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Production of Long Chain Alkyl Esters from Carbon Dioxide and Electricity by a Two-Stage Bacterial Process

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Abstract

Microbial electrosynthesis (MES) is a promising technology for the reduction of carbon dioxide into value-added multicarbon molecules. In order to broaden the product profile of MES processes, we developed a two-stage process for microbial conversion of carbon dioxide and electricity into long chain alkyl esters. In the first stage, the carbon dioxide is reduced to organic compounds, mainly acetate, in a MES process by Sporomusa ovata. In the second stage, the liquid end-products of the MES process are converted to the final product by a second microorganism, Acinetobacter baylyi in an aerobic bioprocess. In this proof-of-principle study, we demonstrate for the first time the bacterial production of long alkyl esters (wax esters) from carbon dioxide and electricity as the sole sources of

carbon and energy. The process holds potential for the efficient production of carbon-neutral chemicals or biofuels.

Keywords: Microbial electrosynthesis, Acinetobacter baylyi ADP1, Carbon neutral, Oleochemicals, Carbon dioxide fixation

1. Introduction

In a carbon neutral future, the energy and carbon used in the fuels and chemicals must come from the sun and CO_2, respectively. Advances in photovoltaic technologies enable efficient harvesting of solar energy (Polman et al., 2016). However, the usability of electricity as an energy source is limited in many applications, including transportation. Production of renewable electricity (wind or solar) is also intermittent by nature, which leads to large fluctuations in production. Consequently, an efficient storage method for the electrical energy is required in order to utilize the full potential of these green technologies.

Acetogenic bacteria (acetogens) are capable of reducing CO_2 into multicarbon molecules, such as acetate and ethanol, using hydrogen as the electron donor (Drake et al., 2008). Besides the hydrogen gas, some acetogens are able to accept electrons from a cathode of a bioelectrochemical system in a process called microbial electrosynthesis (MES) (Lovley & Nevin, 2013; May et al., 2016; Nevin et al., 2011; Tremblay & Zhang, 2015). Thus, the reduction of CO_2 to value-added multicarbon molecules by microbial catalysts is seen as an attractive possibility. Compared with photosynthesis-derived biomass production strategies, the advantages of MES are high energy efficiency and

versatility (Tremblay & Zhang, 2015). The reducing power (electrons) for MES can be obtained from various renewable and sustainable sources, such as solar or wind electricity, sulfide oxidation, or wastewater treatment (Tremblay & Zhang, 2015; Tremblay et al., 2017).

In MES systems, ca. 80-95% of consumed electrons are diverted to the end products (Nevin et al., 2010; Tremblay & Zhang, 2015). Thus, MES coupled with electricity from solar cells could be an efficient way of storing the solar energy in chemical bonds. The process can be considered as a kind of artificial photosynthesis, involving the conversion of carbon dioxide, water and sunlight into oxygen and multicarbon molecules, but with significantly greater efficiency compared with natural photosynthesis (Lovley & Nevin, 2011).

The main product of MES is typically acetate (Aryal et al., 2017; Aryal et al., 2016; Bajracharya et al., 2017; Chen et al., 2016; Jourdin et al., 2015; LaBelle et al., 2014), which itself has value as an industrial chemical. However, in order to exploit the MES system to produce a greater variety of chemicals and biofuels, and especially products that are formed in pathways thermodynamically 'uphill', a more biochemically versatile production platform needs to be developed. Efforts have been made to alter the product profile of the acetogens by metabolic engineering or process design (Phillips et al., 2015; Ueki et al., 2014). However, introducing complex non-native pathways to acetogens may be difficult because of limited knowledge about their genetics and lack of established gene editing tools.

