A two-staged bacterial process coupling methanotrophic and heterotrophic bacteria for 1-alkene production from methane

Ramita Khanongnuch[a,*], Rahul Mangayil[a], Ville Santala[a], Anne Grethe Hestnes[b], Mette Marianne Svenning[b], Antti J Rissanen[a]

[a]Faculty of Engineering and Natural Sciences, Tampere University, P.O. Box 541, 33014 Tampere, Finland

[b]Department of Arctic and Marine Biology, UiT, The Arctic University of Norway, 9037 Tromsø, Norway

*Corresponding author:

Ramita Khanongnuch, Faculty of Engineering and Natural Sciences, Tampere University, P.O. Box 541, 33014 Tampere, Finland; email: ramita.khanongnuch@tuni.fi; Phone: +358 442415042

Abstract

Methane (CH_4) is a sustainable carbon feedstock source for aerobic CH_4-oxidizing bacteria (methanotrophs) to produce value-added chemicals. Under O_2-limiting conditions, CH_4 oxidation results in the production of various short-chain organic acids and platform chemicals. However, these CH_4-derived products are still limited to C2-C6 and could be extended by utilizing them as a feedstock for heterotrophic bacteria. A two-stage system for CH_4 abatement and 1-alkene production was developed in this study. Gamma- and alphaproteobacterial methanotrophs, i.e. *Methylobacter tundripaludum* SV96 and *Methylocystis rosea* SV97, respectively, were investigated in batch tests under different gas supplementation schemes. Under O_2 limiting conditions (O_2/CH_4 molar ratio ~0.3), *M. tundripaludum* SV96 could potentially produce formate, acetate, succinate, and malate, accounted for ~7.4% of mol-CH_4 consumed. For the first time, the organic acids-rich spent media derived from O_2 limited-methanotrophic cultivation were successfully used for 1-alkene production using engineered *Acinetobacter baylyi* ADP1 (‘*tesA-undA*) cells.

Keywords: methane, methanotrophs, organic acids production, 1-alkene production, *Acinetobacter baylyi* ADP1

1. Introduction

Methane (CH_4) is the second most important greenhouse gas (GHG) after CO_2, having a global warming potential of 28-34 times higher than CO_2 (Voigt et al., 2017). Its emission from anthropogenic activities has been continuously increasing, being approximately as high as 60% of total CH_4 emission to the atmosphere (based on top-down estimates of Saunois et al., 2020). Hence, keeping on a stringent climate policy for the next several decades, particularly the use of CH_4 as an energy source, would significantly reduce CH_4 emissions (Harmsen et al., 2020). In environmental carbon flux, CH_4 oxidizing bacteria are the key regulators for CH_4 abatement (Hanson and Hanson, 1996; Saarela et al., 2020). Due to its abundance and potentiality as a sustainable carbon feedstock, the development of the

biological processes for CH_4 conversion to bio-based chemicals/liquid fuel is promising and attractive research area (Liu et al., 2020; López et al., 2013; Sun et al., 2018).

Aerobic methane oxidizing bacteria (methanotrophs) containing methane monooxygenases (MMO) use CH_4 as the sole carbon and energy source. Methanotrophs are classified into gammaproteobacterial (Type I and X) and alphaproteobacterial (Type II) methanotrophs based on their different formaldehyde assimilation pathways, i.e., the ribulose monophosphate (RuMP) and the serine cycles, respectively (Kalyuzhnaya et al., 2015) (Fig. 1). Methanotrophs potentially produce various value-added products including liquid fuels, single cell protein, ectoine and soluble metabolites (Cantera et al., 2018; Ge et al., 2014; Sheets et al., 2016; Strong et al., 2016). Under O_2 limiting conditions, both gamma- and alphaproteobacterial methanotrophs can convert CH_4 to organic acids, such as formate, acetate, lactate and succinate, in order to maintain the cellular redox balance, resulting in a carbon loss in the form of excreted by-products (Fig. 1) (Costa et al., 2000; Gilman et al., 2017; Kalyuzhnaya et al., 2013; Vecherskaya et al., 2009). Microaerobic CH_4 oxidation have been coupled with other biological processes as methanotrophs' microaerobic fermentative metabolism provides organic carbon sources to other bacteria, e.g. in heavy metal bioremediation (Lai et al., 2016) and wastewater denitrification (Cao et al., 2019). On the other hand, organic acids produced from bacterial fermentation could also provide important feedstocks (platform chemicals) for biotechnological production of value-added products in biorefineries (Atasoy et al., 2018; Novak and Pflügl, 2018). But to our knowledge, the recovery and utilization of organic acids obtained from CH_4 oxidation process in such applications has not been previously studied. Moreover, the CH_4-derived value-added products are limited to short carbon chain compounds (C2-C6) and could be extended by utilizing the methanotroph-derived platform chemicals as a feedstock for heterotrophic bacteria that can be easily engineered.

Fig. 1.

Acinetobacter baylyi ADP1, a naturally competent gram-negative gammaproteobacteria, have been recently gained interest in bioengineering. The bacterium's capacity to metabolize a wide range of carbon sources and genetic tractability has led to its application in long-chain alkyl ester (wax ester, WE) and 1-alkene compound (i.e. 1-undecene) biosynthesis from aromatic lignin-derived monomers and lignocellulose (Luo et al., 2019; Salmela et al., 2020). As a platform chemical, 1-undecene can be used in the chemical synthesis of medium-chain poly-α-olefins (C33) which are commonly used as lubricants (Salmela et al., 2020). Recently, its ability to metabolize fermentation by-products has been taken advantage to broaden the metabolic landscape in biological production processes (Mangayil et al., 2019; Salmela et al., 2018). Hence, *A. baylyi* ADP1 would provide a promising, testable candidate for the bioconversion of CH_4–derived organic acids. However, it is critical to find a suitable methanotroph partner for such application. Currently, the organic acid production of methanotrophs is not widely studied (Gilman et al., 2017, 2015; Kalyuzhnaya et al., 2013; Vecherskaya et al., 2009). Furthermore, to our knowledge, no previous study has compared the quantity as well as yields of produced organic acids between gamma- and alphaproteobacterial methanotrophs.

With the aim to redirect the 'overflow carbon' from microaerobic methanotrophic fermentation, organic acids obtained from CH_4 oxidation process could potentially provide as important feedstocks towards high-value product formation. Thus, a two-step bioprocess setup was designed for successful integration of microaerobic fermentation with aerobic synthesis. Towards the two-stage process, the growth and metabolite production profiles of gamma- and alphaproteobacterial methanotrophs, i.e., *Methylobacter tundripaludum* SV96 and *Methylocystis rosea* SV97, respectively, were investigated under different gas supplementation schemes. Next, the possibility of using the spent medium from the

methanotrophs were tested as the growth substrate for the cultivation of wild type *A. baylyi* ADP1 for wax ester production. Finally, the synthesis of 1-alkenes from CH_4 was demonstrated by a two-stage process with previously engineered *A. baylyi* ADP1 (*tesA-undA*) strain (Luo et al., 2019) and a methanotroph, i.e. *M. tundripaludum* SV96 and *M. rosea* SV97.

2. Materials and Methods

2.1. Strains and cultivation conditions

M. tundripaludum SV96 and *M. rosea* SV97, isolated from Arctic wetland soil, Norway (Wartiainen et al., 2006a, 2006b), were used in this study. The methanotrophs were cultivated in nitrate mineral salts (NMS) medium (DSMZ 921 medium; initial pH ~6.80) supplemented with 1 µM lanthanum chloride ($LaCl_3$). The pre-inoculum was statically grown in 120-mL serum bottles containing 10 mL NMS medium and 20% CH_4 and 80% air in headspace at 20 °C.

For cocultivation studies, wild type and the engineered *A. baylyi* ADP1 strain (Luo et al., 2019) carrying the plasmid pBAV1C-*'tesA-undA* (*A. baylyi* ADP1 *tesA-undA*) were used. For pre-inoculum, *A. baylyi* ADP1 cells were inoculated in 10 mL culture tubes containing LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone and 5 g L^{-1} NaCl) supplemented with 0.5% glucose and 25 µg mL^{-1} chloramphenicol (for *A. baylyi* ADP1 *tesA-undA*). The inoculated tubes were grown overnight at 30 °C and 300 rpm. For 1-undecene synthesis, *A. baylyi* ADP1 *tesA-undA* was induced with 0.5 mM cyclohexanone (Luo et al., 2019).

