Journal of Biotechnology 178 (2014) 67–72

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Selective enhancement of autotrophic acetate production with genetically modified *Acetobacterium woodii*

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ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 21 February 2014
Accepted 1 March 2014
Available online 15 March 2014

Keywords:
Acetate
Acetobacterium woodii
Autotrophic
Genetic modification
$CO_2 + H_2$

ABSTRACT

Great interest has emerged in the recent past towards the potential of autotrophic acetogenic bacteria for the sustainable production of fuels and chemicals. This group of microorganisms possesses an ancient pathway for the fixation of carbon dioxide in the presence of hydrogen, making them highly attractive for the utilization of gas mixtures as a cheap and abundant carbon and energy source. As more and more genome sequence data of acetogens becomes available, the genetic tools are being developed concomitantly. Here, we demonstrate for the first time the genetic modification of the well-characterized acetogen *Acetobacterium woodii*. This microorganism selectively produces acetate under autotrophic conditions, but seems to be limited at high acetate concentrations. To increase the carbon flow through the Wood–Ljungdahl pathway and therefore increase the efficiency of CO_2 fixation, genes of enzyme groups of this pathway were selectively overexpressed (the four THF-dependent enzymes for the processing of formate as well as phosphotransacetylase and acetate kinase to enhance an ATP-generation step). Acetate production with genetically modified strains was increased in a batch process under pH-controlled reaction conditions in a stirred-tank reactor with continuous sparging of H_2 and CO_2. Final acetate concentrations of more than 50 g L^{-1} acetate were thus measured with the recombinant strains at low cell concentrations of 1.5–2 g L^{-1} dry cell mass in less than four days under autotrophic conditions.

1. Introduction

Acetate is a commodity chemical, which is mainly produced catalytically via carbonylation of methanol. It is a precursor for the production of a variety of chemical products such as polyvinyl acetates. About 2×10^6 t acetate per year are also produced biotechnologically with aerobic acetic acid bacteria, e.g. *Acetobacter* and *Gluconobacter*, via oxidative fermentation of molasses (Steinborn, 2007). Today, biotechnologically produced acetate is used exclusively for the production of vinegar.

Besides acetic acid bacteria, another group of microorganisms is also able to produce acetate under anaerobic conditions with syn(thesis) gas as a substrate. Some of these acetogenic bacteria form ethanol, butanol, butyrate, and 2,3-butanediol under autotrophic conditions, in addition to the main end product acetate (Mohammadi et al., 2011; Köpke et al., 2011).

If acetate is the sole end product, this way of fermentation is called homoacetogenesis. CO_2, which is either produced during glycolysis in the pyruvate:ferredoxin oxidoreductase reaction or used directly in the autotrophic mode of metabolism, is reduced in the Wood–Ljungdahl-pathway with the produced reducing equivalents or hydrogen to another mol of acetate (Ragsdale and Pierce, 2008). So, under heterotrophic conditions there is an increase of acetate production of 50% compared to the oxidative fermentation of ethanol by aerobic acetic acid bacteria, according to the equation:

$$C_6H_{12}O_6 \rightarrow 3CH_3COO^- + 3H^+ \quad \Delta G^{\circ'} = -310.9\,\text{kJ/mol}$$

Globally, acetogens produce about 10^{13} kg acetate annually (Ragsdale and Pierce, 2008). The overall autotrophic process can be described according to the equation:

$$CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O \quad \Delta G^{\circ'} = -95\,\text{kJ/mol}$$

Detailed recent reviews of acetogens and their metabolic features are given by Ragsdale and Pierce (2008) and Drake et al. (2008). In the recent past, the interest in autotrophic production of solvents and acids with acetogenic bacteria increased

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http://dx.doi.org/10.1016/j.jbiotec.2014.03.005
0168-1656/

Nomenclature

ack	acetate kinase
$pCO_{2,in}$	partial pressure of carbon dioxide at the inlet of the reactor (mbar)
$pH_{2,in}$	partial pressure of hydrogen at the inlet of the reactor (mbar)
pta	phosphotransacetylase
THF	tetrahydrofolate

tremendously. This is due to the fact that more and more genome sequence data of acetogens is becoming available. The genome sequences of the acetogens *Moorella thermoacetica* (Pierce et al., 2008), *Clostridium ljungdahlii* (Köpke et al., 2010, 2011), *Clostridium carboxidivorans* (Bruant et al., 2010; Paul et al., 2010), and *Eubacterium limosum* (Roh et al., 2010)) are published. Concomitantly, the development of genetic tools is picking up (Schiel-Bengelsdorf and Dürre, 2012). The first genetically modified acetogen was *C. ljungdahlii*. Both Köpke et al. (2010, 2011) and Leang et al. (2012) suggested this microorganism as a microbial platform for the production of fuels and chemicals based on syngas as substrate. Furthermore, a genetic transformation system was developed for the acetogen *M. thermoacetica* (Kita et al., 2012).