One solution is to employ a second microorganism to convert the end products of MES into more complex molecules. The advantage of this approach is the possibility to use a well-known organism with suitable metabolism and existing tools and know-how for sophisticated genetic engineering. This kind of two-stage approach has been just recently demonstrated by (Liu et al., 2015) and (Hu et al., 2016). Liu et al (Liu et al., 2015) used a hybrid semiconductor nanowire-bacteria system with an acetogen Sporomusa ovata to convert the solar energy and carbon dioxide to acetate and further produced several commodity chemicals from the acetate with genetically engineered Escherichia coli cells. Hu et al. (Hu et al., 2016) produced acetate from CO_2 and H_2 with Moorella thermoaceticum and used a yeast Yarrowia lipolytica to convert the acetate to lipids. However, products with long hydrocarbon chains have not been previously produced with microbial electrosynthesis from CO_2 and electricity.

Wax esters (WEs) are high-value oleochemicals with a chemical structure of a fatty acid esterified with a long chain 1-alcohol. They have various industrial uses, including cosmetics and the production of lubricants or surfactants (Carlsson et al., 2011; Miwa, 1984). In addition, WEs can be hydrolyzed to yield fatty acid and fatty alcohol, both of which have industrial value (Carlsson et al., 2011). Because of the long hydrocarbon tails, WEs have high energy content and can be used for biofuel production (Canoira et al., 2006).

Long carbon chains are energetically expensive to produce, making their overproduction using acetogens challenging (Rabaey et al., 2011). To highlight the power of the two-stage approach, we built a microbial system which produces long alkyl chains (WEs with an average of 32 carbons/molecule) from carbon dioxide and electricity (Figure

1). For the conversion of acetate to WEs, we utilize Acinetobacter baylyi ADP1, which has proven to be a suitable chassis for synthetic biology and especially lipid production (de Berardinis et al., 2009; Elliott & Neidle, 2011; Kannisto et al., 2015; Santala et al., 2011b; Santala et al., 2014). A. baylyi has high specific growth rate on acetate (1.22 h^{-1} (Du Preez et al., 1981), compared with for example that of E. coli (0.3 h^{-1} (Andersen & von Meyenburg, 1980)). Furthermore, its natural ability to produce storage lipids, namely triacylglycerols and WEs, makes it an appealing organism for the upgrading of the end-products of MES.

Towards the two-stage process, we first characterized the wax ester production of A. baylyi ADP1 from acetate. Second, we screened four different acetogenic bacteria grown on CO_2/H_2 for their acetate production and compatibility with A. baylyi ADP1. Finally, we demonstrate WE production from carbon dioxide and electricity by a two-stage process with S. ovata and A. baylyi ADP1.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. baylyi ADP1 (DSM 24193) was used in the study. The acetogenic bacteria (S. ovata, DSM 2662; Clostridium ljungdahlii, DSM 13528; Clostridium aceticum, DSM 1496; Acetobacterium woodii, DSM 1030) were ordered from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and revived in their DSMZ recommended media. For routine maintenance, the acetogens were cultivated in DSM 311 medium without organic carbon substrate and with a headspace of 80% H_2 and 20% CO_2. All

cultivations were performed at 30°C, except the microbial electrosynthesis, which was performed at ambient temperature (25°C).

2.2. Bacterial cultivations

2.2.1. Wax ester production from acetate by A. baylyi

For the initial acetate utilization experiment, A. baylyi was cultivated in 50 ml of MA/9 minimal salts medium (Kannisto et al., 2014) supplemented with 0.2% casein amino acids and 0-200 mM acetate as carbon and energy sources. The cells were grown for 24 or 48 hours in 250 ml flasks and the samples were collected for HPLC and NMR analyses.

2.2.2. Comparison of the different acetogens

For the comparison of the acetogens, the four different acetogenic strains were cultivated in DSM 311 medium without organic carbon substrate. Three replicates of 50 ml cultures were initiated with a 5 ml inoculum, the headspace pressurized with H_2/CO_2 at 1.5 bar and incubated at 30°C and 150 rpm shaking. The bottles were repressurized daily and acetate formation followed by HPLC. After 8 days, the cells were centrifuged (7000 x g, 15 min) and the supernatants (50 ml) transferred to 250 ml flasks and used for A. baylyi cultivation. The pH of the medium was adjusted by adding 5 ml of 400 mM MOPS (3-(N-morpholino)propanesulfonic acid) buffer (pH 7.2). Casein amino acids (0.2%) were also added to counter the growth inhibition caused by the cysteine present in the anaerobic medium. A. baylyi was grown in the flasks and acetate consumption was followed with HPLC. At the end of the cultivation, samples were collected for the NMR analysis. For control, A. baylyi was grown in unused DSM 311 medium (i.e. the same medium where

