 min. The longer hydrolysis time might facilitate the hydrolysis of coagulated biomass to release entrapped lipids. For the two-stage pretreatment a high recovery of the fatty acids was obtained with a short period of pretreatment time (Fig. 3B), providing another advantage for two-stage pretreatment. Even though a high fatty acid recovery was obtained after a two-stage pretreatment process with 2.5 min alkali pretreatment followed by 2.5 min acid pretreatment (abbreviated as "2.5 min + 2.5 min"), it was observed that the emulsion layer under pretreatment condition with longer times ("5 min + 5 min") was remarkably thinner. The hexane phase could be totally separated from the aqueous phase for condition "5 min + 5 min" after low speed centrifugation (200*g* 5 min). Thus, acid pretreatment was abandoned in favor of the two-stage pretreat-

Table 2
Major fatty acid composition of the lipid produced by *M. buryatense* 5GB1 (fatty acids present at <1% are not listed).

Time	Relative amount of total fatty acids, %						
	C14:0	C15:0	C16:0	C16:1n9	C16:1n7	C16:1n6	C16:1n5
24 h	2.9 ± 0.2	0.7 ± 0.1	18.6 ± 0.2	18.3 ± 0.4	38.8 ± 0.5	18.1 ± 0.1	1.5 ± 0.1
48 h	4.6 ± 0.1	1.0 ± 0.2	18.3 ± 0.4	23.1 ± 0.5	28.3 ± 0.1	21.1 ± 0.1	2.5 ± 0.2
72 h	6.0 ± 0.1	1.1 ± 0.1	18.5 ± 0.3	24.0 ± 0.2	24.6 ± 0.4	21.4 ± 0.2	3.1 ± 0.2

