

Impact of formate on the growth and productivity of *Clostridium ljungdahlii* PETC and *Clostridium carboxidivorans* P7 grown on syngas

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Received 14 September 2014 · 15 November 2014

Summary. The current energy model based on fossil fuels is coming to an end due to the increase in global energy demand. Biofuels such as ethanol and butanol can be produced through the syngas fermentation by acetogenic bacteria. The present work hypothesizes that formate addition would positively impact kinetic parameters for growth and alcohol production in *Clostridium ljungdahlii* PETC and *Clostridium carboxidivorans* P7 by diminishing the need for reducing equivalents. Fermentation experiments were conducted using completely anaerobic batch cultures at different pH values and formate concentrations. PETC cultures were more tolerant to formate concentrations than P7, specially at pH 5.0 and 6.0. Complete growth inhibition of PETC occurred at sodium formate concentrations of 30.0 mM; however, no differences in growth rates were observed at pH 7.0 for the two strains. Incubation at formate concentrations lower than 2.0 mM resulted in increased growth rates for both strains. The most recognizable effects of formate addition on the fermentation products were the increase in the total carbon fixed into acids and alcohols at pH 5.0 and pH 6.0, as well as, a higher ethanol to total products ratio at pH 7.0. Taken all together, these results show the ability of acetogens to use formate diminishing the energy demand for growth, and enhancing strain productivity. [Int Microbiol 2014; 17(4):195-204]

Keywords: *Clostridium carboxidivorans* · *Clostridium ljungdahlii* · syngas fermentation · biofuels · formate

Introduction

The current energy model based on fossil fuels is coming to an end due to the increase in global energy demand, the depletion of primary oil reserves and its large price fluctuation [8]. Another important issue of fossil fuels as energy source is the emission of greenhouse gases and their negative impact on

global warming [1]. In recent decades, this growing concern has led to the development of alternative fuel sources. Currently, ethanol and butanol are considered two of the most promising alternative biofuels.

Biofuels can be obtained from renewable raw materials, such as molasses, starch, cellulose and lignin through hydrolysis and subsequent fermentation. However, the low efficiency of the cellulose and lignin conversion processes, as well as the high feedstock cost of molasses and starch and the ethical issues arising from their use for fuel production, call the viability of these technologies into question [23]. An alternative to these processes is the stepwise process of gasification and microbial fermentation. In gasification, organic matter from a

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variety of sources, i.e. residual agricultural biomass, municipal solid waste or tires, is gasified to synthesis gas (or syngas: a mixture of primarily CO, CO₂, and H₂) [22]. Some studies have highlighted the ability of some acetogenic bacteria to produce volatile fatty acids and alcohols using solely syngas in a fermentation metabolism [5,17].

Acetogens are anaerobic bacteria that use the autotrophic Wood-Ljungdahl pathway (WL) for the production of acetyl-CoA. Depending on the metabolic demand of the cell, acetyl-CoA can be used as a precursor of cellular biomass or further converted into organic acids, such as acetate and butyrate, and alcohols, such as ethanol and butanol, through a pathway similar to the classical acetone-butanol-ethanol (ABE) fermentation pathway [13,28]. The WL pathway consists of two separate branches, the methyl and the carbonyl branch. One molecule of CO₂ is reduced by several steps to a methyl group in the methyl branch, while the carbonyl branch involves the incorporation of a carbon monoxide molecule. The bound methyl group and the carbonyl are condensed with coenzyme A (CoA) to make acetyl-CoA. Among acetogens, *Clostridium carboxidivorans* P7 and *Butyribacterium methylotrophicum* produce a mixture of acetate, butyrate, ethanol and butanol from syngas; however, the production of alcohols by *B. methylotrophicum* is scarce [5,10,18]. *Clostridium ljungdahlii* strains PETC and ERI2, as well as *Alkalibaculum bacchi* strains CP11, CP13 and CP15 are also acetogens that transform syngas into acetate and ethanol, but none of these strains have been shown to produce butyrate or butanol [19,27,34].

The first steps in the methyl and carbonyl branches of the WL pathway, formate synthesis and the carbon monoxide formation, are recognized as reducing equivalent sinks that may diminish the growth capacity of cells and the conversion of inorganic carbon into valuable chemicals [7]. The use of hydrogen and carbon monoxide as sources of reducing equivalents is maximized during autotrophic growth. In this condition, the assimilation of formate as a partially reduced carbon source has the potential to reduce the hydrogen/CO demand for formate dehydrogenase activity in the WL pathway [4,31]. We hypothesize that the excess hydrogen can further be diverted to acetate reduction, increasing biofuel production. The synthesis of formic acid from CO₂ has been accomplished in a bioelectrochemical system (BES) using a purified formate dehydrogenase enzyme [33]. Electrosynthesis cou-

pled to fermentation by carboxydophilic bacteria, has been proven but not studied in detail [25]. However, the concept of enzymatic electrocatalysis involving energy applications is gaining in prominence, especially in the direction of enzymatic electrosynthesis of desired chemicals and fuels under non-limiting reducing power supply.

