Appl Microbiol Biotechnol (2016) 100:4231–4240
DOI 10.1007/s00253-016-7389-8

BIOENERGY AND BIOFUELS

# Carbon monoxide bioconversion to butanol-ethanol by *Clostridium carboxidivorans*: kinetics and toxicity of alcohols

**Ánxela Fernández-Naveira[1] · Haris Nalakath Abubackar[1] · María C. Veiga[1] · Christian Kennes[1]**

Received: 30 December 2015 / Revised: 7 February 2016 / Accepted: 10 February 2016 / Published online: 27 February 2016


**Abstract** Butanol production from carbon monoxide-rich waste gases or syngas is an attractive novel alternative to the conventional acetone-butanol-ethanol (ABE) fermentation. Solvent toxicity is a key factor reported in ABE fermentation with carbohydrates as substrates. However, in the gas-fermentation process, kinetic aspects and the inhibition effect of solvents have not thoroughly been studied. Therefore, different batch bottle experiments were carried out with the bacterial species *Clostridium carboxidivorans* using CO as carbon source for butanol-ethanol fermentation. A maximum specific growth rate of 0.086 ± 0.004 $h^{-1}$ and a biomass yield of 0.011 $g_{biomass}/g_{CO}$ were found, which is significantly lower than in other clostridia grown on sugars. Besides, three assays were carried out to check the inhibitory effect of butanol, ethanol, and their mixtures. Butanol had a higher inhibitory effect on the cells than ethanol and showed a lower $IC_{50}$, reduced growth rate, and slower CO consumption with increasing alcohol concentrations. A concentration of 14–14.50 g/L butanol caused 50 % growth inhibition in *C. carboxidivorans*, and 20 g/L butanol resulted in complete inhibition, with a growth rate of 0 $h^{-1}$. Conversely, 35 g/L ethanol decreased by 50 % the final biomass concentration respect to the control and yielded the lowest growth rate of 0.024 $h^{-1}$. The inhibitory effect of mixtures of both alcohols was also checked adding similar, near identical, concentrations of each one. Growth decreased by 50 % in the presence of a total concentration of alcohols of 16.22 g/L, consisting of similar amounts of each alcohol. Occasional differences in initially added concentrations of alcohols were minimal. The lowest growth rate (0.014 $h^{-1}$) was observed at the highest concentration assayed (25 g/L).

**Keywords** *Clostridium carboxidivorans* · Butanol · Ethanol · Inhibitory effect · Batch experiment · $IC_{50}$

✉ Christian Kennes
Kennes@udc.es

[1] Chemical Engineering Laboratory, Faculty of Sciences, University of La Coruña, Rúa da Fraga 10, 15008 La Coruña, Spain

## Introduction

Nowadays, the instability of the oil price as well as recent concerns about the increasing scarcity of fossil fuels and their negative environmental impact have led to a growing interest in biofuels such as ethanol and butanol (Gowen and Fong 2011; Abdehagh et al. 2014). Butanol has recently been recognized as a highly promising biofuel (Bellido et al. 2014) and has several advantages. It is less hygroscopic and less corrosive and has a higher caloric content than ethanol (Qureshi et al. 2001; Wallner et al. 2009). For those reasons, in recent years, butanol has been considered a chemical of great industrial importance with a high potential to replace gasoline (Dürre 2007; Lee et al. 2008).

Ethanol and butanol can be obtained through fermentation of different sugars available in food crops, which is the conventional and most common commercial technology nowadays, known as first generation process. However, this process leads to food-fuel competition. Recently, in order to avoid such drawback, a new alternative has been developed using lignocellulosic feedstocks from agricultural waste and energy crops, which are inexpensive and renewable starting materials for biofuels production and do not adversely affect food supplies (Gowen and Fong 2011). Extracting simple, carbohydrates from the polymeric lignocellulosic structure is a complex process (Balat and

Balat 2009) requiring physical, chemical, or enzymatic pretreatments in order to hydrolyze the biomass into fermentable sugars in the so-called second generation process. Carbohydrates can be converted to butanol by clostridial strains, together with other side products, i.e., ethanol and acetone, through the acetone-butanol-ethanol (ABE) fermentation. Based on advances in biotechnology and process engineering, new fermentation processes are being developed, using renewable carbon sources, for a more efficient production of butanol (Dürre 2007; Lee et al. 2008; Papoutsakis 2008).