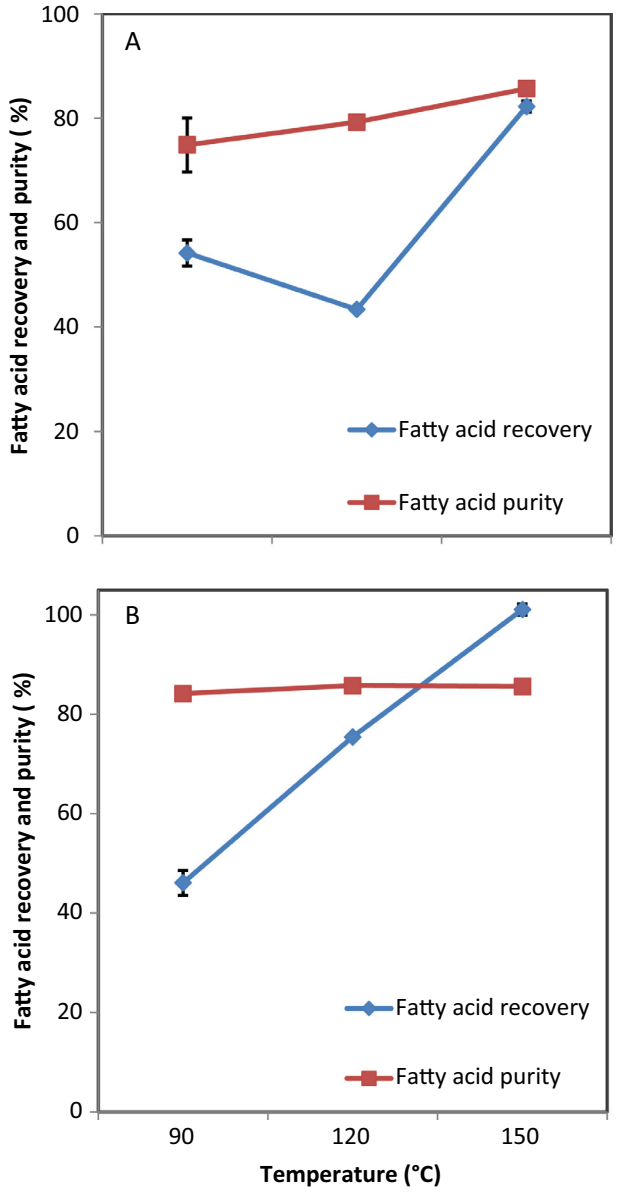

Fig. 2. The effects of temperature on acid & two-stage pretreatments. Pretreatment condition: 2% H_2SO_4, 20 min for acid pretreatment (A); 1% NaOH 10 min followed by 2% H_2SO_4, 10 min for two-stage pretreatment (B). Oil purity is expressed by the concentration of fatty acid in the extracted oil.

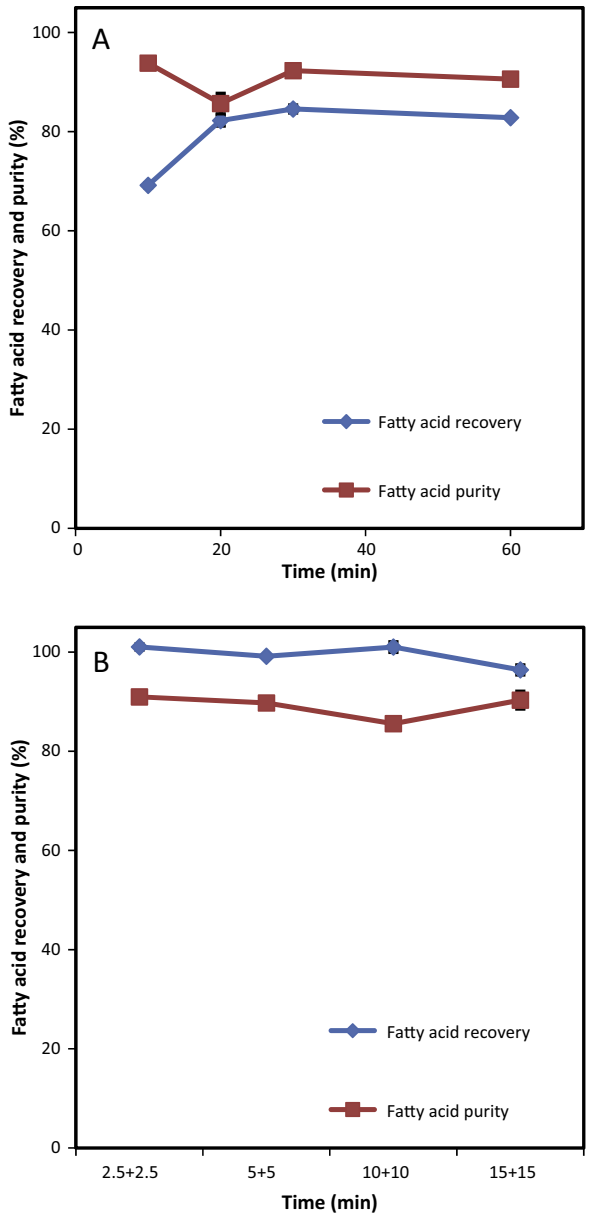

Fig. 3. The effects of time on acid & two-stage pretreatment. Pretreatment condition: 2% H_2SO_4, 150 °C for acid pretreatment (A); 1% NaOH followed by 2% H_2SO_4, 150 °C for two-stage pretreatment (B).

ment process with 5 min alkali pretreatment followed by 5 min acid pretreatment.

The cell wall of gram-negative bacteria consists of a peptidoglycan layer, constructed of an N-acetylglucosamine and N-acetylmuramic acid oligomeric linear chains [48]. The linear chains are cross-linked by peptidic linkages. Our hypothesis is that the integrity of the glycoprotein-rich cell wall of gram-negative bacteria is susceptible to alkaline hydrolysis, which can aid with the accessibility of the lipid-rich membrane layers. Alkali first hydrolyzes the peptidic linkages and makes the linear acetylglucosamine and acetylmuramic acid chain exposed to the following acid pretreament. Thus, the linear chain can be easily hydrolyzed by acid, reducing the emulsification effect that is observed with only alkaline treatment.