Besides *M. thermoacetica* and *Clostridium aceticum*, *Acetobacterium woodii* (Balch et al., 1977) has already been studied for the autotrophic production of acetate as well (e.g. Parekh and Cheryan, 1991; Sim and Kamaruddin, 2008; Demler and Weuster-Botz, 2011). *A. woodii* produces acetate specifically either on fructose or on $CO_2 + H_2$ as carbon sources. With this microorganism, up to 44 g L^{-1} acetate could be produced with $H_2 + CO_2$ as substrates in a batch-operated stirred-tank reactor by controlling the pH at pH 7.0 (Demler and Weuster-Botz, 2011). One possibility to overcome the decrease of acetate production of *A. woodii* at high acetate concentrations may be the enhancement of the carbon flow through the Wood–Ljungdahl pathway by overexpression of enzyme groups of this pathway. Peters et al. assumed that the processing of formate by tetrahydrofolate (THF)-dependent enzymes in the Wood–Ljungdahl pathway constitutes a bottleneck in the catabolism of CO_2 and H_2 (Peters et al., 1999).

Therefore, one of our approaches to enhance acetate productivity was the concomitant overexpression of the four THF-dependent enzymes of this pathway. An ATP-generating step in this CO_2-fixing pathway is the conversion of acetyl-CoA to acetate that is catalyzed by phosphotransacetylase and acetate kinase (Ragsdale and Pierce, 2008). Our second approach to enhance acetate productivity thus was the common overexpression of the two respective genes. As genome sequence data of *A. woodii* was not available at the time of this work, the DNA sequences for the genes of the required enzymes were taken from the acetogen *C. ljungdahlii* (Köpke et al., 2010, 2011) and were heterologously transformed into *A. woodii*. For *A. woodii*, both electroporation and conjugation systems have already been described (Strätz et al., 1993). In the present study, optimized shuttle vectors were successfully developed and the transformation protocol was modified. So we were able to transform *A. woodii* with different overexpression plasmids, resulting in a higher acetate production.

2. Materials and methods

2.1. Bacterial strains and plasmids

All bacterial strains used in this study are listed in Table 1.

Escherichia coli stocks were stored in 50% (v/v) glycerol at −80 °C. *A. woodii* DSM1030 stocks were stored anaerobically in hungate type tubes in 50% (v/v) glycerol at −80 °C. *A. woodii* DSM1030 was provided from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Chemically competent *E. coli* XL1 blue MRF′ cells were used for all cloning experiments and vector maintenance. *E. coli* JM109 pMCljS cells were used to methylate plasmid DNA *in vivo* before transformation into *A. woodii*.

2.2. Media and growth conditions

E. coli strains were cultivated in lysogeny broth (Luria–Bertani) medium at 37 °C. For plates, we used LB medium with agar (15 g L^{-1}) (Sambrook and Russell, 2001).