the acetogens were grown in the first phase). A. baylyi was pregrown overnight at 30°C in MA/9 supplemented with 20 mM acetate and 5 ml was used for inoculation.

2.2.3. Acetate production by microbial electrosynthesis

Microbial electrosynthesis was performed as described earlier (Nevin et al., 2010; Tremblay et al., 2015). A graphite stick cathode (36 cm^2) and anode (36 cm^2) were suspended in 250 ml of DSM 311 medium in two chambers separated by a Nafion 115 ion-exchange membrane (Ion Power, Inc., New Castle, DE, USA). A S. ovata strain (met-T18-2) previously developed by adaptive laboratory evolution (Tremblay et al., 2015) was used in this experiment. The cells were pregrown with H_2/CO_2 and transferred to the cathode chamber of the reactor. The cathode was poised at -690 mV (versus standard hydrogen electrode) with a potentiostat (ECM8, Gamry Instruments, USA). At this cathode potential, it is likely that a large part of the electrons transferred from the cathode to S. ovata are shuttled via H_2 (May et al., 2016). During the experiment both cathode and anode chambers were continuously flushed with N_2/CO_2 (80:20). The potential, acetate formation, and optical density of the reactor were monitored daily.

2.2.4. Conversion of acetate from MES to wax esters

After 10 days, the MES reactor was stopped and the medium from the cathode chamber was used for A. baylyi cultivation. The medium was centrifuged (7000 x g, 10 min) and sterile filtered, and the pH adjusted by adding 10% (vol/vol) 400 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 7.2). Three 50 ml aliquots of the medium were transferred to flasks and used for A. baylyi cultivation at 30°C and 300 rpm shaking. A. baylyi was grown in the flasks and acetate consumption was followed with HPLC. At the

end of the cultivation, samples were collected for the NMR analysis. For control, A. baylyi was grown in unused DSM 311 medium (the same as used in the MES reactor). A. baylyi was pregrown overnight at 30°C in MA/9 supplemented with 20 mM acetate and 5 ml was used for inoculation.

2.3. Metabolite analysis

Acetate formation and consumption were monitored with high performance liquid chromatography (HPLC) using a LC-20AC prominence liquid chromatograph (Shimadzu, Japan) and Rezex RHM-Monosaccharide H + (8%) column (Phenomenex, USA) with 5 mM H_2SO_4 as the mobile phase.

2.4. Wax ester analysis with NMR spectroscopy

Wax esters were quantitatively analyzed by nuclear magnetic resonance (NMR) spectroscopy as described previously (Santala et al., 2011a). Briefly, the cells from 40 ml of the culture were collected by centrifugation, freeze-dried and lipids extracted with methanol-chloroform extraction as described previously (Santala et al., 2011b). The lipid fraction was analyzed with ^{1}H NMR, which allows the quantitative detection of the protons of α-alkoxy-methylene groups specific for WEs.

2.5. Calculations

Palmityl palmitate $C_{32}H_{64}O_2$ (a typical size of WEs in A. baylyi (Fixter et al., 1986 and our unpublished data)) was used as an average WE molecule in the calculations. The production of acetate from two CO_2 molecules requires 8 electrons, and the conversion of 16 acetate molecules to palmityl palmitate requires 60 electrons. Thus, in total the production of a WE molecule from CO_2 requires 188 electrons. The coulombic efficiency of

MES was calculated by dividing the electrons required for acetate (8 per acetate molecule) with the electrons consumed in the cathode. In the case of WE production from acetate, the efficiency was calculated by dividing the electrons required for the production of WE (188 per WE molecule) with the electrons in the consumed acetate (8 per acetate molecule).Yields (carbon/carbon) were calculated as the ratio of carbon atoms in the consumed acetate and in the produced WEs. For calculations of WE content in milligrams, the molar mass of palmityl palmitate (480 g/mol) was used.