As shown in Fig. 4, three alkali/acid concentration levels were tested. The results illustrated that higher levels correlated with

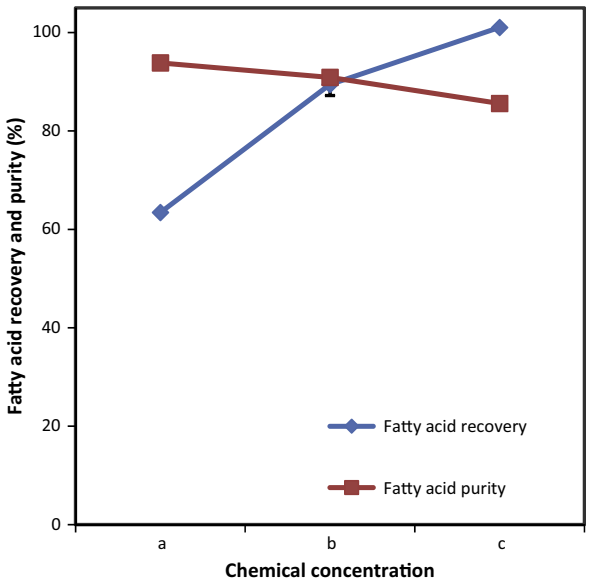

Fig. 4. The effects of chemical concentration on two-stage pretreatment. Pretreatment condition: 150 °C, 5 min alkali pretreatment followed by 5 min acid pretreatment. (a) 0.25% NaOH followed by 0.5% H_2SO_4; (b) 0.5% NaOH followed by 1% H_2SO_4; (c) 1% NaOH followed by 2% H_2SO_4.

better fatty acid recovery. Our results show that an almost complete recovery of fatty acids can be obtained by using 1% of NaOH and 2% H_2SO_4 in a two-stage pretreatment within 10 min. We chose to have better yields even if that resulted in slightly lower purity based on concurrent progress made in catalysis process development (see below).

3.4. Scaled-up biomass pretreatment and lipid extraction

The scaled-up biomass pretreatment and extraction process was carried out to generate sufficient lipids for catalytic upgrading. The optimum two-stage pretreatment (1% NaOH, 5 min followed by 2% H_2SO_4, 5 min at 150 °C) was applied to 160 g of wet biomass (10% dry cell weight) in a Parr reactor (Parr Instruments 4560 Mini Stirred Reactor), and 97.3% of the fatty acids were recovered with hexane extraction. The LC-MS analysis (Fig. S2) shows that lipids in the original biomass were in phospholipid form (mainly PG and PE). After the two-stage pretreatment, all the phospholipids were converted into free fatty acids (FFAs), which are preferred feedstocks for catalytic upgrading compared to phospholipids. The low P content (4 ppm) in the extracted oil is consistent with the phospholipids being hydrolysed to FFAs and phosphorous left in the aqueous phase (Table 3). Thus this approach significantly mitigates the potential issue of catalyst poisoning by phosphorus through the nearly complete hydrolysis of phospholipids to FFAs. The FFA purity was as high as 97%, with C16:0, C16:1 and C14:0 as the dominant fatty acids in the extracted oil. Thus, a very clean oil stream with high FFA content was produced by our two-stage pretreatment followed by solvent extraction.

3.5. Lipid upgrading toward hydrocarbon via catalytic HDO process

The last step of the process is the deoxygenation of the FFAs extracted from the bacterial oil to hydrocarbons. The extraction process developed results in a feed with a low phosphorus content, but still at a level (ppm) that will result in catalyst deactivation due to poisoning and reduce operational lifetime. A catalyst screening study (data not shown) was done on model feed systems (mixture of FAME or FFA and a phosphorus source) using increased levels of

Table 3
Elemental analysis of extracted oil.