The standard medium for *A. woodii* contained per liter: NH_4Cl 1.0 g: KH_2PO_4 0.33 g; K_2HPO_4 0.45 g; $MgSO_4 \times 7H_2O$ 0.16 g; yeast extract 2.0 g (Roth Chemie GmbH, Karlsruhe, Germany); $NaHCO_3$ 10.0 g; cysteine–HCl × H_2O 0.5 g; $Na_2S \times 9H_2O$ 0.5 g; nitrilotriacetic acid 30 mg; $MnSO_4 \times H_2O$ 10 mg; NaCl 20 mg; $FeSO_4 \times 7H_2O$ 2 mg; $CoSO_4 \times 7H_2O$ 3.6 mg; $CaCl_2 \times 2H_2O$ 2 mg; $ZnSO_4 \times 7H_2O$ 3.6 mg; $CuSO_4 \times 5H_2O$ 0.2 mg; $KAl(SO_4)_2 \times 12H_2O$ 0.4 mg; H_3BO_3 0.2 mg; $Na_2MoO_4 \times 2H_2O$ 0.2 mg; $NiCl_2 \times 6H_2O$ 0.5 mg; $Na_2SeO_3 \times 5H_2O$ 6×10^{-3} mg; biotin 4×10^{-5} mg; folic acid 4×10^{-5}; pyridoxine–HCl 0.2 mg; thiamine–HC × $2H_2O$ 0.1 mg; riboflavin 0.1 mg; nicotinic acid 0.1 mg; D-Ca–pantothenate 0.1 mg; cyanocobalamine 2×10^{-6} mg; *p*-aminobenzoic acid 0.1 mg; lipoic acid 0.1 mg. Batch experiments were performed in 125-mL Müller–Krempel bottles (Bülach, Switzerland) filled with 50 mL sterile medium at 30 °C. Growth experiments on fructose contained 10 g L^{-1} fructose. Autotrophic batch experiments contained a mixture of H_2:CO_2 (67:33) at atmospheric pressure. Thiamphenicol or clarythromycin were used as antibiotics in a concentration of 25 μg mL^{-1} or 5 μg mL^{-1}. The 125-mL Müller–Krempel bottles (Bülach, Switzerland) were inoculated with 10% inoculum. The inoculum stemmed from a preculture, grown on the same medium.

2.3. Autotrophic cultivation in a stirred-tank reactor

For autotrophic cultivations on a liter-scale, medium without fructose was used. Furthermore, the concentrations of yeast extract, vitamins, and trace elements were doubled to avoid any growth limitation. As the concentration of Fe^{2+} was shown to have a supportive influence on growth and productivity of *A. woodii* (Demler and Weuster-Botz, submitted), the concentration of Fe^{2+} was increased to 5.4 mg L^{-1}. Thiamphenicol was used as an antibiotic in a concentration of 20 mg L^{-1}. Cultivations were carried out in a 2-L stirred tank bioreactor (Labfors, Infors, Bottmingen, Switzerland) with a working volume of 1 L at a temperature of 30 °C under anaerobic conditions. The reactor was equipped with twofold six-bladed Rushton turbines, which were operated at 1200 min^{-1}. A pH electrode (Mettler Toledo, Giessen, Germany) was used for online measurement of pH and a self-made rapid sampling device without dead volume (Lehrstuhl für Bioverfahrenstechnik, Technische Universität München, Garching, Germany, Patent DE 102005049226) was applied for off-line sampling. The individual gas mixtures of N_2, H_2, and CO_2 were adjusted and controlled by three separate mass flow controllers (WMR, Westphal Mess- und Regelungstechnik, Ottobrunn, Germany). Gas was continuously purged through the fermenter with a constant flow rate of 30 L h^{-1} (0.5 vvm). The partial pressures for H_2 and CO_2 at the inlet of the reactor were 400 mbar and 167 mbar, respectively, which accounts for a gas mixture of 40% H_2, 16.7% CO_2, and 43.3% N_2. The sterilized culture medium was sparged with the gas mixture for at least 12 h before inoculation to ensure oxygen-free medium and thermodynamic equilibrium with respect to carbon dioxide. Prior to inoculation the pH was adjusted to pH 7.0 with sterile H_2SO_4 and controlled at pH 7.0 during autotrophic cultivation by the addition of 10 M NaOH. Polypropylene glycol (100 μL L^{-1}) was used as

Table 1
Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Relevant genotype	Remark	Source
Bacterial strains			
A. woodii DSM1030	WT	Type strain	DSMZ, Braunschweig
E. coli XL 1 blue MRF'	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]*		Stratagene, La Jolla, (USA)
E. coli JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14- [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^-m_K^+$)*		Yanisch-Perron et al. (1985)
Plasmids			
pMCljS	pACYC184, methyltransferase gene of *C. ljungdahlii*, Sp^R	Methylation strain	This study
pJIR750	Cm^R, pMB1 (rep), *oriCP*, *oriEC*, *lacZ*		Bannan and Rood (1993)
pJIR750_pta-ack	pJIR750, phosphotransacetylase gene (*pta*) and acetate kinase gene (*ack*) of *C. ljungdahlii*, ErmC		This study
pJIR750_THF	pJIR750, genes of formyl-THF-synthetase, methenyl-THF-cyclohydrolase, methylene-THF-dehydrogenase and methylene-THF-reductase of *C. ljungdahlii*, ErmC		This study
pANS1	pACYC148/p15A *ori (rep)*, Sp^R, methyltransferase gen of phage 3T	Used for spectinomycin resistance cassette in pMCljS	Böhringer (2002)

antifoam agent. The batch experiments were initiated with a cell dry weight concentration of $0.56 \pm 0.08\ g\ L^{-1}$.