Besides butanol production from carbohydrates, a novel production route has been suggested, consisting of converting biomass or any other carbonaceous feedstocks into CO-rich gases, such as syngas. The gaseous substrate can then be fermented into ethanol and/or butanol by some bacterial species, mainly clostridia. Interestingly, this alternative route can also use CO-rich waste gases as substrates (Abubackar et al. 2011; Kennes and Veiga 2013). Under optimized conditions, a mixture of butanol and ethanol (B-E) is obtained as end-products (Fernández-Naveira et al. 2016). Only very few bacteria have been isolated so far and shown to produce butanol from carbon monoxide. *Clostridium carboxidivorans* is one such bacterium able to grow on synthesis gas, by using CO, $CO_2$, and $H_2$ to produce the liquid biofuels ethanol and butanol using a variation of the classical Wood-Ljungdahl pathway (Ukpong et al. 2012).

In the more extensively studied conventional ABE fermentation from carbohydrates, solvent toxicity is a critical problem. Under normal conditions, the clostridial cellular activity decreases significantly in the presence of 20 g/L or more solvents (Woods 1995). This is one of the most important factors to be considered in butanol fermentation as bacterial cells rarely tolerate concentrations exceeding 2 % butanol (Liu and Qureshi 2009). This should thus be taken into account in order to get a more efficient production process. Although studies have been performed on the inhibitory effect of butanol on cell growth of specific bacteria with sugars as a carbon source in the conventional ABE fermentation (Moreira et al. 1981), no data are yet available in the literature on the kinetics and the inhibition effect of solvents (butanol, ethanol) in the more recently developed clostridial butanol-ethanol (B-E) fermentation from CO-rich gases as carbon source.

In order to increase butanol production from gaseous substrates, it is necessary to know its inhibitory effect as well as the inhibitory effect of other end-product solvents (i.e. ethanol) on the B-E fermentation process. Therefore, this work focussed on evaluating the inhibitory effect of end-products of the B-E fermentation on the growth kinetics and bioconversion of CO to valuable metabolites, by *C. carboxidivorans* in batch bottle experiments.

## Material and methods

### Microorganism and culture media

*C. carboxidivorans* P7 DSM 15243 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was maintained anaerobically on modified basal medium (Liou et al. 2005; Tanner 2007) at pH 5.75 with CO (100 %) as the sole gaseous substrate.

The medium composition (per liter distilled water) was as follows: Yeast extract, 1 g; mineral solution 25 mL; trace metal solution, 10 mL; resazurin, 1 mL; cysteine-HCl, 0.60 g.

The mineral stock solution contained (per liter distilled water): 80 g sodium chloride, 100 g ammonium chloride, 10 g potassium chloride, 10 g potassium monophosphate, 20 g magnesium sulfate, and 4 g calcium chloride.

The trace metal stock solution contained (per liter distilled water) the following: 2 g nitrilotriacetic acid, 1 g manganese sulfate, 0.80 g ferrous ammonium sulfate, 0.20 g cobalt chloride, 0.20 g zinc sulfate, and 20 mg each of cupric chloride, nickel chloride, sodium molybdate, sodium selenate, and sodium tungstate.

### Bottle batch experiments

Batch experiments were carried out in order to check the inhibitory effect of ethanol, butanol, and mixtures of both ethanol and butanol.