Elements[a]	TX[b]	P	Na	S	N
Concentration (ppm)	68	4	11	1250	546

[a] The concentrations of Ca, Fe, Mg, and K in the extracted oil were lower than 2 ppm (not shown in this table).
[b] Total halogens (Cl + Br + I) as equivalent chlorine.

Table 4
Fatty acid distribution in extracted oil and hydrocarbon distribution in the finished biofuel product.[a] Errors are estimated to be ±5%.

Extracted oil		Biofuel product	
Fatty acids	Content %	Hydrocarbons	Content %
C14:0	6.1	C13	6.2
C15:0	1.1	C14	1.8
C16:0	18.2	C15	87.9
C16:1	73.6	C16	2.8
C18:0	0.5	C17	1.3
C18:1	0.3		

[a] Fatty acids with concentrations lower than 0.1% were not shown in this table.

phosphorus to accelerate catalyst deactivation. This study revealed that Ni- and Cu-based catalysts, generally suitable for HDO reactions, are rapidly deactivated in the presence of phosphorus, leading to very low yields of hydrocarbon products. Platinum group metals (PGM) such as palladium, platinum and ruthenium were found to be phosphorus resistant. Although perceived as higher cost alternatives than base metal catalysts, the improved performance and stability of the PGM-based catalysts, plus the fact that the metal component can be effectively recovered and recycled from the catalyst after use, makes these both technically and economically preferred. Based on these results and continuous runs carried out with model feed systems, a 5% Pd on silica catalyst was selected for the catalytic upgrading step. The experiment was conducted with the extracted bacterial lipids. The composition of this lipid is given in Table 4. FFA contents add up to 97% of the total, with the balance of organic components comprising mainly C18-amides (0.7%), squalene (0.8%) and hopanoids (1.5%) (Fig. S3).

Upgrading of fatty acids consists of deoxygenation reactions which yield hydrocarbons. In HDO reactions, the fatty acid can be reduced sequentially to fatty aldehydes, fatty alcohols and then to the fully reduced n-alkane with the same chain length as the starting fatty acid. Alternatively, in hydrodecarboxylation/decarbonylation (HDC) reactions, n-alkanes are formed with chain lengths one carbon shorter than the starting fatty acid (Fig. 5). These two reaction pathways and the formation of these intermediates are well documented in the literature using different types of catalysts [38,49,50].

Upgrading was carried out in an autoclave reactor using 0.5 mL of bacterial oil dissolved in 25 mL of solvent (decane) and using 100 mg of catalyst. The upgrading reaction was carried out over a period of 6 h under an initial atmosphere of hydrogen (4 MPa pressure) at 360 °C. GC analysis of the resultant product (Fig. S4) shows full conversion of the starting fatty acids. The product was dominated by linear hydrocarbons ranging from C13 to C17 in chain length. The formation of C13 to C17 hydrocarbons is as expected from the distribution of fatty acid chain lengths (C14 to C18) in the starting oil based on a combination of HDC and HDO pathways. The yield of C17 linear hydrocarbon was higher than would be expected based on the fatty acid distribution given in Table 4; the increased level of this product is likely due to the conversion of longer chain amides (octadecanamide and octadecenamide) that were present in the starting oil (0.7%). These amides were no longer detected in the hydrocarbon product after the upgrading