Formate has been reported as an inducer of acetate production in *Clostridium acetobutylicum*. Maximum effects of formate on acetate production in *C. acetobutylicum* were obtained under acidic conditions (at pH = 4.8) [2]. Despite this example, growth on weak organic acids is rather difficult for most microorganisms and inhibition occurs at very low concentrations. Inhibition effects are higher at low pH values where higher concentrations of the undissociated acid forms exist, which can freely diffuse to the cytoplasm of the cell eventually causing the dissipation of energy gradients built across the cell membrane [13]. Additionally, formate can cause sub-lethal damage in some bacteria and has been used as an antibacterial agent [35].

PETC has been grown chemoorganotrophically in a medium containing 5 g/l of formate and 1 g/l of yeast extract [34]. However, similar experiments have never been done autotrophically with this strain. Moreover, *B. methylotrophicum* can also use formate as substrate for growth, but it is unclear whether other acetogenic bacteria, including P7, can use formate when growing either organo- or autotrophically [15,18]. In this light, the present work hypothesizes that the addition of formate, as a partially reduced C1 compound, would positively impact kinetic parameters for growth and alcohol production in *C. ljungdahlii* PETC and *C. carboxidivorans* P7 by diminishing the need for external reducing equivalents. The aim of this work was to provide experimental evidence to evaluate formate addition as a potential enhancer of alcohol production in *C. ljungdahlii* PETC and *C. carboxidivorans* P7.

Materials and methods

Bacterial strains. *Clostridium ljungdahlii* PETC (DSM13528^T) and *C. carboxidivorans* P7 (DSM15243^T) strains were obtained from DSMZ [www.dsmz.de].

Media and culture conditions. Bacteria were cultured in an anaerobic mineral medium similar to ATCC1754 [34]. The used medium differed from ATCC1754 in: (i) all soluble carbon sources, i.e., yeast extract, fructose,

and NaHCO_3 , were excluded from the original formulation; and (ii) 2-(N-morpholino)ethanesulfonic acid (100 mM, final concentration) was used as pH buffer. Resazurin (1 mg/l) was used as an indicator of anaerobic conditions, and the pH of the medium was initially adjusted to 5.0, 6.0 or 7.0 with 1 M NaOH or HCl. Liquid medium was prepared and distributed anaerobically in Hungate tubes. Formate was added to the medium as sodium formate (Merck, Darmstadt, Germany) at different concentrations as indicated below. In all cases, tubes head-space were flushed with synthetic syngas consisting of a mixture of 32% CO , 32% H_2 , 28% N_2 , and 8% CO_2 of high purity (Praair Technology Ltd, Spain). All culture manipulations and inoculation of freshly prepared media were done inside an anaerobic chamber (Coy Lab Products, Michigan, USA). PETC and P7 cultures were incubated in 125 ml serum bottles containing 25 ml of modified ATCC1754 medium and syngas in the head-space at an overpressure of 100 kPa. Cultures were maintained active by a 4% weekly transfer into new serum bottles.

Fermentation experiments. Exponentially growing *C. ljungdahlii* PETC and *C. carboxidivorans* P7 cultures were used as inocula for batch experiments to test for formate effects on growth and alcohol production. Fermentation experiments were conducted in 25 ml anaerobic tubes containing 6 ml of organic-carbon-free ATCC1754 medium. A 10% inoculum of either PETC or P7 strains was used in all experiments. Culture tubes were inoculated in anaerobic conditions and thoroughly flushed with syngas mixture reaching a final headspace overpressure of 100 kPa. Syngas was injected only at the beginning of the experiment. Sodium formate solutions adjusted at the desired pH were aseptically added to the medium at final concentrations of: 0.1, 1.0, 2.2, 5.5, 7.6, 10.9, 15.0, 20.0, 27.2, 54.5, and 109.0 mM. In all batch tests, tubes containing no sodium formate were included as controls for growth kinetics under fully autotrophic conditions. Experiments were carried out for the two bacterial species at three pH values, 5.0, 6.0 and 7.0. The cultures were incubated at 35°C under mild agitation on a rotary shaker Stuart incubator SI500 at 100 rpm (Bibby Scientific Ltd., OSA, UK). Tubes were placed horizontally to enhance gas-liquid mass transfer. All experimental conditions were assayed in triplicate using three independent inoculated cultures.

Growth was monitored on a daily basis by measuring the absorbance at 600 nm using a CE1021 spectrophotometer (CECIL, Cambridge, UK). Growth experiments finished once cultures reached the stationary growth phase, which was considered to occur 48 to 72 h after growth cessation. Samples for the determination of organic acids (formate, acetate and butyrate) and alcohols (ethanol and butanol) concentrations were obtained at the beginning and at the end of the incubation experiments, filtered using nylon filters (0.2- μm diameter, Millipore, Germany) and stored at 4°C until analyzed. Finally, the pH of the medium was measured using a BASIC 20 pHmeter (Crison, Spain).