All media used for the batch experiments were prepared with the same methods and under the same conditions. For the experiments, 10 % seed culture in the early exponential growth phase, grown with CO as sole carbon source, was aseptically inoculated into 200-mL serum vials containing 100 mL medium at pH = 5.0 ± 0.1. The bottles were maintained under anaerobic conditions. They were pressurized to 1.2 bar with 100 % CO and were agitated at 150 rpm inside an orbital incubator at 30 °C. The experimental setup and the method used for media preparation as well as sampling details are described elsewhere (Abubackar et al. 2011; Abubackar et al. 2015; Fernández-Naveira et al. 2016). All the batch experiments were carried out in duplicate, reaching statistically highly reproducible results, and some were even repeated in order to confirm data whenever needed.

Three separate experiments were carried out in order to analyze the inhibitory effect of each compound separately. Butanol was checked at the following concentrations: control (0 g/L), 1, 5, 10, 15, and 20 g/L. The effect of ethanol, which appeared to be somewhat less inhibitory, was checked at the following concentrations: control (0 g/L), 1, 5, 20, 25, and 35 g/L. Similarly, the combined inhibitory effect of both alcohols together was analyzed, using the following total final concentrations: control (0 g/L), 2, 6, 15, and 25 g/L, using

identical concentrations of each alcohol. There was only a slight difference in the initial concentrations at the highest values assayed of 15 and 25 g/L, but always below 10 %.

### Growth measurement

One milliliter liquid sample was withdrawn daily in order to avoid affecting too much the total liquid volume. Two daily samples were occasionally taken, mainly during the exponential growth phase, in order to have more data points allowing to calculate the exponential growth rate. The optical density ($OD_{\lambda = 600\ nm}$) was measured for each sample, in order to estimate the biomass concentration, using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa and Giralt, Madrid, Spain). The measured absorbance allowed to estimate the biomass concentration (mg/L) by comparing it with a previously generated calibration curve.

Growth rates, ($\mu$), expressed in hour$^{-1}$, were calculated using the following formula:

$$\mu = [\mathrm{Ln}(N_t) - \mathrm{Ln}(N_0)]/(t - t_0)$$

Where Nt is the cell density (g/L) at time t (expressed in hours) and $N_0$ is the cell density at time 0 ($t_0$).

Such maximum growth rate was estimated during the exponential growth phase based on the best slope fit to the experimental data and making sure reproducible results were obtained. Whenever needed, an additional experiment was performed to confirm reproducibility.

One of the most common parameters used in toxicity assays is the $IC_{50}$ (Leboulanger et al. 2001), i.e., the concentration of the tested substance that decreases the growth by 50 %. $IC_{50}$ values were calculated using non-linear regression analysis (four parameters sigmoidal) of transformed alcohol concentration as natural logarithm data versus percentage of growth inhibition. The regression analysis was performed using the regression Wizard software (Sigma-Plot 12.5, SPSS Inc.).

Cell yield coefficient ($Y_{X/S}$) is defined as the amount of cell mass produced per amount of substrate consumed (CO). It was estimated through the following equation:

$$Y_{x/s} = \frac{N - N_o}{S - S_o}$$

Where $N$ is the cell density at the end of the exponential growth phase (g/L), $N_0$ is the cell density at time 0, S is the final substrate concentration at the end of the exponential growth phase, and $S_0$ is the substrate concentration at time 0.

### Gas-phase CO concentrations

Gas samples of 1 mL were taken from the headspace of the bottles to monitor the CO concentrations.

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5A column (ID 0.53 mm, film thickness 50 μm). The oven temperature was initially kept constant at 50 °C, for 5 min, and then raised by 20 °C·min$^{-1}$ for 2 min, to reach a final temperature of 90 °C. The temperature of the injection port and the detector were maintained constant at 150 °C. Helium was used as the carrier gas.