2.2. Organic acid production by *M. tundripaludum* SV96 and *M. rosea* SV97

To evaluate the organic acid production from methanotrophs, *M. tundripaludum* SV96 and *M. rosea* SV97 were cultivated in batch under three different gas supplementation schemes (Fig. 2). Batch tests were performed in 120-mL serum bottles with a working volume of 15 mL NMS medium. The precultures were inoculated with an initial optical

density at 600 nm (OD_{600nm}) of 0.02 and the growth, CH_4 utilization and organic acid profiles were monitored every two days for 14 days at 20°C and static conditions. All the batch bottles were initially filled with 20% CH_4 and 80% air into headspace and incubated for 7 days (days 0-7). On day 7, the batch bottles were supplemented with three different gas compositions into headspace, (i) both CH_4 and air (20% CH_4 and 80% air), (ii) without CH_4 (20% N_2 and 80% air), and (iii) without air (20% CH_4 and 80% N_2). The bottles containing only NMS without bacterial cells were used as the control. The bottles were incubated further for 7 days (days 8-14). The biomass growth (OD_{600}), gas compositions in headspace and organic acid accumulation in liquid medium were monitored every two days.

To test CH_4 oxidation under completely anaerobic conditions, *M. tundripaludum* SV96 and *M. rosea* SV97 were incubated in 120-mL serum bottles with a working volume of 15 mL NMS medium and 20% CH_4 and 80% N_2 in headspace for 7 days. The cultivation with 20% CH_4 and 80% air in headspace was used as control 1, while those with 100% air and 100% N_2 were used as controls 2 and 3, respectively.

Fig. 2.

2.3. Conversion of organic acids from microaerobic methanotrophic fermentation to wax esters

In this test, *M. tundripaludum* SV96 and *M. rosea* SV97 were cultivated in 500-mL airtight bottles with 60 mL NMS medium in conditions as described previously (Section 2.2). To maximize the organic acid titer, the bottle headspace was initially filled with 20% CH_4 and 80% air and cultivated for 28 days at optimal conditions for *M. tundripaludum* SV96 and *M. rosea* SV97, individually. Gaseous composition in headspace and organic acid concentrations in liquid medium were monitored twice a week.

After 28 days, the methanotroph cultivation was stopped and the culture medium was used for *A. baylyi* ADP1 cultivation. The spent medium was collected by centrifugation at

12000 rpm for 10 min. Two 50-mL aliquots were transferred to 250 mL sterile flasks and used for *A. baylyi* ADP1 cultivation. Pre-grown *A. baylyi* ADP1 cells, washed using phosphate buffered saline (PBS: 137 M NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4), were added to an initial OD_{600nm} of 0.02 and grown under aerobic conditions at 30 °C and 300 rpm. After 4 h cultivation, the liquid culture was collected to monitor cell growth, organic acid concentration and wax ester production. The spent media from methanotrophs fermentation without *A. baylyi* ADP1 was used as contamination control (control 1) and *A. baylyi* ADP1 cells in NMS (fresh medium) was used to determine their background growth (control 2).

2.4. Conversion of organic acids from microaerobic methanotrophic fermentation to 1-alkene synthesis

In this test, the liquid culture samples from *M. tundripaludum* SV96 and *M. rosea* SV97 microaerobic fermentation were collected and directly used to cultivate *A. baylyi* ADP1 *tesA-undA* strain. The tests were conducted in quadruplicate in a sealed 20 mL glass tube containing 5 mL of the spent medium. Subsequently, *A. baylyi* ADP1 *tesA-undA* cells (initial OD_{600nm}, 0.02) were added into the vials. After 1 h of incubation at 30 °C and 300 rpm, 0.5 mM cyclohexanone was added to the vials to induce 1-undecene synthesis, and the vials were further incubated for 23 hours. The liquid culture samples from *M. tundripaludum* SV96 and *M. rosea* SV97 cultivation without *A. baylyi* ADP1 *tesA-undA* were included as the experimental control. The growth of *A. baylyi* ADP1 *tesA-undA* was estimated based on difference of OD_{600nm} between the tests and controls at 24-h incubation (ΔOD_{600nm}).

2.5. Analytical methods

Liquid samples were filtered through a 0.2 µm membrane (Chromafil® Xtra PET-20/25, Macherey-Nagel, Germany) prior to the analysis of liquid metabolites using a Shimadzu high-performance liquid chromatograph (HPLC) equipped with Rezex RHM-

Monosaccharide H^+ column (Phenomenex, USA) as described in Okonkwo et al. (2018). The gas samples in the headspace (CH_4, CO_2, O_2 and N_2) were measured using a Shimadzu gas chromatograph GC-2014 equipped with a thermal conductivity detector (TCD) and a Carboxen-1000 60/80 column (Agilent Technologies, USA). The oven temperature was held at 35 °C for 3.75 min, increased 30 °C min^{-1} to 150 °C for 3 min. The injector and detector were 155 °C and 160 °C, respectively. Helium was used as a carrier gas at 30 mL min^{-1}.

The production of 1-undecene in headspace was detected using gas chromatography-mass spectrometry (GC-MS) as described by Luo et al. (2019). Compounds were identified using the NIST/EPA/NIH Mass Spectral Library (NIST 05). The bacterial growth was measured as OD_{600nm} using an Ultrospec 500 pro spectrophotometer (Amersham Biosciences, UK) and as cell dry weight (CDW) using gravimetric method. The conversion factor between CDW and OD_{600nm} obtained from the experimental measurement was 0.2914 g L^{-1} OD^{-1} (R^2 = 0.9948) and 0.3015 g L^{-1} OD^{-1} (R^2 = 0.9892) for *M. tundripaludum* SV96 and *M. rosea* SV97, respectively. Growth rate was calculated as $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_1 and N_2 are the OD at time t_1 and t_2, respectively.

Wax ester production was detected by using lipid extraction and thin layer chromatography (TLC) analysis. Lipid extraction was done by using methanol-chloroform extraction described by Santala et al. (2011) and the obtained lipid phase layer was used for TLC analysis to visualize the total lipid composition. TLC analysis was done as described by Santala et al. (2011). Briefly, 60 µl of lipid phase of a sample was applied to TLC plate (10 × 20 cm HPTLC Silica Gel 60 F_{254} glass plates with 2.5 × 10 cm concentrating zone, Merck, USA) using n-hexane:diethyl ether:acetic acid of 90:15:1 as a mobile phase. The visualization was done by dyeing with iodine and Jojoba oil was used as a standard.

The statistical analysis was performed using Minitab16.0 (USA). The significant differences between treatments in the data sets (e.g., growth of the tested strains, gas

concentrations and utilization, concentrations and yields of the products produced by each strain) were analyzed using one-way of variance (ANOVA) with Tukey's multiple comparison tests at the 95% confidence interval, where *P-value* ≤ 0.05 was considered statistically significant.

3. Results and discussion

3.1. CH_4-derived organic acid production by *M. tundripaludum* SV96 and *M. rosea* SV97

Both *M. tundripaludum* SV96 and *M. rosea* SV97 used CH_4 as the sole carbon and energy sources and O_2 as an electron acceptor. Both the methanotrophs did not grow without CH_4 and in anaerobic conditions with CH_4 (shown in Supplementary data). Evaluation of CH_4-derived organic acid production were carried out for 14 days. Three different supplementation schemes of CH_4 and O_2 were applied after 7-day incubation of both *M. tundripaludum* SV96 and *M. rosea* SV97 (Fig. 3). During days 0-7, the growth rate of *M. tundripaludum* SV96 (0.04 h^{-1}) was higher than *M. rosea* SV97 (0.02 h^{-1}) in all tests (*P-value* < 0.05) (calculated based on Fig. 3). In the supplementation with both CH_4 and O_2 on day 14, *M. tundripaludum* SV96 and *M. rosea* SV97 showed the highest biomass production of 0.60 ± 0.03 and 0.48 ± 0.05 g CDW L^{-1}, respectively (Fig. 3a, d). However, the production yields of biomass and CO_2 were similar in all supplementation schemes (Table 1).