Additionally, an independent experiment was conducted at five sodium formate concentrations: 13.7, 17.2, 21.9, 30.0 and 97.5 mM at pH 6.0 using PETC strain to test the consumption of formate throughout growth. The samples for the determination of formate concentration were obtained every 48–72 h, as well as, at the beginning and at the end of the incubation experiment.

Determination of growth variables. Linear regression of transformed absorbance readings ($\ln A_t$) at time intervals of 72 h (t) were used to estimate the changes of growth rate, according to equation (1):

$$\ln A_t = \ln A_{\text{init}} + \mu \cdot t \quad (1)$$

Growth rates (μ , h^{-1}) were calculated for each incubation experiment as a measure of the growth capacity of the bacterial cultures at the conditions set in the experiment. Duration of the lag phase (days) was estimated as the time interval between the inoculations of tubes and the time at which the calculated maximum growth rate was observed.

Analytical methods. The total amount of formic acid/formate (sum of formic acid and formate) was measured by using a spectrophotometric method [32]. Formate concentrations were measured at the beginning and the end of incubation experiments. Undissociated formic acid (HCOOH) concentration at initial conditions was calculated based on the measured pH and the total formic acid/formate measurements according to the equilibrium equation (2).

$$\text{HCOOH} = F_t - (10^{(\text{pH}-\text{pKa})} \cdot F_t) / (10^{(\text{pH}-\text{pKa})} + 1) \quad (2)$$

Where F_t is the concentration of sodium formate added in each experiment and pKa is the equilibrium constant. In this study, a value of 3.76 was used, corresponding to the equilibrium constant at 35°C [16].

The fermentation products (acetate, ethanol, butyrate, and butanol) were analyzed quantitatively using a gas chromatograph (Agilent 7890A GC system, Agilent Technologies, Spain) equipped with a fused-silica capillary column (DB-FFAP, 30 m \times 0.32 mm \times 0.5 μm) and a flame ionization detector (FID) using helium as carrier gas. The injector and detector temperatures were set at 250°C and 275°C, respectively. The oven temperature was initially kept at 40°C for 1 min, and subsequently increased following a ramp of 5°C min^{-1} until temperature reached 70°C, at 10°C min^{-1} from 70°C to 180°C, and at 35°C min^{-1} from 180°C to 250°C. Finally, the temperature was maintained at 250°C for 5 min.

Statistical analyses. All statistical analyses were conducted using SPSS 15.0 statistical package for Windows (LEAD Technologies Inc., EEUU). Significance levels were established for $P \leq 0.05$. ANOVA tests were used to analyze differences of maximum growth rate in relation to the initially added formic acid concentration, as well as, the alcohol to total product ratio and the products concentration in relation to the initially sodium formate concentration. Multiple comparisons between initial formic acid and sodium formate concentrations were further analyzed using a T3 of Dunnet post-hoc test assuming not equal variance or Bonferroni post-hoc test assuming equal variance between treatments. Pearson correlation tests were used to analyze the correlation of the acids and solvents production, as well as, alcohols to total products ratio with the initial sodium formate concentration.

Results and Discussion

Growth in the presence of formate. *Clostridium ljungdahlii* PETC was able to grow under all experimental conditions tested except at sodium formate concentrations of 54.5 and 109.0 mM at pH 6.0 (Table 1). On the contrary, *C. carboxidivorans* P7 showed a more restricted range of growth conditions and no increase in absorbance was observed in many of the experimental conditions, especially at low pH values. Growth of P7 was restricted to formate concentrations lower than 10.9 mM at pH 6.0, and no growth was observed at any of the formate concentrations tested at pH 5.0. Main differences observed in growth curves for the two strains were the decrease in the optical density at the stationary phase and the duration of the lag phase depending on the formate concentration (Fig. 1).

The rapid decrease in absorbance values during the stationary phase was observed for both *Clostridium* species when incubated at pH 7.0. Reasons for this decrease were not inves-

Table 1. Concentration of formate and formic acid at the beginning of the experiments; mean values \pm SD (n = 3) of lag phase, maximum growth rate attained and formate concentration consumed during the experiment of *Clostridium ljungdahlii* PETC and *Clostridium carboxidivorans* P7