3. Results and discussion

3.1. Wax ester production from acetate

Several Acinetobacter species have been reported to efficiently utilize acetate as carbon source. Additionally, WE production from acetate has been reported for some strains (Fixter et al., 1986). As the first step towards the two-phase production system, we characterized the growth and WE production of A. baylyi ADP1 in different acetate concentrations. The cells were cultivated in 100 or 200 mM acetate for 48 hours, and growth, acetate consumption, and WE production were analyzed. The results are presented in Table 1. A baylyi tolerated both acetate concentrations without any adaptation phase, albeit less biomass was obtained with 200 mM acetate. The introduced acetate concentrations are in the same range as the amounts of the acetate produced with various MES approaches (May et al., 2016), underlining the suitability of A. baylyi ADP1 for the acetate upgrading. The WE production from acetate was analyzed after 24 and 48 hours. The highest WE titer, 190 µmol/L (corresponding to approximately 91 mg/L) was observed with 100 mM initial acetate concentration after 24 hours. At that point, 69

mM of acetate had been consumed, resulting in a 4.4% yield (carbon/carbon). After the acetate had been completely utilized, the WEs were degraded, as has been observed previously with other carbon sources (Fixter et al., 1986; Santala et al., 2011a).

3.2. Wax ester production from carbon dioxide and hydrogen

Next, we studied the WE production from carbon dioxide and hydrogen in a two-stage process, where acetate is produced by the acetogens in the first phase and consumed by A. baylyi in the second phase. In order to demonstrate the simplicity and applicability of the set-up, we studied the possibility to inoculate A. baylyi directly to the same medium that has been used for the acetogenic cultivation. Specifically, we wanted to find an acetogenic strain that is compatible with A. baylyi, in terms of culture medium and possible side products potentially produced by the acetogens. We evaluated the applicability and performance of four different acetogenic species for the two-stage process. The acetogens were cultivated on H_2/CO_2, and the medium and growth temperature were chosen to be close to the optimum for A. baylyi. The acetate production was measured (Figure 2 A) and the suitability of the supernatant for A. baylyi cultivation and WE production was tested (Figure 2 B-D). Of the tested strains, S. ovata and A. woodii performed better in terms of acetate production and A. baylyi growth and WE production than C. aceticum. C. ljungdahlii exhibited poor growth and negligible acetate production in the studied conditions (data not shown).

Interestingly, we found that the cysteine used in the anaerobic medium induces a long lag-phase in the A. baylyi cultivation, even if the medium was aerated before the aerobic phase. The effect could be reduced with the addition of 0.2% casein amino acids

to the A. baylyi cultivation (data not shown). A similar effect has been reported with E. coli (Kari et al., 1971).

When provided with the casein amino acids, A. baylyi grew well and produced WEs from the acetate-containing supernatant of S. ovata or A. woodii (Fig. 2 B-D). Because of the tendency of A. baylyi to degrade the WEs in carbon-limited conditions, the consumption of acetate was monitored during the cultivation and the experiment stopped before the acetate was completely utilized from the medium. The highest WE production, 38 µmol/L (corresponding to approximately 18 mg/L) was observed with the supernatant of A. woodii cultivation. At that point, 17 mM of acetate had been consumed, resulting in a 3.6% yield (carbon/carbon). A small amount of WEs (12 µmol/L) was also detected from the control samples (A. baylyi cultivation in unused medium) due to the use of the casein amino acids in the medium. The supernatant of C. aceticum did not support A. baylyi growth or WE production efficiently, potentially because C. aceticum produced little acetate, but likely consumed the casein amino acids from the medium.