Fig. 3.

Table 1.

In all tests, the initial CH_4 and air composition in headspace was 20% and 80% corresponding to molar ratio of O_2/CH_4 of ~1.2 (Fig. 4). From days 10-14, the supplementation of both CH_4 and air resulted in the final CH_4 and O_2 concentrations of 6-9% and 1-3%, respectively (Fig. 3a and d) as well as the O_2/CH_4 molar ratio in the system remained about 0.2-0.3 (Fig. 4a and d). This condition induced CH_4 oxidation under O_2 limiting conditions to occur in the system and it showed the difference of organic acid

accumulation in different methanotroph types. After incubation under O_2 limiting conditions from days 10-14, *M. tundripaludum* SV96 accumulated the highest concentrations of formate (1.9 mM) and malate (0.04 mM) in the cultivation medium compared to other gas supplementation schemes (*P-value* < 0.05), while acetate and succinate concentrations were not significantly different (Fig. 4a-c). In the cultivation of *M. rosea* SV97, the succinate concentration (0.02 mM) was significantly higher under O_2 limiting conditions compared to other schemes (*P-value* < 0.05) (Fig. 4d). Besides, high concentrations of excreted formate and acetate were observed under O_2 limiting conditions (Fig. 4d and e), while their concentrations showed significant lower under excess O_2 (only air added scheme, Fig. 4f) (*P-value* < 0.05). However, malate was not detected in *M. rosea* SV97 culture. *M. tundripaludum* SV96 culture under both CH_4 and air supplementation scheme showed the highest yield of organic acid production (7.4 ± 1.3% carbon per mol CH_4) after the 14-day incubation, comparing to other supplementation schemes as well as to those observed in *M. rosea* SV97 tests (*P-value* < 0.05) (Table 1). This circumstance corresponded to the pH reduction only observed under both CH_4 and air supplementation scheme (shown in Supplementary data). The results suggested that O_2 limiting conditions could redirect the methanotroph metabolism turning into a glycolysis-based CH_4 fermentation mode, thereby secreting the CH_4-derived organic acids (Gilman et al., 2015; Kalyuzhnaya et al., 2013). Kalyuzhnaya et al. (2013) have reported that the genome of *M. tundripaludum* SV96 contains genes encoding enzymes involved in fermentation (e.g., pyruvate kinase py*kA*, acetate kinase *ackA*, malate dehydrogenase *mdh*). In *M. rosea* SV97 cultivation, low concentration of excreted organic acids was detected compared to *M. tundripaludum* SV96 (Fig. 4). It could be due to the less efficient carbon assimilation via the serine cycle in alphaproteobacterial methanotrophs than via the RuMP cycle in gammaproteobacterial methanotrophs. This circumstance contributed less to organic acid production in alphaproteobacterial

methanotrophs than in gammaproteobacterial methanotrophs resulting also in slightly higher %CO_2 production in *M. rosea* SV97 than in *M. tundripaludum* SV96 at all tests (Table 1).

Fig. 4.

Comparing to the studies of other gammaproteobacterial methanotrophs, *M. tundripaludum* SV96 used in our study showed higher organic acid yields, i.e., formate, acetate, and succinate, corresponding to its higher biomass concentration (CDW) at the end of the tests (Table 2). One reason could be the time-point for initiating the O_2 limiting conditions. Those previous studies of organic acid production from microaerobic CH_4 oxidation cultivated the methanotrophs with the initial CH_4 and O_2 concentrations in headspace of 20% and 5%, respectively (Gilman et al., 2017, 2015; Kalyuzhnaya et al., 2013). However, in this study, *M. tundripaludum* SV96 was prior cultivated aerobically before allowing O_2 limiting condition to occur. Whether the prior cultivation in aerobic conditions enhanced organic acid production in the subsequent microaerobic conditions requires further studies to compare organic acid production between totally microaerobic and the aerobic-microaerobic treatments.

Table 2.

3.2. The application of organic acids produced by methanotrophs for the biomass and wax ester productions by *A. baylyi* wild type ADP1

In this study, wild type *A. baylyi* ADP1 cells were grown in the organic acid-rich spent media of both methanotrophs, containing formate, acetate, and succinate (and malate for *M. tundripaludum* SV96). During 4-h incubation, *A. baylyi* ADP1 cells incubated in the spent media from *M. tundripaludum* SV96 and *M. rosea* SV97 grew to an OD_{600nm} of 0.14 ± 0.01 and 0.12 ± 0.03, respectively (Fig. 5a). Except for formate, acetate, succinate, and malate were utilized as carbon sources for biomass assimilation (Fig.5b and c). *A. baylyi* ADP1 cells do not utilize formate as a sole carbon source, but it is rather used for maintaining the cellular

redox balance (Kannisto et al., 2015). The growth of *A. baylyi* ADP1 was also confirmed by the present of fatty acid fractions derived from *A. baylyi* ADP1 biomass on TLC plates; however, wax ester production was not detected in this study (shown in Supplementary data). This could be due to the low concentration of organic acids in the spent methanotrophic fermentation media. Another reason could be due to rapid degradation of the accumulated wax ester during carbon limiting conditions. Wax esters are carbon storage compounds. Under carbon limiting conditions *A. baylyi* ADP1 cells utilize the stored carbon for maintaining cellular activities (Fixter et al., 1986; Salmela et al., 2018). Leading from the observations, wild-type ADP1 cells could utilize the spent media from methanotroph fermentation but required higher quantity of organic acids for wax ester production. Thus, the subsequent study investigated the application potential of the spent methanotroph fermentation media for 1-undecene production by engineered ADP1 strain.

Fig. 5.

3.3. 1-alkene synthesis from organic acids generated by methanotrophs for

Except formate, *A. baylyi* ADP1 *tesA-undA* completely utilized acetate (0.3 mM), succinate (0.05 mM), and malate (0.2 mM), present in the spent fermentation media (Fig. 6a). The production of 1-undecene from *M. tundripaludum* SV96 spent medium (14.1 ± 2.7 µg L^{-1}) was higher than that from *M. rosea* SV97 cultivations (1.0 ± 0.5 µg L^{-1}) (Fig. 6c), corroborating with the organic acid concentrations (Fig. 6a and b). Likewise, the growth of *A. baylyi* ADP1 *tesA-undA* was higher in spent medium from *M. tundripaludum* SV96 (0.115 ± 0.06 OD_{600nm}) than from *M. rosea* SV97 (0.070 ± 0.054 OD_{600nm}) (Fig. 6c). The production of 1-undecene was not detected in the control cultivations (shown in Supplementary data). In addition, the growth occurred during the co-cultivation was from solely *A. baylyi* ADP1 *tesA-undA* as both methanotrophs could not grow without CH_4 as a carbon source.

The obtained 1-undecene production in this study was lower than that reported for *A. baylyi* ADP1 *tesA-undA* (Luo et al., 2019; Salmela et al., 2020). This phenomenon can be attributed towards the higher substrate concentrations used in those studies. For example, Salmela et al. (2020) obtained 1-undecene concentration of up to 107 ± 8 µg L^{-1} from a two-stage system for 1-undecene production from cellulose which was converted into organic acids by *Clostridium cellulolyticum* (containing 5.2 mM glucose, 4.9 mM acetate and 6.8 mM lactate). The authors reported the highest 1-undecene production yield of ~35 µmol mol $C_{substrate}^{-1}$ using lactate as a substrate. Besides, Luo et al. (2019) developed a high ferulate-tolerant strain for 1-undecene production. The authors tested the use of high concentration of ferulate (100 mM) as a sole carbon source and obtained the 1-undecene production titer of 72 ± 7.5 µg L^{-1} (yield of 1.0 µmol mol $C_{substrate}^{-1}$). Interestingly, the use of the spent media from methanotrophs provided higher 1-undecene production yield than those previous studies, resulting 60.6 ± 14.9 and 40.6 ± 19.8 µmol mol $C_{substrate}^{-1}$ for *M. tundripaludum* SV96 and *M. rosea* SV97, respectively. The spent media of methanotrophs likely did not contain intermediate compounds being toxic to growth and 1-undecene production of *A. baylyi* ADP1 *tesA-undA*. Furthermore, the media could be directly used for the cultivation without purification or additional downstream processing.