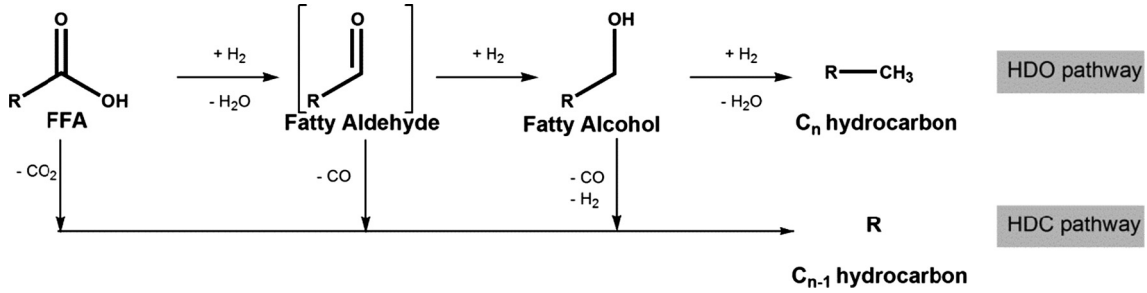

Fig. 5. Reaction pathways for the deoxygenation of FFA to C_n (HDO pathway) or C_{n-1} (HDC pathway) hydrocarbons. HDO represents hydrodeoxygenation and HDC represents hydrodecarbonylation/hydrodecarboxylation.

step, consistent with full conversion of these minor components in this step (see Fig. S4).

Octane was also detected in the product mix. The presence of octane has been confirmed by GC–MS analysis. The source of this product is not immediately obvious - it may be derived from cracking of longer chain molecules, but a lack of other cracked products and isomerization in the product distribution is consistent with low levels of these reactions occurring. GC analysis of the product from hydrotreating also showed full conversion of the squalene and hopanoids present in the bio-oil (Fig. S4). These were either converted to squalane and hydrogenated derivatives or were potentially degraded to shorter chain compounds and may account for the presence of octane in the chromatogram.

Since the starting oil was composed of 97% of FFAs, these side products (squalane, hydrogenated hopanoids and octane) were not present in significant amounts. The main products of the catalytic upgrading reaction were the linear saturated hydrocarbons. The distribution of these hydrocarbons (Table 4) shows that pentadecane (87.9%) is the major product, which was expected since C16 fatty acids represent the main components of the bacterial oil and the HDC pathway, which leads to the formation of the C_{n-1} hydrocarbon, is the dominant deoxygenation pathway. No significant isomerisation occurred under the upgrading conditions employed.

In summary, bacterial oil, composed dominantly of FFAs, has been catalytically upgraded to linear saturated hydrocarbons. The main reaction pathway observed is HDC, which leads to the loss of one carbon from the initial fatty acids. At this scale of experimentation, no significant effect of the impurities on the catalyst was found and full conversion was achieved with the extracted bacterial oil.

4. Conclusions

This study, for the first time, demonstrates an integrated biorefinery process to convert CH_4 into diesel blendstock product. *M. buryatense* 5GB1 was grown in a CSTR with continuous sparging of air plus CH_4 to provide biomass with high membrane lipid content. A two-stage pretreatment process was developed which could efficiently release membrane-bound lipids primarily as FFAs, which were then recovered by hexane directly from aqueous environment without energy-intensive dehydration. The extracted oil stream was converted into n-alkanes with 99% efficiency via a catalytic HDO using Pd/SiO_2 as a catalyst. At the moment this process is limited in utility due to the low lipid content of the wild-type *M. buryatense* biomass, but it demonstrates in principle, the feasibility of this approach, and establishes successful upgrading of phospholipid-derived fatty acids, something of a worst-case scenario based on conventional wisdom. Work is in progress to improve the lipid content in *M. buryatense* through metabolic engineering, but even with a successful outcome in these efforts, membrane lipids are likely to remain a significant fraction of the total lipids and so our success in extraction and upgrading is likely to remain relevant even with improved production strain. Compared to traditional FT process, this biorefinery concept enjoys the major advantages of flexible scale and mild conversion conditions, offering the opportunity for deployment at the locations where sufficient sources of currently wasted natural gas or biogas are generated offering an opportunity to reduce GHG emissions and produce liquid biofuels.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enconman.2017.02.075.

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