Initial pH	<i>C. ljungdahlii</i> PETC				<i>C. carboxidivorans</i> P7			
	Sodium formate (mM)	Formic acid (mM) ^a ($\times 10^2$)	Lag phase (days)	Maximum growth rate (h ⁻¹)	Δ formate concentration (mM)	Lag phase (days)	Maximum growth rate (h ⁻¹)	Δ formate concentration (mM)
5.0	0.0	0	1.7 \pm 0.6	0.027 \pm 0.005	0.76 \pm 0.42	7.0 \pm 3.0	0.023 \pm 0.005	nm ^c
	1.0	6	1.0 \pm 0.0	0.060 \pm 0.010	-1.19 \pm 0.21	ng ^c	na ^c	na
	2.2	10	1.0 \pm 0.0	0.042 \pm 0.002	-1.64 \pm 0.13	ng	na	na
	5.5	30	2.3 \pm 1.2	0.033 \pm 0.005	-5.26 \pm 0.42	ng	na	na
	7.6	40	3.0 \pm 0.0	0.032 \pm 0.006	-5.95 \pm 0.30	ng	na	na
	10.9	60	6.5 \pm 0.7	0.036 \pm 0.003	-8.83 \pm 0.11	ng	na	na
6.0	0.0 ^b	0	2.2 \pm 0.4	0.049 \pm 0.008	1.10 \pm 0.30	1.0 \pm 0.0	0.051 \pm 0.009	1.26 \pm 0.30
	0.1	0.1	3.0 \pm 0.0	0.050 \pm 0.003	0.09 \pm 0.24	1.7 \pm 1.2	0.055 \pm 0.008	0.18 \pm 0.08
	1.0	1.1	1.0 \pm 0.0	0.058 \pm 0.004	-0.70 \pm 0.20	1.0 \pm 0.0	0.062 \pm 0.008	-0.41 \pm 0.22
	2.2 ^b	2	1.6 \pm 0.6	0.049 \pm 0.002	nm	1.0 \pm 0.0	0.053 \pm 0.004	-1.73 \pm 0.15
	5.5 ^b	5.7	1.8 \pm 0.8	0.035 \pm 0.003	nm	5.8 \pm 2.9	0.043 \pm 0.012	nm
	7.6	6.5	1.7 \pm 0.6	0.030 \pm 0.018	-7.71 \pm 0.32	4.0 \pm 1.4	0.034 \pm 0.008	-7.34 \pm 0.52
7.0	10.9 ^b	11	6.0 \pm 0.0	0.063 \pm 0.020	nm	ng	na	na
	15.0	15	7.0 \pm 4.4	0.028 \pm 0.006	-13.21 \pm 0.08	ng	na	na
	20.0	19	9.5 \pm 0.7	0.037 \pm 0.003	-16.75 \pm 0.05	ng	na	na
	27.2	30	9.0 \pm 1.4	0.037 \pm 0.002	-21.29 \pm 0.00	ng	na	na
	54.5	60	ng	na	na	ng	na	na
	109.0	120	ng	na	na	ng	na	na
	0.0	0	1.0 \pm 0.0	0.045 \pm 0.007	0.23 \pm 0.14	1.0 \pm 0.0	0.053 \pm 0.005	0.36 \pm 0.49
	1.0	0.081	1.0 \pm 0.0	0.035 \pm 0.002	-0.95 \pm 0.35	1.0 \pm 0.0	0.050 \pm 0.006	-0.87 \pm 0.07
	2.2	0.17	2.0 \pm 0.0	0.025 \pm 0.008	-2.11 \pm 0.08	1.0 \pm 0.0	0.049 \pm 0.010	-2.31 \pm 0.04
	5.5	0.4	1.0 \pm 0.0	0.036 \pm 0.003	-5.23 \pm 0.19	1.0 \pm 0.0	0.053 \pm 0.005	-4.92 \pm 0.39
	7.6	0.6	2.3 \pm 1.2	0.029 \pm 0.007	-6.87 \pm 0.27	1.0 \pm 0.0	0.063 \pm 0.010	-6.67 \pm 0.34
	10.9	0.8	1.7 \pm 1.2	0.027 \pm 0.008	-7.59 \pm 0.12	1.0 \pm 0.0	0.055 \pm 0.002	-7.01 \pm 1.21

^a Values calculated according to equation 2; ^b n = 6; ^c na-not applicable, nm-not measured, ng-no growth.