### Ethanol and butanol concentrations

The concentrations of ethanol and butanol were analyzed for each bottle from liquid samples (1 mL) using an HPLC (HP1100, Agilent Co., USA) equipped with a 5 μm × 4 mm × 250 mm Hypersil ODS column and a UV detector at a wavelength of 284 nm. The mobile phase was a 0.1 % ortho-phosphoric acid solution fed at a flow rate of 0.5 ml/min. The column temperature was set at 30 °C. The samples were centrifuged (7000*g*, 3 min) using a centrifuge (ELMI Skyline Ltd. CM 70M07) before analyzing the concentration of water-soluble compounds by HPLC.

## Results

### Growth parameters of *C. carboxidivorans*

Growth parameters of *C. carboxidivorans* were estimated on pure carbon monoxide in experiments repeated in sextuplicate. The maximum specific growth rate ($\mu$) was found to reach 0.086 ± 0.004 h$^{-1}$. This value is significantly lower than for clostridial strains grown on sugars in ABE fermentation (Table 1). Each experiment corresponds to the duplicate controls of the three alcohols inhibition experiments (i.e., a total of six assays) described in the next section and summarized in Tables 2, 3, and 4. Detailed experimental data can thus be found in the next sections. Besides, the biomass yield ($Y_{X/S}$) was also estimated based on the amount substrate consumed and generated biomass and appeared to reach 0.011 g biomass per gram carbon monoxide.

### Butanol toxicity experiment

In the experiments with added butanol, *C. carboxidivorans* started growing immediately after inoculation (Fig. 1a) at all the concentrations assayed, with the exception of the bottle with the highest butanol concentration (20 g/L) in which no growth at all was observed. Butanol affected the growth of *C. carboxidivorans* and the negative effect on growth was concentration-dependent (Fig. 1a, Table 2). The control reached its maximum biomass concentration (0.135 g/L) after

**Table 1** Specific growth rates of *Clostridium carboxidivorans* and *Clostridium acetobutylicum* grown, respectively, on CO or carbohydrates (glucose, lactose)

| Carbon source | Specific growth rate ($h^{-1}$) | Microorganism | References |
|---|---|---|---|
| Carbon monoxide | 0.086 ± 0.004 | *C. carboxidivorans* | This study |
| Glucose | 0.48 | *C. acetobutylicum* | Srivastava and Volesky 1990 |
| Lactose | 0.23–0.28 | *C. acetobutylicum* | Napoli et al. 2012 |

30 h (Fig 1a), and the maximum growth rate was reached in the control bottles, in the absence of any added butanol, with a value of 0.084 $h^{-1}$ (Table 2). All butanol concentrations assayed provoked a decrease in the growth rate of that strain (Table 2). The highest concentration assayed (20 g/L) completely inhibited the bacterial growth, with a growth rate of 0 $h^{-1}$.

The $IC_{50}$ of butanol for growth was 14.50 g/L after 48 h of butanol exposure and 14.20 g/L after 72 h of exposure.

Carbon monoxide consumption was monitored during the experiment and is shown in Fig. 1b. In the control bottles and with 1 g/L of butanol, 50 % CO was already consumed after 24 h and it was totally consumed after 77 h. In the case of butanol concentrations of 5 and 10 g/L, 50 % CO consumption was reached after 28 h of butanol exposure, and total CO consumption was observed after 92 h exposure. Only in the bottles with the highest alcohol concentrations (15 and 20 g/L) was total substrate removal not possible, observing 20 % CO consumption, after 99 h of butanol exposure. This was related to the fact that in those bottles, biomass had scarcely grown and could not consume CO, whereas significant growth was found in the control bottles as well as in the presence of butanol at 1, 5, and 10 g/L, where growth took place at different rates, and reaching different final biomass concentrations. The highest final, total amount biomass was reached in the control bottle. The total maximum biomass concentration gradually decreased at increasing added butanol concentrations.