The preculture was grown in preculture medium with $10\ g\ L^{-1}$ fructose in 1-L septum bottles with a filling volume of 0.5 L under non-shaking conditions. Cells were harvested in the early stationary phase by centrifugation (30 min at 4500*g*). Batch experiments were initiated by transferring the inoculum anaerobically into 10 mL fresh medium without fructose with a syringe into the stirred-tank reactor.

2.4. Analytical methods

A gas chromatograph "clarus 600" (PerkinElmer LAS GmbH; Rodgau, Germany) was used for analysis of product concentrations. Samples were withdrawn with a syringe, centrifuged, and 1 mL of the supernatant was acidified with 0.1 mL of 2 N HCl containing 110 mM isobutanol, which served as an internal standard. 1 μL of this solution was injected into the gas chromatograph and analyzed on a glass column (iØ 2 mm × 2 m) packed with Chromosorb 101, 80–100 mesh. N_2 was the carrier gas ($33.5\ mL\ min^{-1}$), the injector temperature was 200 °C, and the detector temperature was 300 °C. A temperature profile was preset by keeping 130 °C for 1 min, then $4\ °C\ min^{-1}$ raising steps from 130 °C to 200 °C, finally keeping the temperature for 3 min at 200 °C.

Cell growth was monitored offline by measuring the optical density at 600 nm (Genesys 20, Thermo Electron, Dreieich, Germany). An experimentally determined correlation factor of 0.5 was used to estimate the cell dry weight concentrations.

For quantification of acetate concentrations in the stirred-tank bioreactors, samples of the fermentation broth were withdrawn anaerobically and filter sterilized (0.45 μm pore size). Acetate concentrations in the samples were analyzed by HPLC (Smartline HPLC, Knauer, Berlin, Germany) equipped with a RI detector (S2300, Knauer, Berlin, Germany) and an Aminex HPX-87H column (Bio-Rad, München, Germany). The column was operated at 50 °C with a constant flow rate of $0.7\ mL\ min^{-1}$ (eluent: 5 mM sulfuric acid).

2.5. DNA isolation and transformation

Standard molecular cloning techniques were performed according to established protocols (Sambrook and Russell, 2001). Genomic DNA of *A. woodii* was isolated using a modified protocol of Bertram and Dürre (1989). A 5-mL overnight grown culture was centrifuged at 6000*g* and 4 °C for 15 min. The cell pellet was suspended in 1 mL potassium phosphate buffer (10 mM, pH 7.5) and centrifuged again (6000*g*, 4 °C, 15 min). The sediment was then suspended in 0.4 mL STE-buffer (50 mM Tris–HCl, pH 8.0; 1 mM EDTA; 200 mM sucrose). 60 μL Lysozym ($20\ mg\ mL^{-1}$, Roche) were added and incubated for 30 min at 37 °C. 56 μL of 10% SDS were given to the samples and incubated for 10 min at 37 °C. Then, 48 μL of EDTA (0.5 M) and 20 μL Tris (1 M, pH 7.5) were added and mixed carefully. 20 μL RNase A ($20\ mg\ mL^{-1}$ (w/v)) were added and incubated for 30 min on ice. 20 μL of proteinase K ($20\ mg\ mL^{-1}$ (w/v)) were added and the mixture was incubated for 3 h at 37 °C. After this period, 120 μL of Na–perchlorate (5 M) were added, followed by phenol/chloroform extraction and isopropanol precipitation. The DNA was suspended in water and stored at 20 °C. Plasmid DNA of *E. coli* was isolated by the Zyppy™ plasmid miniprep kit (Hiss Diagnostics GmbH, Freiburg, Germany). PCR amplification of clostridial DNA was done using ReproFast polymerase (Gennaxxon). For isolation of plasmids from *A. woodii*, first cells of a 5-mL 3-day culture were harvested and lysed according to a modified protocol of Eikmanns et al. (1994). Modifications were use of anaerobic solutions and a pH of 7.5 for a TE buffer. Then, the above mentioned Zyppy™ plasmid miniprep kit was applied. Plasmids could not be visualized in a gel after electrophoresis (probably due to low DNA concentration), but their presence was confirmed by PCR (see below).