3.3. Wax ester production from carbon dioxide and electricity

To demonstrate the production of long chain alkyl esters from electricity and CO_2, we set up a two-stage process in which A. baylyi ADP1 is used to convert the end products of MES into long chain WE molecules. S. ovata was chosen for the electrosynthesis experiment based on its previous high performance in MES (Chen et al., 2016; Nevin et al., 2010; Nevin et al., 2011) and promising results in our experiments with H_2/CO_2. A S. ovata strain (met-T18-2) previously developed by adaptive laboratory evolution (Tremblay et al., 2015) was grown in the MES bioreactor with carbon dioxide and electricity as sources of

carbon and energy, respectively, and the current and acetate formation were monitored (Figure 3A). After the electrosynthesis phase, the medium was collected and used for A. baylyi cultivation as such. As expected, the acetate produced by S. ovata was used by A. baylyi for growth and WE production (Figure 3B-C). A. baylyi utilized approximately 13 mM of acetate and produced 38 µmol/L WEs (carbon/carbon yield 4.6%). Again, the cells were harvested before complete consumption of the acetate. No WEs were detected from A. baylyi cells grown in the control medium (unused DSM 311 medium).

In this study, the production of WEs was studied with three different settings; directly from acetate, from acetate produced from H_2 and CO_2 and from acetate produced with MES. The molar yields (carbon/carbon) for WE production from acetate were similar (from 3.6% to 4.6%) in each of the three approaches, suggesting that the supernatant of acetogenic cultivations is an equally good a substrate for A. baylyi as acetate provided in the defined media. The variation in the WE titers is potentially caused by the different acetate concentration at the end of the cultivation, as A. baylyi tends to degrade the WEs in low-carbon conditions.

The MES approach also enabled the calculation of the coulombic efficiencies (the fraction of the supplied electrons that end up in the end product). The average coulombic efficiency of the acetate production (the MES phase) was approximately 70%. The coulombic efficiency of the WE production from acetate was 6.6%, resulting in the total coulombic efficiency of 4.6% from CO_2 and electricity to WE. These values serve as a benchmark for future development of the system.

The critical aspects to be improved include the acetate productivity and titer of the MES system, and the efficiency of WE production from the acetate by A. baylyi. In particular, it would be beneficial to improve the WE production from relatively low acetate concentrations. The wild type A. baylyi ADP1 was used in this study, but potentially higher productivities could be achieved with genetically engineered strains (Kannisto et al., 2017; Santala et al., 2011b). Furthermore, A. baylyi has been recently engineered to produce long chain n-alkanes, which could serve as a drop-in replacement for current traffic fuels (Lehtinen et al., 2017). Thus, this system holds potential for the production of carbon neutral traffic fuel directly from carbon dioxide and electricity. Additionally, the productivity of the system could potentially be improved by optimizing the medium and culture parameters, such as the carbon/nitrogen ratio and temperature (Fixter et al., 1986). The two parts of the system work in a modular fashion, which enables them to be further developed independently of each other. Microbial electrosynthesis is a novel, rapidly developing field and advances made in the efficiency would likely benefit the overall process.

This study demonstrates for the first time the bacterial production of long chain neutral lipids from electricity and carbon dioxide as the sole sources of carbon and energy. The dual-culture approach enables the production of versatile end products, depending on the characteristics of the production strain. Production of commodity chemicals such as n-butanol and polyhydroxybutyrate has been demonstrated with a similar system with E. coli (Liu et al., 2015). In this study, the production of lipid compounds with >30 carbon

chains was demonstrated, further emphasizing the system potential and the possibility to produce a broader range of products.

4. Conclusions

In this study, a proof of principle for long chain alkyl ester production from electricity and carbon dioxide was demonstrated. The process holds potential for producing carbon-neutral biofuels or chemicals. The two-stage cultivation process allows for the optimization of both parts individually and further development is required for both parts in order to enhance the overall productivity of the system.

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Table 1. Wax ester production from acetate. A. baylyi ADP1 was cultivated in minimal salts medium with two different acetate concentrations (initial concentrations approximately 100 mM (low) and 200 mM (high)) and without acetate. The cell dry weight (CDW), acetate concentration and wax ester (WE) production were measured. The average and standard deviation from two replicates are shown. n.a; not analyzed.