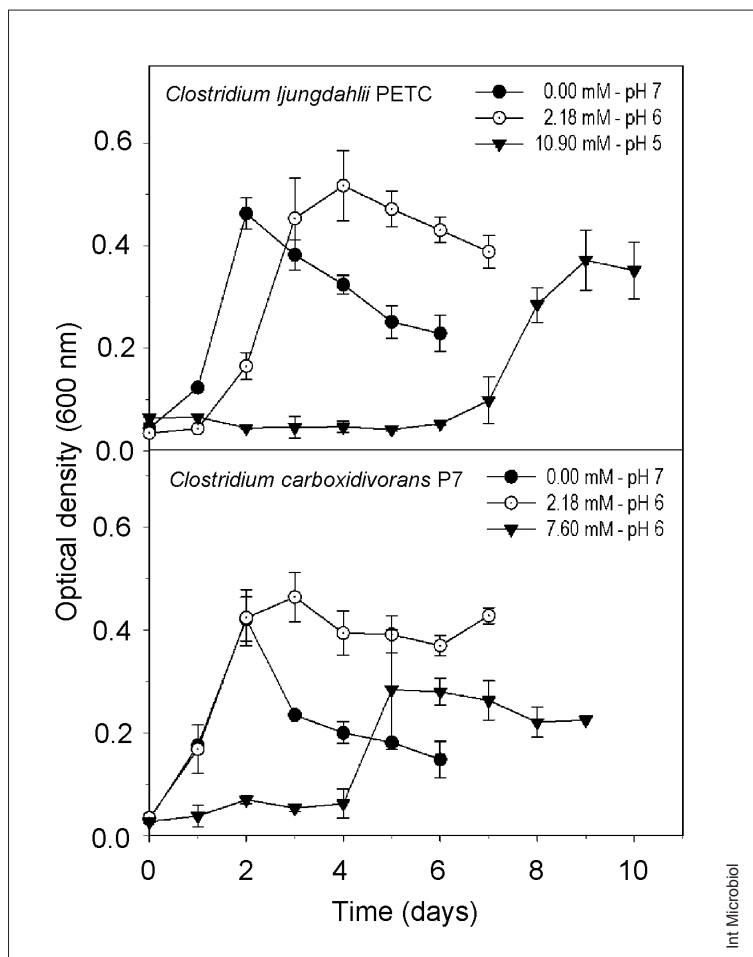


Fig. 1. Selected growth curves (mean values and SD, $n \geq 3$) of *Clostridium ljungdahlii* PETC (top) and *Clostridium carboxidivorans* P7 (bottom) at different pH and formate concentrations.

tigated in detail but a thorough inspection of those cultures under phase-contrast microscopy revealed the presence of cell clumps and lysed cells (results not shown), both contributing to the decrease in the absorbance.

The duration of the lag phase varied from 1 to 12 days for both P7 and PETC, and was directly correlated with the increase in the sodium formate concentration of the culture, especially at low pH values (Table 1). The increase on the lag phase of bacteria is generally recognized as an adaptation phase, during which bacteria stimulate transcription of new genes to resume growth under the new environmental conditions [30]. For instance, it has been reported that 10 mM of formic acid at pH 5.0 caused bacteriostasis in *Escherichia coli*, and growth resumed only after a 2 h incubation period proving its adaptation to formic acid [6]. However, adaptation to increasing formate concentrations may be complex since organic acids can serve as both additional carbon substrates and inhibitory compounds, depending on the concentration, pH of the media and/or the cell resistance to the acid, which

could explain such long lag phases. Moreover, the addition of sodium formate, particularly at high concentrations, could have caused a significant increase in the ionic strength of the culture medium thus causing an additional stress for cell growth. The effect of sodium chloride concentration on the growth and alcohol production of *Clostridium autoethanogenum* in a completely autotrophic medium has been previously tested using a Plakett-Burmann experimental design; even if that work reports a positive effect of NaCl in ethanol production, it is not significant in the range of 0.4 to 1.0 g/l [9].

The maximum estimated growth rates for PETC and P7, were $0.063 \pm 0.020 \text{ h}^{-1}$ and $0.063 \pm 0.010 \text{ h}^{-1}$, respectively (Table 1). The calculated maximum growth rates agree with the values obtained in previous works using the same strains [15,17,27]. In both strains at low pH values, low concentrations of sodium formate (<2.2 mM) resulted in a slight increase in the growth rate compared to the formate free media, although the observed differences were only significant for PETC cultures at pH 5.0 ($P < 0.05$, Bonferroni test, $n \geq 3$).

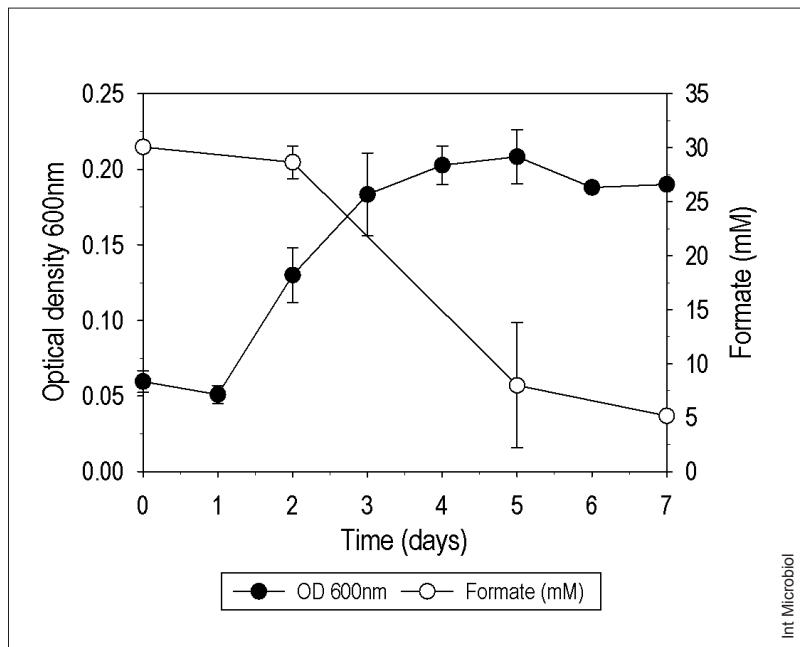


Fig. 2. Formate consumption during growth of *Clostridium ljungdahlii* PETC. Optical density (black dots) and formate concentration (white dots) are shown as mean values of two replicates. Error bars indicate SD.