For transformation of *A. woodii* with different plasmids, a modified protocol of Köpke et al. (2010) was used. 200 mL medium in a 500-mL Müller–Krempel bottle supplemented with 8 mL DL-threonine (0.5 M) were inoculated with an overnight grown culture of *A. woodii*. At an OD_{600} of 0.4–0.6, the culture was centrifuged (RT, 6000*g*, 10 min) in the anaerobic chamber in four separate 50-mL Falcon tubes. The pellets were suspended and washed two times with 20 mL SMP buffer (sucrose 270 mM, $MgCl_2 \times 6H_2O$ 1 mM, NaH_2PO_4 7 mM) and were centrifuged again (RT, 6000*g*, 10 min). The pellets were suspended in 600 μL SMP buffer. The complete cell suspension (600 μL) was pipetted into an electroporation cuvette (4 mm, Biozym Scientific GmbH, Oldendorf, Germany), which contained 1 μg of plasmid DNA. The electroporation was performed at 2.5 kV, 25 μF, and 600 Ω. After electroporation, the cells were transferred into 5 mL fresh medium and incubated at 30 °C for 3 days. After 3 days, 1 mL of the culture was transferred into new medium with antibiotics, until growth was visible. Transformation success was checked by PCR with genomic as well as plasmid DNA as template. Primers were used amplifying the plasmid origin of replication plus the erythromycin resistance cassette.

2.6. Plasmid construction and manipulation

Novel clostridial expression plasmids (pJIR750_pta-ack and pJIR750_THF) were constructed using pJIR750 as a backbone (Bannan and Rood, 1993): For pJIR750_pta-ack, the *pta-ack* operon with its native promoter was amplified from *C. ljungdahlii* DNA

Table 2
Primers used for the amplification of *pta-ack* and the genes coding for THF-dependent enzymes.

Primer	Sequence (5′ → 3′)
pta-ack_SalI_forward	GCAGTCGACGTACAAAGTATAGGGCATTTGA
pta-ack_SalI_reverse	GCAGTCGACCCCTTTTCTTCTACTGTAACAT
THF_BamHI_forward	GCAGGATCCGCTTCACCTTCAACATTAATTC
THF_BamHI_reverse	GCAGGATCCCATCGAAATAGCTCAAGAATG

fad: restriction enzymes

(CLJU_c12770, CLU_c12780) using a standard PCR reaction and then ligated into the vector pJIR750 (restriction sites used: *Sal*I, primers used see Table 2).

For pJIR750_THF, the genes encoding formyl-THF-synthetase, methenyl-THF-cyclohydrolase, methylene-THF-dehydrogenase, and methylene-THF-reductase of *C. ljungdahlii* (CLJU_c37610, CLJU_c37630, CLJU_c37640, CLJU37650) were amplified using a standard PCR reaction and then ligated into the vector pJIR750 (restriction sites used: *Bam*HI, primers used see Table 2).

Before transformation of *A. woodii*, plasmids were *in vivo* methylated. This was essential, as all attempts to transform the three plasmids pJIR750, pJIR750_pta-ack, and pJIR750_THF without prior methylation into *A. woodii* failed. First, the methylation strain *E. coli* XL1 blue MRF′ pMCljS was constructed. The methylase gene, using the vector pAYC184 as a backbone, was synthesized by DNA 2.0 (USA). pMCljS contains in addition to the methylase gene of *C. ljungdahlii* (CLJU_c03310) a spectinomycin antibiotic resistance gene (amplified from the plasmid pANS1). For methylation, the plasmids pJIR750, pJIR750_pta-ack and pJIR750_THF were transformed into *E. coli* XL1 blue MRF′ pMCljS. After selection on agar plates with spectinomycin (50 μg μL^{-1}) and chloramphenicol (15 μg μL^{-1}), recombinant colonies were grown and used for plasmid preparation.