		0 hours	24 hours	48 hours
CDW (g/L)	No Acetate	n.a.	0.43 ± 0.01	0.46 ± 0.08
	Low Acetate	n.a.	1.48 ± 0.02	1.72 ± 0.08
	High Acetate	n.a.	0.37 ± 0.03	0.89 ± 0.17
Acetate (g/L)	No Acetate	0	0	0
	Low Acetate	6.50 ± 0.06	2.41 ± 0.32	0
	High Acetate	13.06 ± 0.20	13.64 ± 0.04	12.73 ± 1.3
WE (µmol/L)	No Acetate	n.a.	0	0
	Low Acetate	n.a.	185 ± 10	0
	High Acetate	n.a.	17.9 ± 1.2	136 ± 1.8

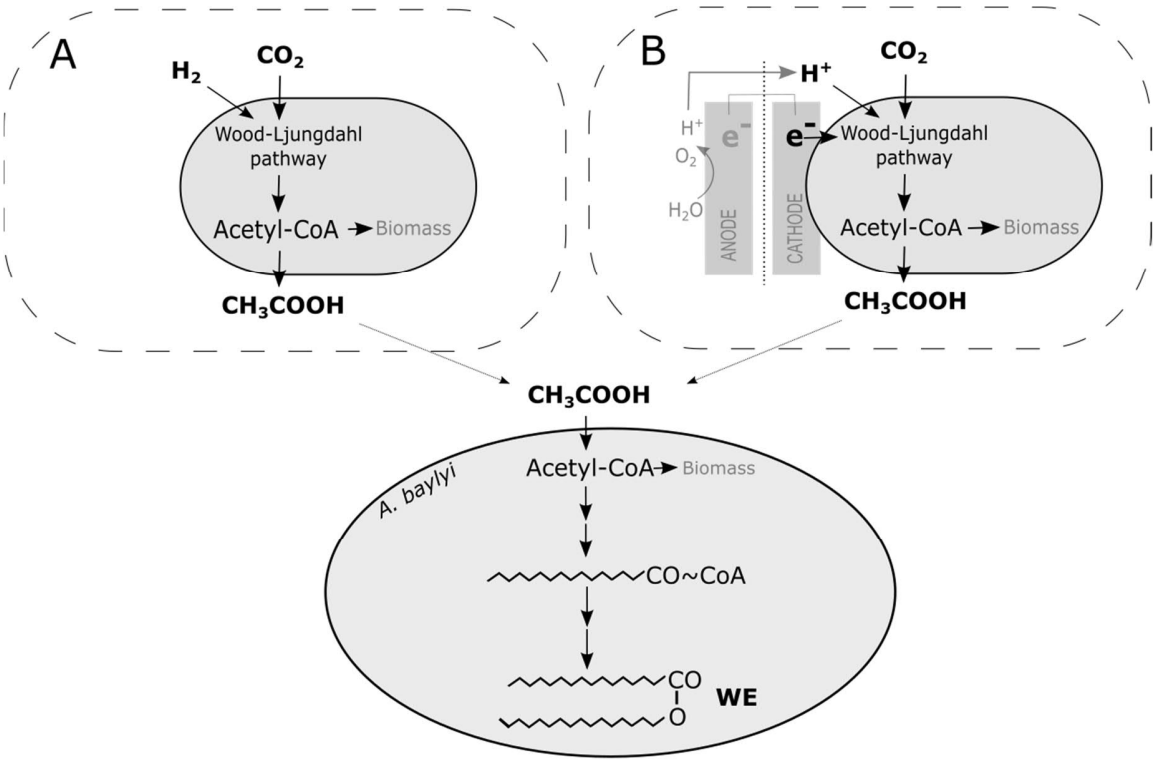

Figure 1. The principle of the production system. The conversion of acetate to value-added long chain oleochemicals (wax esters, WE) was established using Acinetobacter baylyi ADP1 (bottom). The WE production module was connected with an upstream acetate production module, where acetogenic bacteria were used to produce acetate form CO_2 and H_2 gas (A) or CO_2 and electricity (B). In the case (A), the substrates are provided in gaseous form. In the case (B), the acetogenic cells are grown in the cathode chamber of a bioelectrochemical system, and electrical energy is used to provide electrons through the cathode. When either A or B is connected to the WE production module, a net production of wax esters from CO_2 is established, the sole source of energy being either H_2 gas or electricity. CoA; Coenzyme A, WE; Wax ester.