## Ethanol toxicity experiment

In case of the ethanol inhibition study, ethanol showed a negative effect on the bacterial growth of *C. carboxidivorans*, and this effect was here also concentration-dependent (Fig. 2a and Table 3) similarly as for butanol. The maximum biomass concentration was observed in the bottles with 1 g/L of ethanol (0.153 g/L) as well as in the control bottles (0.150 g/L), resulting in statistically similar values.

It is noteworthy that in bottles with an added ethanol concentration of 1 g/L, in assays performed under exactly the same conditions, and with the same preculture inoculum, a somewhat higher growth rate was found than in the control bottles, with growth rates of 0.090 and 0.082 $h^{-1}$, respectively. The study was repeated and that effect was observed again in a second experiment, where the growth rate in bottles with 1 g/L added ethanol was slightly higher than the value found in the control bottles. Although the highest ethanol concentration added (35 g/L) was higher than for butanol (20 g/L), growth was detected in all the bottles with ethanol and reached growth rates higher than 0.024 $h^{-1}$ in all cases, even in the most concentrated bottles (25 and 35 g/L) (Table 3).

The $IC_{50}$ of ethanol for growth could not be estimated with the statistical software (SigmaPlot) because the most concentrated bottle had only reached 51 % growth inhibition at the end of the experiment, suggesting a much lower inhibitory effect of ethanol compared to butanol. However, a rather accurate estimation of the $IC_{50}$ could be done based on the experimental data obtained, that way the $IC_{50}$ of ethanol for growth appeared to be very close to 35 g/L, where 51 % inhibition was found.

Carbon monoxide consumption is shown in Fig. 2b. Maximum fast CO consumption was observed after a similar time period in all the assays up to an ethanol concentration of 5 g/L. In the bottles with 20 g/L of ethanol, carbon monoxide did not disappear completely, and the maximum consumption was 84 % after 329 h of ethanol exposure. Similarly, in the

**Table 2** Batch experiments with butanol. Maximum specific growth rates in the presence of different butanol concentrations, expressed in $h^{-1}$

| Butanol concentration (g/L) | Growth rate ($h^{-1}$) |
|---|---|
| 0 | 0.084 |
| 1 | 0.076 |
| 5 | 0.043 |
| 10 | 0.026 |
| 15 | 0.019 |
| 20 | 0.000 |

**Table 3** Batch experiments with ethanol. Maximum specific growth rates in the presence of different ethanol concentrations, expressed in $h^{-1}$

| Ethanol concentration (g/L) | Growth rate ($h^{-1}$) |
|---|---|
| 0 | 0.082 |
| 1 | 0.090 |
| 5 | 0.066 |
| 20 | 0.055 |
| 25 | 0.028 |
| 35 | 0.024 |

**Table 4** Batch experiments with mixtures of alcohols. Maximum specific growth rates in the presence of different total concentrations of butanol and ethanol (1:1), expressed in $h^{-1}$

| Total concentration of alcohols (1:1 mixtures) (g/L) | Growth rate ($h^{-1}$) |
|---|---|
| Control | 0.090 |
| 2 | 0.072 |
| 7 | 0.033 |
| 15 | 0.018 |
| 25 | 0.014 |

bottles with the highest alcohol concentrations (25 and 35 g/L), total substrate consumption was not possible either, observing 42 and 39 % CO consumption, respectively, at the end of the experiment after 230 h of ethanol exposure. This was related to the fact that in those bottles, biomass had scarcely grown and could thus not consume CO, whereas in the control bottles and with 1, 5, and 20 g/L added ethanol, growth took place at different rates, and different final biomass concentrations were reached. The final total amount accumulated biomass gradually decreased at increasing ethanol concentrations.