2.7. Measuring specific activity of acetate kinase

For determination of enzyme activity of acetate kinase, a modified protocol of Nakajima et al. (1978) was used. The cells were harvested in the exponential growth phase and centrifuged at 5000*g* for 10 min at 4 °C. Then, the sediment was suspended in buffer (Tris–HCl 0.1 M [pH 7.2], $MgCl_2 \times 7H_2O$ 5 mM) and cell disruption was performed with a ribolyser (RiboLyser™, Hybaid GmbH, Heidelberg) (5-times, step 6.5, 30 s). For the measurement of acetate kinase activity, we used a dilution of crude extract of 1:100. Protein concentration, for calculation of specific activity (U mg^{-1}), was determined using a "BCA™ Protein Assay Kit" (Perbio Science Deutschland GmbH, Bonn).

3. Results

3.1. Acetate production with fructose

Growth and metabolic profiles of *A. woodii* with plasmid pJIR750 and of the overexpression strains *A. woodii* pJIR750_pta-ack and *A. woodii* pJIR750_THF were compared with 55 mM fructose as substrate (Table 3). These uncontrolled batch experiments were repeated at least three times in Müller–Krempel bottles. The reference strain *A. woodii* pJIR750 reached a higher OD_{600} (4.78) after 78 h than the overexpression strains (maximum OD_{600} of 3.86). The reference strain produced significantly more acetate in anaerobic bottles (7.48 g acetate L^{-1} after 168 h) than the overexpression strains *A. woodii* pJIR750_pta-ack (5.68 g acetate L^{-1}) and *A. woodii* pJIR750_THF (5.26 g acetate L^{-1}). Higher acetate production correlated with the increased OD_{600} of the reference strain.

Table 3
Maximum optical density (600 nm) and acetate production of genetically modified *A. woodii* during heterotrophic batch cultivations.

Strain	Max. OD_{600}	Acetate production [g L^{-1}]
A. woodii pJIR750	4.78	7.48
A. woodii pJIR750_pta-ack	3.85	5.68
A. woodii pJIR750_THF	3.95	5.26

Table 4
Specific activity of acetate kinase of genetically modified *A. woodii* during heterotrophic batch cultivations.

Strain	Specific activity of acetate kinase [U mg^{-1}]
A. woodii pJIR750	6.41 ± 0.08
A. woodii pJIR750_pta-ack	7.79 ± 0.22
A. woodii pJIR750_THF	9.98 ± 0.24

3.2. Acetate kinase activity

Enzyme activity was measured in *A. woodii* pJIR750_pta-ack overexpressing acetate kinase and compared to *A. woodii* pJIR750_THF and *A. woodii* pJIR750 as references. The cells were harvested in the exponential growth phase and disrupted in a ribolyser. Table 4 shows that the overexpression of the *pta-ack* operon had significant effects on the acetate kinase activity. The strain *A. woodii* pJIR750_pta-ack (7.79 ± 0.22 U mg^{-1}) showed higher acetate kinase activities compared to the reference strain with plasmid pJIR750 (6.41 ± 0.08 U mg^{-1}), but *A. woodii* pJIR750_THF showed the highest acetate kinase activity (9.98 ± 0.24 U mg^{-1}). It might well be that these increased activities are the result of increasing the metabolic flux through the THF-dependent enzymes, suggesting that there is a regulatory link which serves for increasing expression of *pta* and *ack* genes.

3.3. Acetate production with $CO_2 + H_2$

Growth and metabolic profiles of *A. woodii* with plasmid pJIR750 and of the overexpression strains were compared with $CO_2 + H_2$ as substrate (Table 5). These uncontrolled batch experiments were repeated at least three times in Müller–Krempel bottles. All strains reached nearly the same maximum optical density OD_{600} of 0.362–0.381 after a process time of 216 h. Afterwards, OD_{600} dropped continuously to 0.2 until the end of the batch experiments (t = 931 h). Both overexpression strains produced more acetate (9.52 g L^{-1} with *A. woodii* pJIR750_THF and 9.61 g L^{-1} with *A. woodii* pJIR750_pta-ack) compared to the reference strain (8.16 g L^{-1}) at a process time of 667 h.

Autotrophic growth and acetate formation of genetically modified *A. woodii* was also studied in batch-operated, fully controlled stirred-tank bioreactors with continuous gas sparging.