Fig. 6.

The results indicate that the spent media from methanotrophs microaerobic fermentation is an excellent carbon source for heterotrophs. This two-stage bacterial process enhanced and extended the conversion of CH_4 to 1-undecene, a C11 compound. However, the quantity of the CH_4-derived organic acids produced by methanotroph requires to be enhanced in order to also improve the production efficiency of *A. baylyi* ADP1. In further studies, the optimization and scale-up of the system for methanotroph cultivation will be developed.

4. Conclusion

This study shows a proof of concept for integrating greenhouse gas (GHG) utilization and biofuel production: the application of organic acids produced by methanotrophs for 1-undecene (C11) production. A gammaproteobacterial methanotroph, *M. tundripaludum* SV96, showed potential for various organic acid production under O_2 limiting conditions. The organic acids-rich spent media of methanotrophs could be directly used as medium for the cultivation of the wild type *A.baylyi* ADP1 and the engineered *A.baylyi* ADP1 *tesA-undA* without additional downstream processes or purification. Acetate, succinate, and malate contained in the spent media were completely utilized by *A.baylyi* ADP1 *tesA-undA* for their 1-undecene production.

Appendix A. Supplementary data

E-supplementary data of this work can be found in online version of this paper.

Acknowledgement

The research work was supported by Kone foundation [grant number 201803224] and Academy of Finland [grant number 323214]. The authors would like to acknowledge Jin Luo for helping with using GC-MS for 1-undecene measurement.

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.

CRediT author statement

Ramita Khanongnuch: Conceptualization, Writing – Original Draft, Formal analysis, Investigation, Visualization **Rahul Mangayil:** Supervision, Conceptualization, Writing – Review & Editing, Formal analysis, Investigation **Ville Santala:** Methodology, Writing – Review & Editing **Anne Grethe Hestnes:** Methodology, Writing – Review & Editing **Mette Marianne Svenning:** Supervision, Methodology, Writing – Review & Editing **Antti J**

Rissanen: Supervision, Conceptualization, Formal analysis, Investigation, Writing – Review & Editing, Project administration, Funding acquisition

References

1. Atasoy, M., Owusu-Agyeman, I., Plaza, E., Cetecioglu, Z., 2018. Bio-based volatile fatty acid production and recovery from waste streams: Current status and future challenges. Bioresour. Technol. 268, 773–786. https://doi.org/10.1016/j.biortech.2018.07.042

2. Cantera, S., Muñoz, R., Lebrero, R., López, J.C., Rodríguez, Y., García-Encina, P.A., 2018. Technologies for the bioconversion of methane into more valuable products. Curr. Opin. Biotechnol. https://doi.org/10.1016/j.copbio.2017.12.021

3. Cao, Q., Liu, X., Ran, Y., Li, Z., Li, D., 2019. Methane oxidation coupled to denitrification under microaerobic and hypoxic conditions in leach bed bioreactors. Sci. Total Environ. 649, 1–11. https://doi.org/10.1016/j.scitotenv.2018.08.289

4. Costa, C., Dijkema, C., Friedrich, M., Garcõâ A-Encina, P., Fernaâ Ndez-Polanco, F., Stams, A.J.M., 2000. Denitrification with methane as electron donor in oxygen-limited bioreactors. Appl Microbiol Biotechnol 53, 754–762.

5. Fixter, L.M., Nagi, M.N., Mccormacks, J.G., Fewson, C.A., 1986. Structure, distribution and function of wax esters in *Acinetobacter calcoaceticus*. J. Gen. Microbiol. 132, 3147–3157.

6. Ge, X., Yang, L., Sheets, J.P., Yu, Z., Li, Y., 2014. Biological conversion of methane to liquid fuels: Status and opportunities. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2014.09.004

7. Gilman, A., Fu, Y., Hendershott, M., Chu, F., Puri, A.W., Smith, A.L., Pesesky, M., Lieberman, R., Beck, D.A.C., Lidstrom, M.E., 2017. Oxygen-limited metabolism in the methanotroph *Methylomicrobium buryatense* 5GB1C. PeerJ 2017. https://doi.org/10.7717/peerj.3945

8. Gilman, A., Laurens, L.M., Puri, A.W., Chu, F., Pienkos, P.T., Lidstrom, M.E., 2015. Bioreactor performance parameters for an industrially-promising methanotroph *Methylomicrobium buryatense* 5GB1. Microb. Cell Fact. 14. https://doi.org/10.1186/s12934-015-0372-8

9. Hanson, R.S., Hanson, T.E., 1996. Methanotrophic Bacteria. Microbiol. Rev. 60, 439–471.

10. Harmsen, M., van Vuuren, D.P., Bodirsky, B.L., Chateau, J., Durand-Lasserve, O., Drouet, L., Fricko, O., Fujimori, S., Gernaat, D.E.H.J., Hanaoka, T., Hilaire, J., Keramidas, K., Luderer, G., Moura, M.C.P., Sano, F., Smith, S.J., Wada, K., 2020. The role of methane in future climate strategies: mitigation potentials and climate impacts. Clim. Change 163, 1409–1425. https://doi.org/10.1007/s10584-019-02437-2

11. Kalyuzhnaya, M.G., Yang, S., Rozova, O.N., Smalley, N.E., Clubb, J., Lamb, A., Gowda, G.A.N., Raftery, D., Fu, Y., Bringel, F., Vuilleumier, S., Beck, D.A.C., Trotsenko, Y.A., Khmelenina, V.N., Lidstrom, M.E., 2013. Highly efficient methane biocatalysis revealed in a methanotrophic bacterium. Nat. Commun. 4. https://doi.org/10.1038/ncomms3785

12. Kannisto, M.S., Mangayil, R.K., Shrivastava-Bhattacharya, A., Pletschke, B.I., Karp, M.T., Santala, V.P., 2015. Metabolic engineering of *Acinetobacter baylyi ADP1* for removal of *Clostridium butyricum* growth inhibitors produced from lignocellulosic hydrolysates. Biotechnol. Biofuels 8, 198. https://doi.org/10.1186/s13068-015-0389-6

13. Lai, C.Y., Zhong, L., Zhang, Y., Chen, J.X., Wen, L.L., Shi, L.D., Sun, Y.P., Ma, F., Rittmann, B.E., Zhou, C., Tang, Y., Zheng, P., Zhao, H.P., 2016. Bioreduction of chromate in a methane-based membrane biofilm reactor. Environ. Sci. Technol. 50, 5832–5839. https://doi.org/10.1021/acs.est.5b06177

14. Liu, T., Li, J., Khai Lim, Z., Chen, H., Hu, S., Yuan, Z., Guo, J., 2020. Simultaneous removal of dissolved methane and nitrogen from synthetic mainstream anaerobic effluent. Environ. Sci. Technol. 54, 7629–7638. https://doi.org/10.1021/acs.est.0c00912

15. López, J.C., Quijano, G., Souza, T.S.O., Estrada, J.M., Lebrero, R., Muñoz, R., 2013. Biotechnologies for greenhouse gases (CH_4 , N_2O, and CO_2) abatement: State of the art and challenges. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-013-4734-z

16. Luo, J., Lehtinen, T., Efimova, E., Santala, V., Santala, S., 2019. Synthetic metabolic pathway for the production of 1-alkenes from lignin-derived molecules. Microb. Cell Fact. 18, 48. https://doi.org/10.1186/s12934-019-1097-x

17. Mangayil, R., Efimova, E., Konttinen, J., Santala, V., 2019. Co-production of 1,3 propanediol and long-chain alkyl esters from crude glycerol. N. Biotechnol. 53, 81–89. https://doi.org/10.1016/j.nbt.2019.07.003

18. Novak, K., Pflügl, S., 2018. Towards biobased industry: Acetate as a promising feedstock to enhance the potential of microbial cell factories. FEMS Microbiol. Lett. 365. https://doi.org/10.1093/femsle/fny226