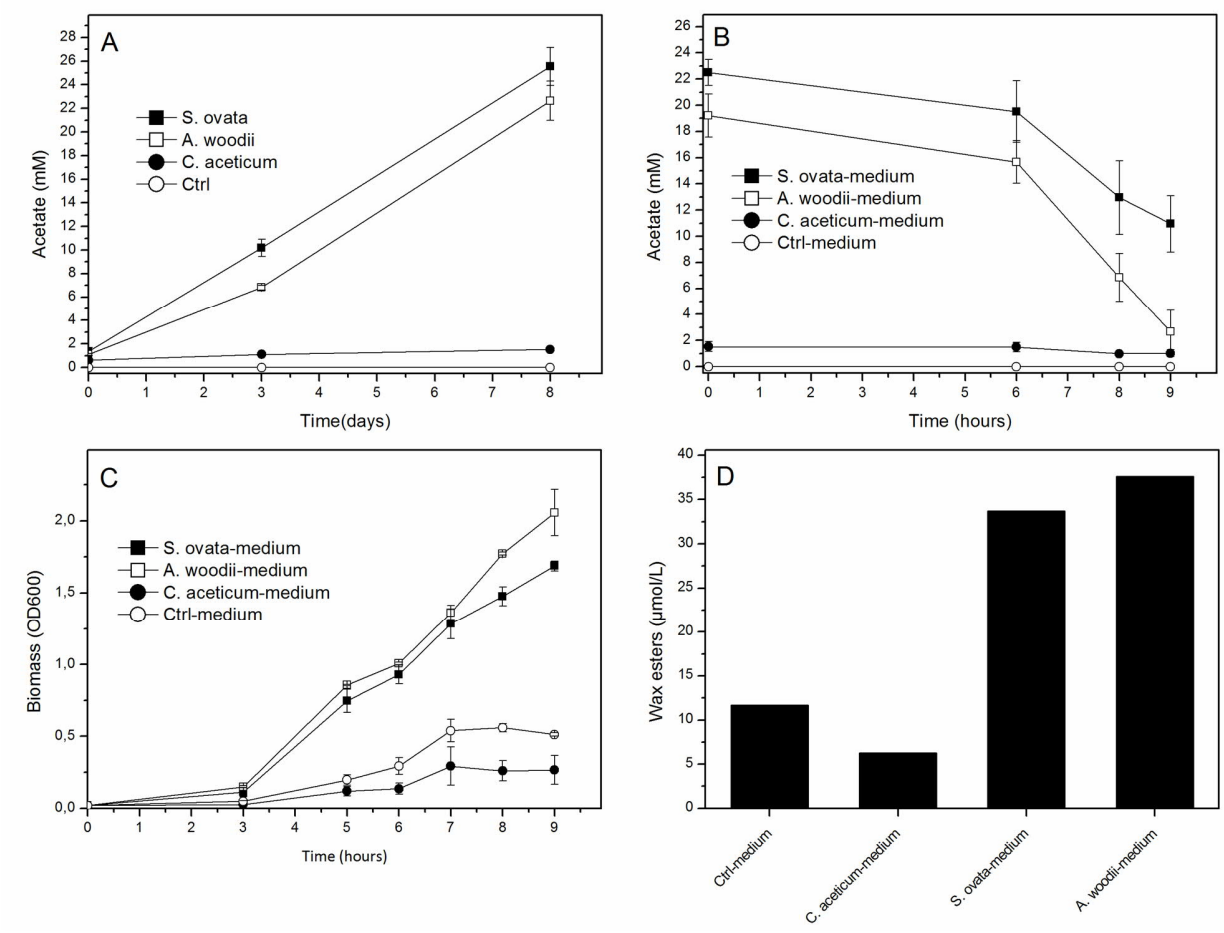

Figure 2. Production of wax esters from carbon dioxide and hydrogen. A two-phase system was established, where in the first phase, three acetogenic strains were used to produce acetate from carbon dioxide and hydrogen (A). In the second phase, A. baylyi was grown in the medium from each acetogenic cultivations. The acetate consumption (B) and optical density (C) were monitored during the A. baylyi cultivation. The cultivations were stopped after nine hours, lipids extracted and wax ester content analyzed by NMR (D). The experiment was run in triplicates, and the average and standard deviation are shown (A-C). For the wax ester analysis, the three samples were pooled together (D). The control in (A) is the unused medium. The control in (B-D) is the culture of A. baylyi grown in the control medium from (A).

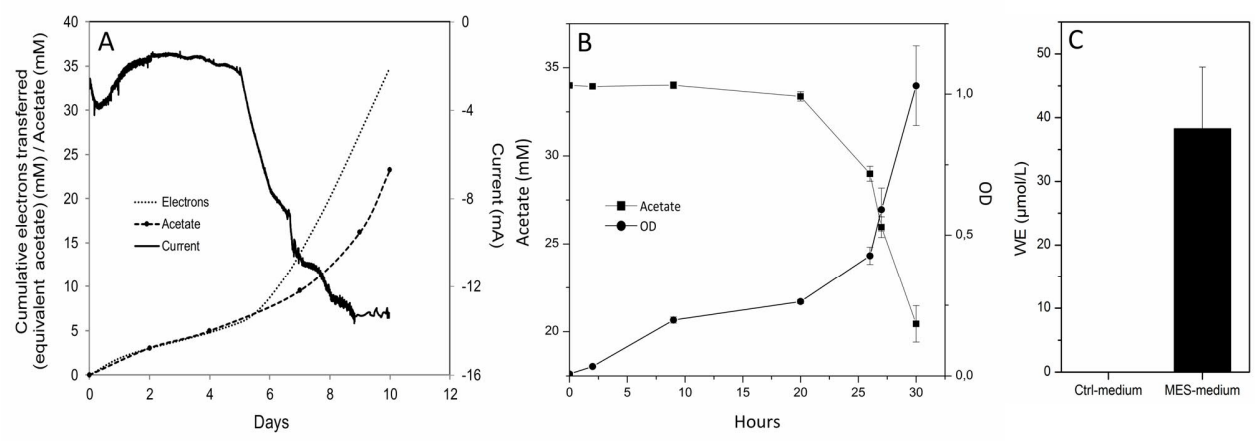

Figure 3. Production of wax esters from carbon dioxide and electricity. A two-phase cultivation was established for the production of wax esters from carbon dioxide and electricity. First, S. ovata was grown in a bioelectrochemical system with carbon dioxide as the sole carbon source and electrons provided by a potentiostat as the sole source of energy. The current consumption and acetate formation were monitored (A). The Electrons curve corresponds to acetate concentration if all the consumed electrons were transferred to acetate, whereas the Acetate curve represents the real acetate concentrations measured with HPLC. After ten days, the medium from the cathode chamber was divided for three A. baylyi cultivations. The optical densities and acetate consumption were monitored and averages and standard deviations are shown in (B). At the end of the cultivation, the cells were collected, lipids extracted and wax esters (WE) analyzed with NMR (C). The average and standard deviation of the WE production for the three replicates is shown. The control cultivation for A. baylyi was performed with unused medium (identical to the medium used in the MES reactor). The Acetate curve in (A) was set to start from zero for illustrative purposes, even though there was approximately 14 mM of residual acetate in the medium in the beginning of the electrosynthesis phase.