Fig. 1 shows the concentrations of cell dry weight and acetate of the two overexpression strains of *A. woodii* compared to the reference strain harbouring solely the plasmid backbone. The cell dry weight concentrations of the mutant strains increased a bit faster and reached a higher maximum concentration of 1.9 g L^{-1} (*A. woodii*

Table 5
Maximum optical density (600 nm) and acetate production of genetically modified *A. woodii* during autotrophic batch cultivations.

Strain	Max. OD_{600}	Acetate production [g L^{-1}]
A. woodii pJIR750	0.381	8.16
A. woodii pJIR750_pta-ack	0.367	9.61
A. woodii pJIR750_THF	0.362	9.52

Table 6
Growth rates, volumetric and cell-specific production rates of genetically modified *A. woodii* during autotrophic batch cultivations in a stirred-tank bioreactor (V = 1.0 L, T = 30 °C, pH = 7.0, $pH_{2,in}$ = 400 mbar, $pCO_{2,in}$ = 167 mbar).

Strain	Growth rate (μ_{max}) [h^{-1}]	Volumetric production rate (Q_P) [$g\,L^{-1}\,d^{-1}$]	Cell-specific production rate (q_P) [$g\,g^{-1}\,d^{-1}$]
A. woodii pJIR750	0.052	25.31	20.54
A. woodii pJIR750_pta-ack	0.050	28.81	21.74
A. woodii pJIR750_THF	0.056	28.75	20.59

pJIR750_pta-ack) and 2.0 $g\,L^{-1}$ (*A. woodii* pJIR750_THF) at a process time of 1.5 days compared to the reference strain (1.7 $g\,L^{-1}$). The maximum growth rate of the reference strain (μ_{max} = 0.052 h^{-1}) was estimated by log-linear regression to be in between the maximum growth rates of the overexpressing strains (μ_{max} = 0.050 h^{-1} with *A. woodii* pJIR750_pta-ack and μ_{max} = 0.056 h^{-1} with *A. woodii* pJIR750_THF, see Table 6).

After reaching stationary phase, cell dry weight decreased in all batch processes to final concentration of 1.58 ± 0.04 $g\,L^{-1}$ after 3.7 days.

Similarly to biomass formation, acetate productivity was enhanced in the batch cultivations with the overexpression strains. Volumetric acetate productivities were estimated by linear regression. For cell-specific acetate productivity, the volumetric acetate productivity was divided by the corresponding mean cell concentration. A maximum volumetric acetate productivity of 28.8 $g\,L^{-1}\,d^{-1}$ was achieved with both recombinant strains compared to 25.3 $g\,L^{-1}\,d^{-1}$ with the reference strain. The cell-specific acetate productivities of the reference strain (20.54 $g\,g^{-1}\,d^{-1}$) and *A. woodii* pJIR750_THF (20.59 $g\,g^{-1}\,d^{-1}$) were the same within the estimation error, only *A. woodii* pJIR750_pta-ack reached a higher productivity of 21.7 $g\,g^{-1}\,d^{-1}$. With both modified strains, higher acetate concentrations of 50.5 $g\,L^{-1}$ (*A. woodii* pJIR750_pta-ack) and 51 $g\,L^{-1}$ (*A. woodii* pJIR750_THF), compared to the reference strain (44.7 $g\,L^{-1}$), were measured after 3.7 days in the controlled batch process.

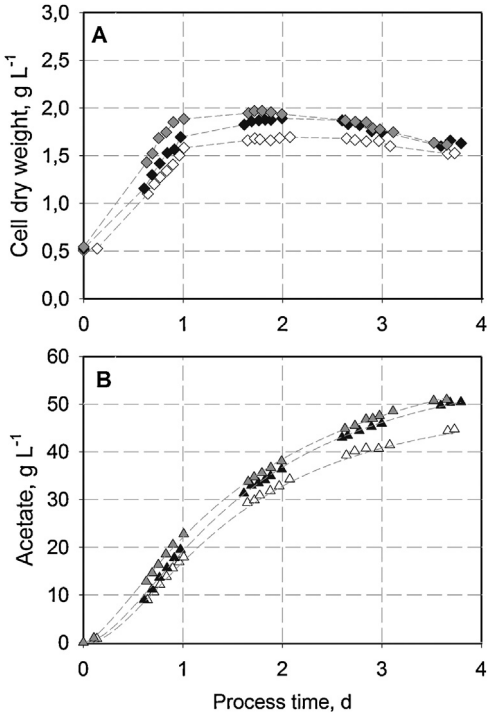

Fig. 1. Autotrophic batch cultivation of genetically modified *A. woodii* in a stirred-tank bioreactor (V = 1.0 L, T = 30 °C, pH = 7.0, $pH_{2,in}$ = 400 mbar, $pCO_{2,in}$ = 167 mbar). Concentration of (A) cell dry weight and (B) acetate. White symbols: *A. woodii* pJIR750; black symbols: *A. woodii* pJIR750_pta-ack; grey symbols: *A. woodii* pJIR750_THF. Dashed lines are only intended as visualization aid.