19. Okonkwo, O., Lakaniemi, A.M., Santala, V., Karp, M., Mangayil, R., 2018. Quantitative real-time PCR monitoring dynamics of *Thermotoga neapolitana* in synthetic co-culture for biohydrogen production. Int. J. Hydrog. Energy 43, 3133–3141. https://doi.org/10.1016/j.ijhydene.2017.12.002

20. Saarela, T., Rissanen, A.J., Ojala, A., Pumpanen, J., Aalto, S.L., Tiirola, M., Vesala, T., Jäntti, H., 2020. CH_4 oxidation in a boreal lake during the development of hypolimnetic hypoxia. Aquat. Sci. 82. https://doi.org/10.1007/s00027-019-0690-8

21. Salmela, M., Lehtinen, T., Efimova, E., Santala, S., Mangayil, R., 2018. Metabolic pairing of aerobic and anaerobic production in a one-pot batch cultivation. Biotechnol. Biofuels 11, 187. https://doi.org/10.1186/s13068-018-1186-9

22. Salmela, M., Lehtinen, T., Efimova, E., Santala, S., Santala, V., 2020. Towards bioproduction of poly-α-olefins from lignocellulose. Green Chem. 22, 5067–5076. https://doi.org/10.1039/d0gc01617a

23. Santala, S., Efimova, E., Kivinen, V., Larjo, A., Aho, T., Karp, M., Santala, V., 2011. Improved triacylglycerol production in *Acinetobacter baylyi* ADP1 by metabolic Engineering. Microb. Cell Fact. 10, 36. http://www.microbialcellfactories.com/content/10/1/36

24. Saunois, M., Stavert, A.R., Poulter, B., Bousquet, P., Canadell, J.G., Jackson, R.B., Raymond, P.A., Dlugokencky, E.J., et al., 2020. The global methane budget 2000-2017. Earth Syst. Sci. Data 12, 1561–1623. https://doi.org/10.5194/essd-12-1561-2020

25. Sheets, J.P., Ge, X., Li, Y.F., Yu, Z., Li, Y., 2016. Biological conversion of biogas to methanol using methanotrophs isolated from solid-state anaerobic digestate. Bioresour. Technol. 201, 50–57. https://doi.org/10.1016/j.biortech.2015.11.035

26. Strong, P.J., Kalyuzhnaya, M., Silverman, J., Clarke, W.P., 2016. A methanotroph-based biorefinery: Potential scenarios for generating multiple products from a single fermentation. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2016.04.099

27. Sun, M.T., Yang, Z.M., Fu, S.F., Fan, X.L., Guo, R.B., 2018. Improved methane removal in exhaust gas from biogas upgrading process using immobilized methane-oxidizing bacteria. Bioresour. Technol. 256, 201–207. https://doi.org/10.1016/j.biortech.2018.02.020

28. Vecherskaya, M., Dijkema, C., Saad, H.R., Stams, A.J.M., 2009. Microaerobic and anaerobic metabolism of a *Methylocystis parvus* strain isolated from a denitrifying bioreactor. Environ. Microbiol. Rep. 1, 442–449. https://doi.org/10.1111/j.1758-2229.2009.00069.x

29. Voigt, C., Lamprecht, R.E., Marushchak, M.E., Lind, S.E., Novakovskiy, A., Aurela, M., Martikainen, P.J., Biasi, C., 2017. Warming of subarctic tundra increases emissions of all three important greenhouse gases – carbon dioxide, methane, and nitrous oxide. Glob. Chang. Biol. 23, 3121–3138. https://doi.org/10.1111/gcb.13563

30. Wartiainen, I., Hestnes, A.G., McDonald, I.R., Svenning, M.M., 2006a. *Methylobacter tundripaludum* sp. nov., a methane-oxidizing bacterium from Arctic wetland soil on the Svalbard islands, Norway (78° N). Int. J. Syst. Evol. Microbiol. 56, 109–113. https://doi.org/10.1099/ijs.0.63728-0

31. Wartiainen, I., Hestnes, A.G., McDonald, I.R., Svenning, M.M., 2006b. *Methylocystis rosea* sp. nov., a novel methanotrophic bacterium from Arctic wetland soil, Svalbard, Norway (78° N). Int. J. Syst. Evol. Microbiol. 56, 541–547. https://doi.org/10.1099/ijs.0.63912-0

Figure Captions

Fig. 1. Aerobic CH_4 oxidation pathway in gamma- and alphaproteobacterial methanotrophs (black arrows) and the possible pathway of CH_4 oxidation under O_2-limiting conditions via glycolysis-based CH_4 fermentation mode (red; dash line).

Fig. 2. Experimental set up for methanotrophs under three different gas supplementation schemes applied on day 7 including (i) both CH_4 and air added, (ii) only air added and (iii) only CH_4 added.

Fig. 3. Gas compositions (CH_4, O_2 and CO_2) in headspace during the 14-day incubation of *M. tundripaludum* SV96 (left row) and *M. rosea* SV97 (right row) under three different gas supplementation schemes applied on day 7: (i) both CH_4 and air added (a,d), (ii) only air added (b,e), and (iii) only CH_4 added (c,f).

Fig. 4. The molar ratio of O_2/CH_4 present in the system and the accumulation of organic acids production during the 14-day incubation of *M. tundripaludum* SV96 (a-c) and *M. rosea* SV97 (d-f) under three different gas supplementation schemes applied on day 7: (i) both CH_4 and air added (a and d), (ii) only air added (b and e) (iii) only CH_4 added (c and f).

Fig. 5. Fig. 5. Growth of *A. baylyi* ADP1 after 4-h cultivation in the methanotroph spent media (a) and concentrations of organic acids contained in the spent media of *M. tundripaludum* SV96 (MT) (b) and *M. rosea* SV97 (MR) (c). Error bars indicate the standard deviation of duplicate samples. The incubations of spent media of MT and MR without *A. baylyi* ADP1 (MT- and MR-controls) and *A. baylyi* ADP1 with NMS fresh medium (ADP1 + NMS) were used as controls.

Fig. 6. Organic acids utilization by *A. baylyi* ADP1 *tesA-undA* in spent media obtained from the cultivation of *M. tundripaludum* SV96 (MT) (a) and *M. rosea* SV97 (MR) (b) under O_2 limiting conditions. Growth and 1-undecene production from *A. baylyi* ADP1 *tesA-undA* after 24 h incubation (c). ΔOD indicates the difference in OD_{600nm} of *A. baylyi* ADP1 *tesA-undA*

between 0 and 24 h. Error bars indicates the standard deviation of four replicate samples. The spent media from methanotrophs fermentation without *A. baylyi* ADP1 was used as control (Control-24 h).

Table 1. The carbon mass balance per 1 mol of CH_4 consumed after 14-day incubation of the experiments of *M. tundripaludum* SV96 (MT) and *M. rosea* SV97 (MR). All tests were incubated with both CH_4 and air (20% CH_4 and 80% air) in headspace on day 0 and three different gas supplementation schemes applied on day 7.

Headspace supplement schemes	**Consumed CH_4 (mmol)**	**Carbon mass (mmol per mol CH_4 consumed)**				**% Excreted carbon mass as metabolites per mol CH_4**	**% CO_2 production per mol CH_4**	**Biomass yield (g CDW g CH_4^{-1})**
		Formate (CH_2O)	**Acetate ($C_2H_3O_2$)**	**Succinate ($C_4H_6O_4$)**	**Malate ($C_4H_6O_5$)**			
(i) both CH_4 and air added (20% CH_4 and 80% air)								
MT	0.70 ± 0.01	27.4 ± 7.4	20.0 ± 3.6	21.5 ± 2.0	4.8 ± 0.8	7.4 ± 1.3	39.1 ± 1.4	0.54 ± 0.04
MR	0.72 ± 0.20	1.2 ± 0.3	10.6 ± 3.0	1.2 ± 0.3	nd.	1.3 ± 0.4	40.6 ± 8.8	0.43 ± 0.07
(ii) only air added (20% N_2 and 80% air)								
MT	0.56 ± 0.02	1.2 ± 0.4	39.5 ± 8.7	5.8 ± 1.3	3.6 ± 1.2	5.0 ± 1.0	40.6 ± 8.8	0.52 ± 0.06
MR	0.44 ± 0.14	0.2 ± 0.3	2.7 ± 2.5	nd.	nd.	0.29 ± 0.27	45.3 ± 9.9	0.45 ± 0.1
(iii) only CH_4 added (20% CH_4 and 80% N_2)								
MT	0.46 ±0.02	2.2 ± 0.2	30.1 ± 2.4	4.7 ± 0.6	1.4 ± 0.2	3.8 ± 0.2	28.5 ± 2.5	0.57 ± 0.02
MR	0.34 ± 0.01	1.9 ± 0.2	14.5 ± 6.3	0.8 ± 0.1	nd.	1.7 ± 0.6	33.2 ± 4.2	0.42 ± 0.05

Table 2. The production of organic acids and other metabolites obtained from different gammaproteobacterial methanotrophs (type I) cultivated under O_2 limiting conditions.