According to the observed growing capacity, PETC was more tolerant to formate concentration than P7, and complete growth inhibition was only observed at 54.5 mM at pH 6.0. As stated above, a potential effect of added salt concentration could also contribute to growth inhibition. However, this is not expected to occur at sodium formate concentrations lower than 10.9 mM, according to the results obtained at pH 7.0, at which no significant growth inhibition occurred in any of both strains. This observation suggests that low sodium formate concentrations at pH 6.0 and 5.0 might enhance the growth of PETC.

Extra-cellular formic acid diffuses across the lipid bilayers and dissociates inside the cell based on the intra-cellular pH [36]. The distribution of dissociated and undissociated forms on the two sides of the cell membrane is proportional to the pH [11]. The most common metabolic processes to circumvent organic acid diffusion into cells includes the use of specific transporters functioning as efflux pumps [14,24]. However, and at least for enterobacteriaceae, several other strategies exist including amino acid decarboxylases and other protective mechanisms [3]. An inspection of public genome sequences of PETC and P7 have confirmed the presence of putative formate transporters, although with differences in the two bacterial species [17,26]. One single gene encoding for a hypothetical formate/nitrite transporter (WP_013240353) was identified in the PETC genome (NC_014328), whereas, the P7 draft genome (PRJNA48985; PRJNA29495; PRJNA55755; PRJNA33115) contains at least three genes coding

for formate transporters, two formate/nitrite transporters (WP_007062507 and WP_007063385) and one oxalate/formate antiport (WP_007061997). The alignment of the four retrieved amino acid sequences revealed that the unique nitrite/formate transporter found in PETC had a highly similar homolog (>80%, Blosum62 matrix) in *C. carboxidivorans* P7 (results not shown). Note that PETC showed a much faster adaptation and higher tolerance to formate, which might be explained to some extent by differences in the other two transport proteins detected.

Formate consumption. Formate concentrations were measured once growth stopped and were compared to the initial concentration to assess its net consumption or production (Table 1). A net production was detected for both strains when incubated under completely autotrophic conditions or at low formate concentration (<1.0 mM). This production was probably due to the activity of formate dehydrogenase (FDH), which converts CO₂ into formate in the first step of the WL pathway. Net production ranged from 1.1 to 0.09 mM in PETC and from 1.26 to 0.18 mM in P7. On the contrary, formic acid consumption was observed in most of the treatments where formate had been added and growth resumed after the lag phase. This net formate consumption was significant for both strains, accounting for more than 80% of the added sodium formate. Time course experiments were carried out to elucidate whether formate consumption occurred during the lag or the exponential phase. No net formate consumption was observed during the

lag phase, it being mostly consumed during the exponential growth phase at any of the concentrations tested (Fig. 2).

The observed formate consumption could be related to its use as an alternative carbon or energy substrate in addition to H_2 , CO_2 and CO , as has been proven for some acetogenic bacteria. Theoretically, formate uptake would partially circumvent the use of hydrogen/ CO and make the first step in the WL pathway unnecessary. Moreover, formate oxidation to CO_2 via formate dehydrogenase (FDH) would provide an

additional reducing power source, which would diminish the total energy requirements to incorporate new carbon molecules [28]. The most favorable reaction to obtain reducing power in acetogenic bacteria is the oxidation of CO to CO_2 by the carbon monoxide dehydrogenase (CODH) [12], but no analyses of the composition of the gas phase were done to confirm this hypothesis in this experiment. Cultivation of PETC and P7 in the same media composition as the used here, but no formate added, resulted in a complete depletion of CO , which was