4. Discussion

Autotrophic acetogens are promising microorganisms for industrial biotechnology. The gases used as substrates (CO, $CO_2 + H_2$) are cheap and easily available as industrial off-gases or from biomass gasification. Meanwhile, several genome sequence data of acetogens are available and more and more genetic tools for these anaerobes are being developed. This opens the possibility to expand the product range of acetogens into platform chemicals and biofuels (Schiel-Bengelsdorf and Dürre, 2012). The first successful genetic modification of an acetogen was reported by Köpke et al. (2010). They introduced genes for the production of butanol in *C. ljungdahlii*, grew the bacterium on the gaseous substrates $H_2 + CO_2$ or syngas, and demonstrated low autotrophic production of butyrate and butanol.

In the present study, an improved protocol for DNA transfer as well as genetic modification of another acetogen, *A. woodii*, was successfully demonstrated for the first time.

A lot of bacteria possess a sequence specific restriction/modification system to protect themselves against foreign DNA. Many authors could show that *in vivo* or *in vitro* methylation of plasmid DNA stimulates the transformation efficiency in some *Clostridium* species (Leang et al., 2012; Kita et al., 2012; Jennert et al., 2000; Mermelstein and Papoutsakis, 1993). To eliminate restriction of foreign DNA in our study, we used the methylase of the acetogen *C. ljungdahlii* for the methylation of plasmids. At the time of this study the genome of *A. woodii* had not been sequenced, so that we used the methylase CLJU_c03310 from *C. ljungdahlii* for the methylation of plasmid DNA. Analysis of the *C. ljungdahlii* genome sequence identified this methylase as a homolog of a type I restriction/modification system. Analysis showed also that this methylase is a member of *N*-6-methyltransferases and therefore should methylate adenine. For determination of the specificity of this methylase, the *C. ljungdahlii* restriction/modification system would have to be characterized in detail.

Genetic modification of *A. woodii* in this work was applied for investigating possible limitations of the autotrophic acetate production at high acetate concentrations. Increasing the carbon flow through the Wood–Ljungdahl pathway by selective overexpression of groups of genes of this pathway led to a higher acetate productivity, a higher enzyme activity of the acetate kinase, and an increased final concentration of acetate (>10% in the stirred-tank bioreactor at low biomass concentrations compared to the reference *A. woodii* strain).

There may be further metabolic bottlenecks in autotrophic acetate production with *A. woodii* which limit acetate production in batch processes to about 50 $g\,L^{-1}$. A possible reason for no further increase in the final acetate concentration may be an inhibition of acetate kinase at high acetate concentrations as it was already assumed by Klemps et al. (1987) for the acetogen *Acetogenium kivui*. An inhibition of the terminal ATP-conserving reaction of the Wood–Ljungdahl pathway would result in the shut-down of the carbon flow through this pathway with no further growth and

acetate production. The genome sequence of *A. woodii* published recently (Poehlein et al., 2012) will facilitate further analyses of metabolic bottlenecks of autotrophic acetate production and will enable purposeful metabolic design of this acetogen. This anaerobic microorganism might thus soon represent a robust platform organism for the production of non-natural products (e.g. fuels or chemicals) with syngas or CO_2 and H_2-containing gas mixtures as substrates.

Acknowledgments

This work was funded by Wacker Chemie AG (Munich, Germany) and the Federal Ministry of Education and Research (BMBF, Berlin, Germany) within the BioIndustry 2021 program (BioM White Biotechnology, cluster Munich, project 0315192) and within the BioIndustry 2021 program (BioM White Biotechnology cluster Munich, project 0315192 and CLIB2021, project 031A100B). The support of Martin Demler by the TUM Graduate School (Munich, Germany) is acknowledged as well.

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