Methanotrophs	**Test mode**	**Volume**	**OD (CDW, g L^{-1})**	**Initial CH_4:O_2 (%)**	**Yield (mmol g CDW^{-1})**				**Note**	**Reference**
					Formate	Acetate	Succinate	Malate		
M. tundripaludum SV96	batch	15 mL in 120 mL vial	~2 (0.6)	20:15 (10:3)*	3.20 ± 0.9	1.15 ± 0.17	0.16 ± 0.01	0.07 ± 0.01		Our study
Methylomicrobium alcaliphilum 20Z	fed-batch/chemostat	1 L in 2 L chemostat	~2 ± 0.2	20:5	1.53 ± 0.27	0.020	0.0025	ND	H_2, lactate detected	Kalyuzhnaya et al. (2013)
Methylomicrobium buryatense 5GB1	continuous	1 L in 2.5 L vessel	(0.45-0.46)	20:5	0.29-0.30	0.036-0.085	ND	ND	lactate detected	Gilman et al. (2015)
	fed-batch	1 L in 2.5 L vessel	(0.31-0.34)	10:5	0.40-0.67	0.096-0.123	ND	ND	lactate detected	
Methylomicrobium buryatense 5GB1C (the aa3 cytochrome oxidase mutant strain)	batch	1 L in 2.5 L vessel	(0.21)	20:5	1.22-3.00	0.081-0.086	ND	ND		Gilman et al. (2017)

Note: CDW, cell dry weight; ND, no data; *The CH_4:O_2 in headspace when O_2 limiting conditions occurring in the system

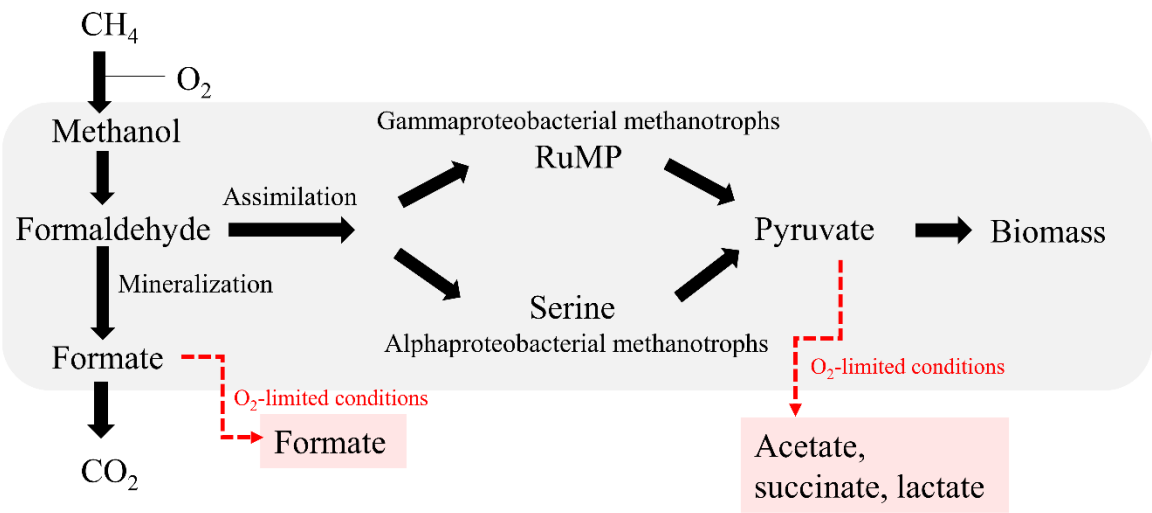

Fig. 1. Aerobic CH_4 oxidation pathway in gamma- and alphaproteobacterial methanotrophs (black arrows) and the possible pathway of CH_4 oxidation under O_2-limiting conditions via glycolysis-based CH_4 fermentation mode (red; dash line).

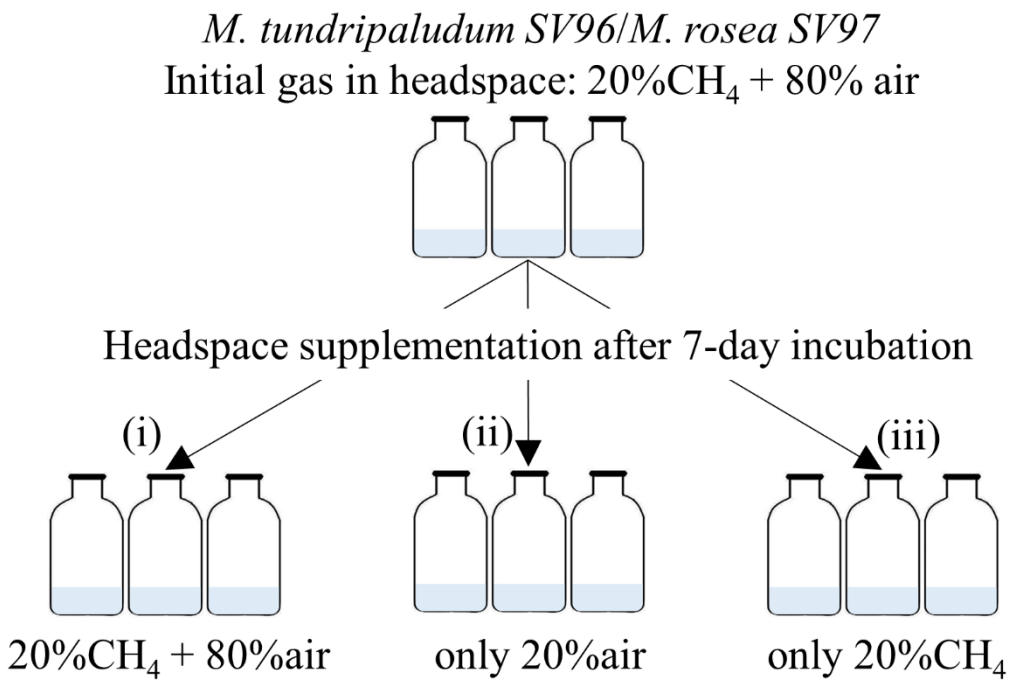

Fig. 2. Experimental set up for methanotrophs under three different gas supplementation schemes applied on day 7 including (i) both CH_4 and air added, (ii) only air added and (iii) only CH_4 added.