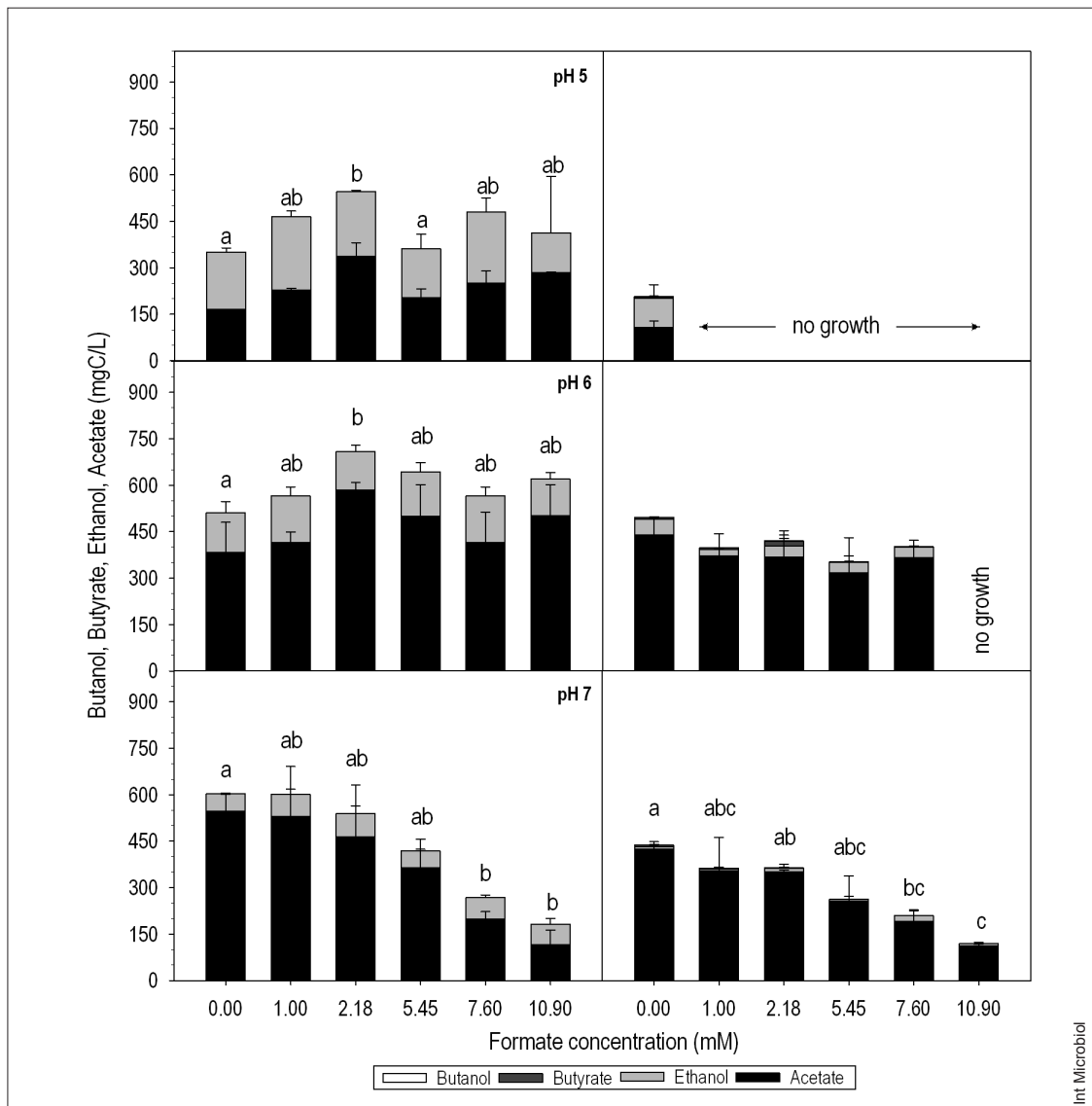


Fig. 3. Concentrations of organic acids (acetate and butyrate) and alcohols (ethanol and butanol) produced by *Clostridium ljungdahlii* PETC (left) and *Clostridium carboxidivorans* P7 (right) according to initial formate concentration. Incubations at different pH values are shown. Different letters above bars indicate significant differences of acetate production between experiments within each bacterial species according to Bonferroni or T3 of Dunnet post-hoc test assuming equal or not equal variance respectively. Bars show mean value of 3 replicates. Error bars indicate SD.

mostly converted into CO₂ and used as the main source of reducing power instead of H₂ (results not shown). In this respect, the incorporation of formate would considerably lower the CO oxidation as a reducing power source and increase the carbon available for fixation into cellular biomass. This probably occurred at low formate concentrations, at which an increase in the growth rate and the growth yield of *C. ljungdahlii* was observed. Most presumably, the positive effect of formate addition on growth was masked by the activation of resistance mechanisms to circumvent potential inhibition effects of either increased formic acid concentration or ionic strength.

Production of acids and alcohols. The concentration of acids (acetate and butyrate) and alcohols (ethanol and butanol) produced by *C. ljungdahlii* PETC and *C. carboxidivorans* P7 measured at the end of the incubation experiments is shown in Fig. 3.

Acetate production by PETC increased with increasing concentrations of formate at pH 5.0 and 6.0. Maximum acetate production (337.9 mg C/l and 584.7 mg C/l, respectively) was observed at 2.18 mM of formate, representing an increase of 104.5% and 52.4% compared to the control. On the contrary, acetate production was negatively affected by increasing concentration of sodium formate at pH 7.0 ($P < 0.05$, Pearson correlation test, $n = 18$). Acetate production of PETC decreased from 546.1 mg C/l (control test) to 116.3 mg C/l (10.9 mM formate). Regarding ethanol, the highest production occurred at pH 5.0, with maximum concentrations slightly over 230.0 mg C/l. Nevertheless, even if differences in alcohol production were observed at different pH values, changes were not related to the initial formate concentration ($P > 0.05$, ANOVA test, $n \geq 3$). Formate concentrations higher than 2.18 mM can positively affect the ethanol production due to the changes in salt concentrations [9].