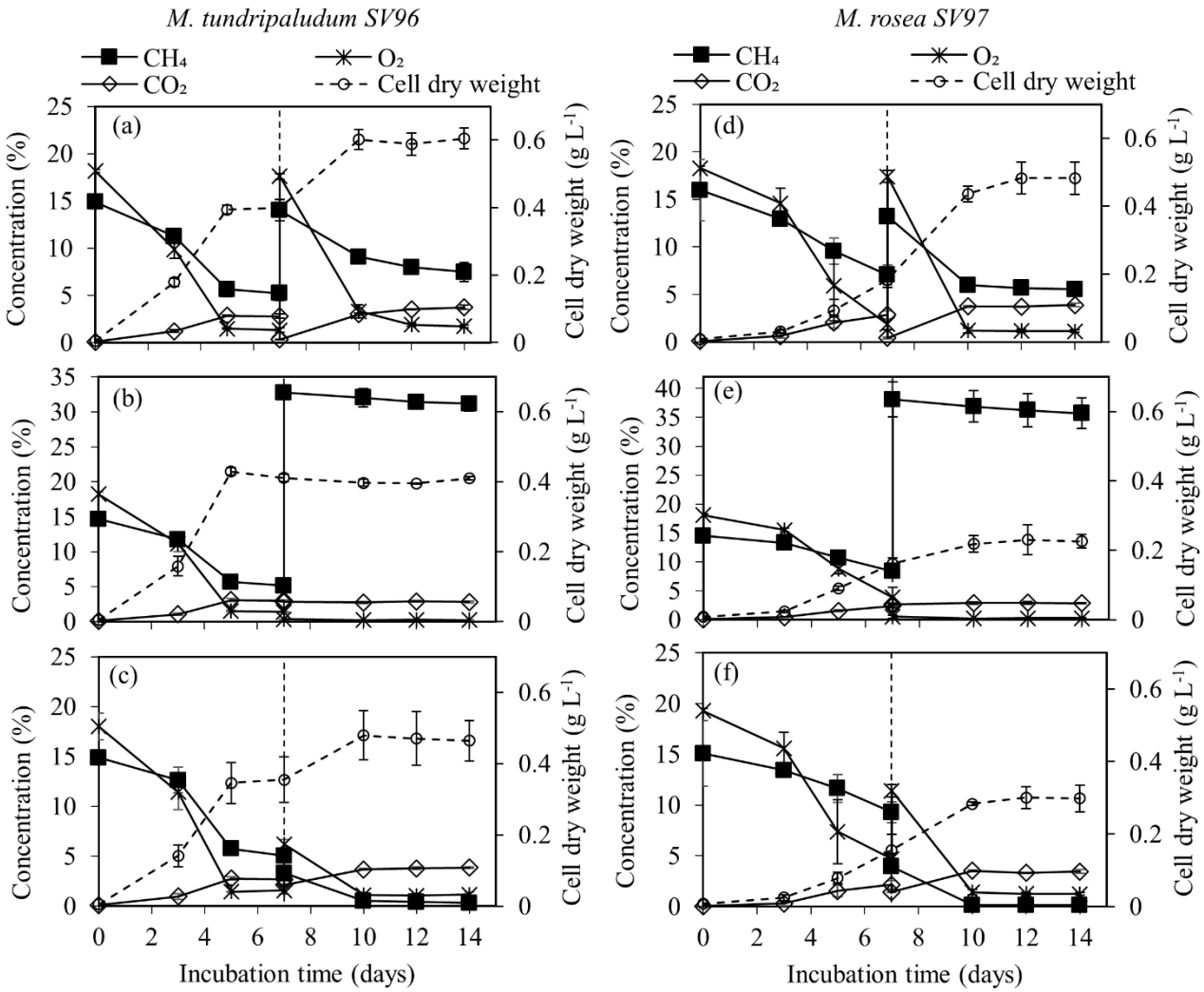

Fig. 3. Gas compositions (CH_4, O_2 and CO_2) in headspace during the 14-day incubation of *M. tundripaludum* SV96 (left column) and *M. rosea* SV97 (right column) under three different gas supplementation schemes applied on day 7: (i) both CH_4 and air added (a,d), (ii) only CH_4 added (b,e), and (iii) only air added (c,f).

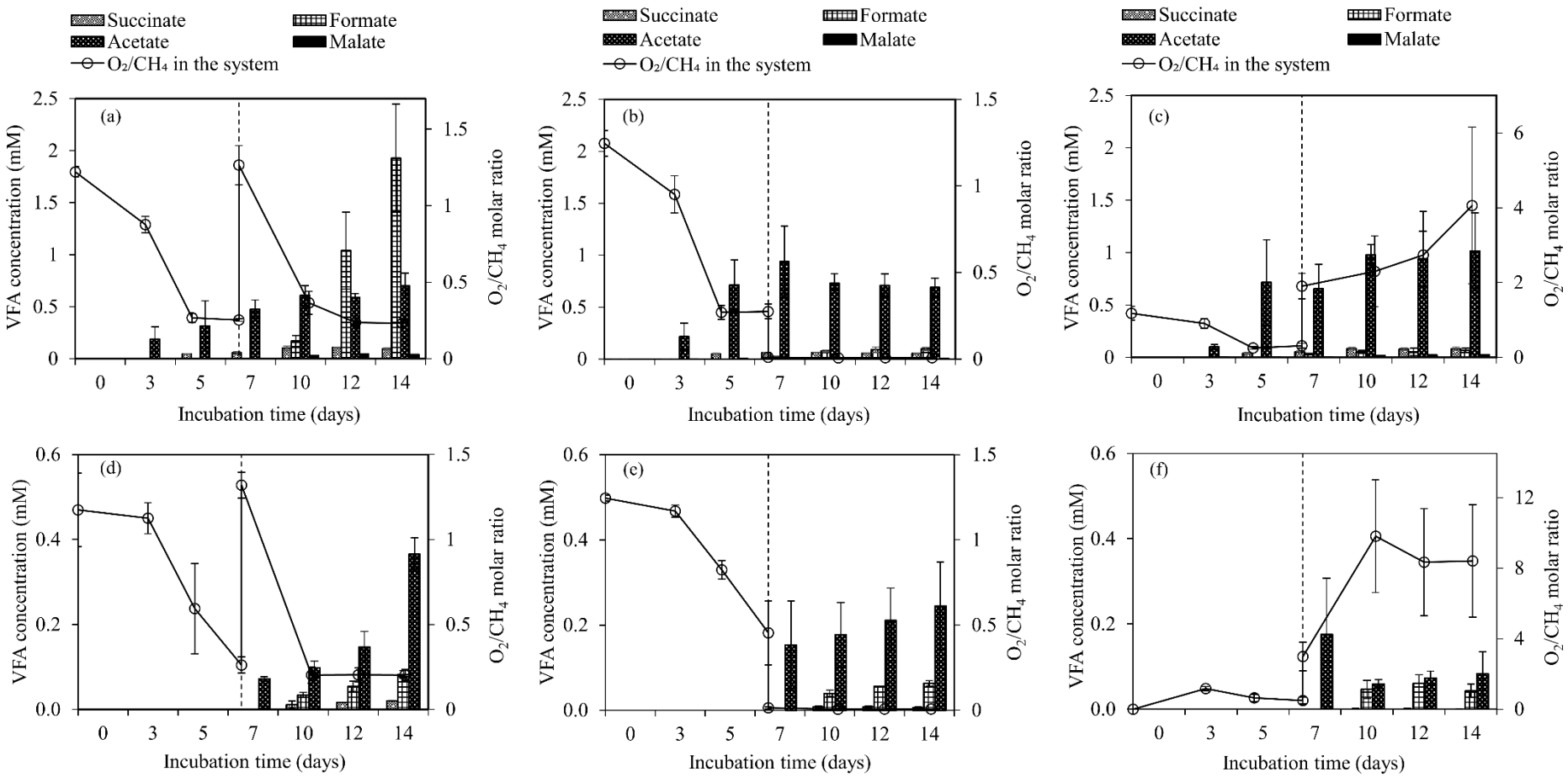

Fig. 4. The molar ratio of O_2/CH_4 present in the system and the accumulation of organic acids production during the 14-day incubation of *M. tundripaludum* SV96 (a-c) and *M. rosea* SV97 (d-f) under three different gas supplementation schemes applied on day 7: (i) both CH_4 and air added (a and d), (ii) only CH_4 added (b and e), and (iii) only air added (c and f).

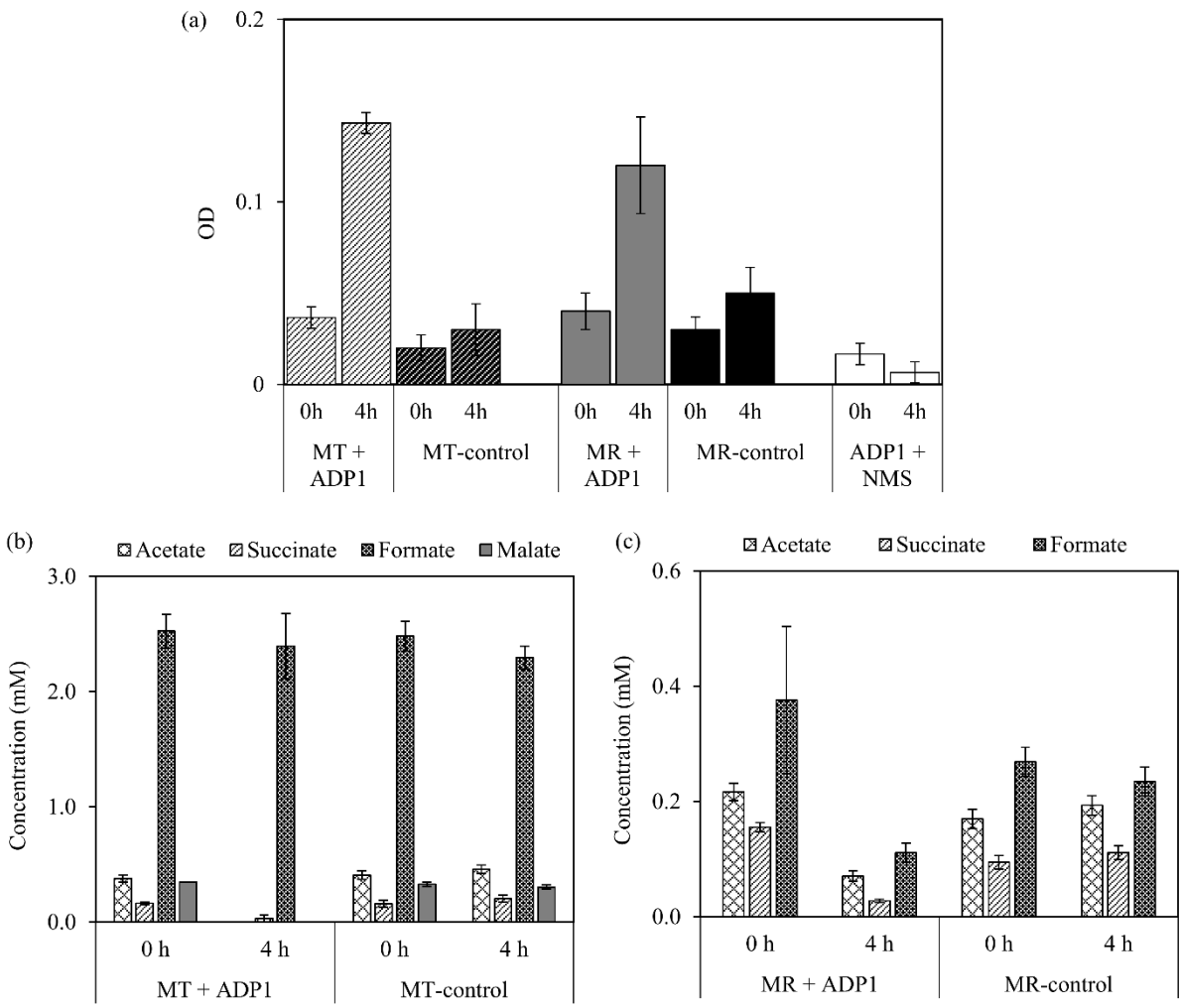

Fig. 5. Growth of *A. baylyi* ADP1 after 4-h cultivation in the methanotroph spent media (a) and concentrations of organic acids contained in the spent media of *M. tundripaludum* SV96 (MT) (b) and *M. rosea* SV97 (MR) (c). Error bars indicate the standard deviation of duplicate samples. The incubations of spent media of MT and MR without *A. baylyi* ADP1 (MT- and MR-controls) and *A. baylyi* ADP1 with NMS fresh medium (ADP1 + NMS) were used as controls.

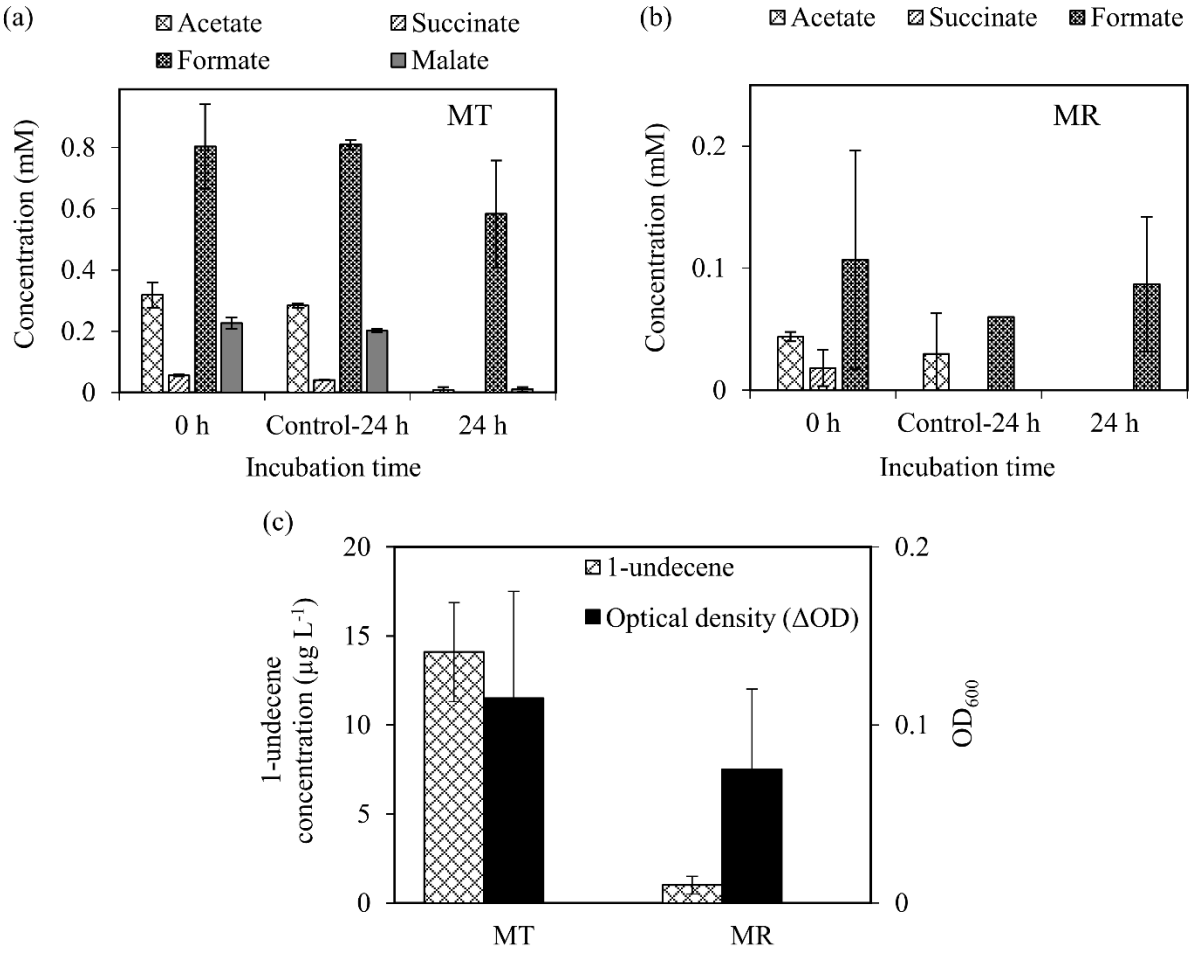

Fig. 6. Organic acids utilization by *A. baylyi* ADP1 *tesA-undA* in spent media obtained from the cultivation of *M. tundripaludum* SV96 (MT) (a) and *M. rosea* SV97 (MR) (b) under O_2 limiting conditions. Growth and 1-undecene production from *A. baylyi* ADP1 *tesA-undA* after 24 h incubation (c). ΔOD indicates the difference in OD_{600nm} of *A. baylyi* ADP1 *tesA-undA* between 0 and 24 h. Error bars indicates the standard deviation of four replicate samples. The spent media from methanotrophs fermentation without *A. baylyi* ADP1 was used as control (Control-24 h).