Acetate and ethanol production in *C. carboxidivorans* P7 was lower than in PETC, but different formate dependence trends were observed depending on pH. At pH 6.0, acetate production remained almost invariable between 0 and 10.9 mM. However, acetate concentration showed a negative correlation ($P < 0.05$, Pearson correlation test, $n = 18$) with added formate at pH 7.0. Maximum butyrate was 14.9 mg C/l, and was obtained at a sodium formate concentration of 2.18 mM and pH 6.0. Neither pH nor initial formate concentration did play a major role in butyrate production, except at pH 7.0 where production decreased more than 50% in the presence of formate. Alcohols production of PETC at pH 6.0 showed some significant differences, although no correlation to the sodium formate addition was observed ($P > 0.05$, ANOVA test, $n \geq 3$) (Fig. 3).

Overall, the addition of formate increased the acid production of *C. ljungdahlii* PETC at pH 5.0 and 6.0, although solvent production remained unaffected. This could be explained by the “acid crash” effect, during which the fast accumulation of acids results in a failure of the switch from acidogenic phase to solventogenic phase to occur, and no solvent are produced by clostridia. Formic acid has been reported to play a major role in triggering the acid crash of ABE fermentations [20,37]. This phenomenon was not observed for *C. carboxidivorans*, as the concentrations of acids produced in the different experiments were never higher than that of the control. The addition of formate at pH 7.0 caused the opposite effect in both strains, and accumulation of acids decreased with increasing formate concentrations. These observations seem to be in disagreement with the measure OD, lag phases and growth rates (Table 1), which shows that, at pH 7.0, undissociated formic acid concentration remained low and inhibitory effects were clearly diminished in both PETC and P7 strains. Ideally, the energy saved by the use of formate as a substrate could be utilized to increase cellular ATP production. It has been long recognized that autotrophic growth by the WL pathway must be linked to an energy-generating anaerobic respiratory process, since during autotrophic growth there is no net ATP synthesis by substrate-level phosphorylation [28]. Thus, the use of partially reduced compounds could have also allowed higher available reducing power and/or ATP, so reducing the need of acetate production. However, this was not clearly confirmed and further work would be needed to test this hypothesis.

The highest alcohol to total product ratios were obtained at pH 5.0 for both strains, and they decreased significantly at higher pH values. The statistical tests ($P > 0.05$, Dunnett T3 test, $n \geq 3$) proved that such differences were not linked to the presence of sodium formate at P7 strain, but to incubation pH. The highest alcohol to total product ratio through all the experiment was 0.53, corresponding to the PETC control experiment at pH 5.0. This ratio was negatively influenced by the addition of formate because the productivity enhancement led to the production of mainly acetate. Finally, alcohols/products ratio at pH 7.0 significantly increased from 0.09 to 0.36 ($P < 0.05$, Dunnett T3 test, $n \geq 3$) in PETC. However, the reason for such an increase was related to the reduction of acetate production rather than to an increase in the net alcohol production. Although the total amount of carbon fixed into synthesized products was lower, such operational conditions could be beneficial when aiming at alcohol production. Downstream separation processes account for a large part of operational costs, therefore the decrease in acetate production in the fermentation medium could ease alcohol separation [29].

Implications and future prospects. The present work assessed the impact of the addition of formate on *C. ljungdahlii* PETC and *C. carboxidivorans* P7. Results showed the higher tolerance of PETC to formate, in addition to the enhancement of its growth rate and productivity at low formate concentrations at pH 5.0 and 6.0. This is of interest from the biotechnological point of view as the ability of the PETC strain to use formate as a feedstock opens up potential to upgrade carboxydrotrophic fermentation process with external formate supply. Of special interest could be the combination of enzymatic electrocatalysis to produce formic acid [33] and microbial electrosynthesis [21]. In fact this could be a major breakthrough in the production of added-value compounds from carbon dioxide via bio-electrochemical fermentation. In this light, PETC could be cultivated at moderately acid pH in a BES with low formic acid production to accelerate its metabolism and enhance carbon fixation into products. However, further studies are required to elucidate the metabolic fate of formate, and to understand the impact of formate assimilation on the cell energy and reducing power balances.

Acknowledgements. The authors thank the Autonomous Government of Catalonia (Generalitat de Catalunya) (2013 FI-DGR) and the Spanish Ministry of Science and Innovation (Best-Energy, CTQ2011-23632, CTM2013-43454-R) for their financial support in this study. LEQUIA and IEA have been recognized as consolidated research groups by the Catalan Government (2014-SGR-1168 and 2014-SGR-2016). RG gratefully acknowledges support from Beatriu de Pinós fellowship (BP-2011-B) and FP7 Marie Curie Career Integration Grants (PCIG13-GA-2013-618593).

Competing interests. None declared.

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