### Toxicity experiment with mixtures of both alcohols

In this experiment, the bacteria started growing soon after inoculation (Fig. 3a). The negative effect of the mixture of ethanol and butanol on the growth of *C. carboxidivorans* is shown in Fig. 3a and in Table 4. This negative effect of the ethanol-butanol mixture is here also concentration-dependent (Fig. 3a and Table 4). The maximum biomass concentration (0.170 g/L) was reached after 48 h in the control bottle. Growth took place in all the bottles and reached growth rates higher than at least 0.014 $h^{-1}$. In the bottles with the highest alcohol concentration (25 g/L), growth started initially and

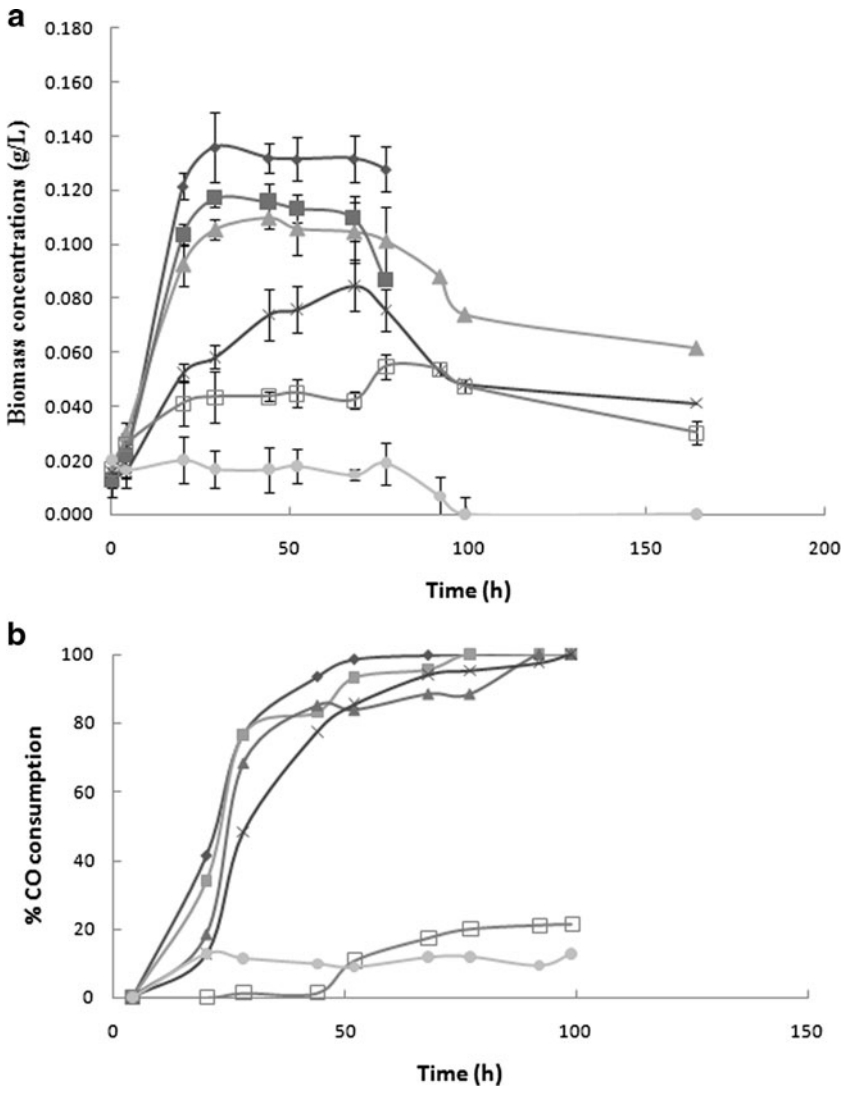


**Fig. 1** **a** Batch experiments with butanol. Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as *filled diamond*, 1 g/L butanol represented as *filled square*, 5 g/L butanol represented as *filled triangle*, 10 g/L butanol represented as X, 15 g/L butanol represented as *square*, and 20 g/L butanol represented as *filled circle*). **b** Batch experiments with butanol. Percentage CO consumption (control bottles represented as *filled diamond*, 1 g/L butanol represented as *filled square*, 5 g/L butanol represented as *filled triangle*, 10 g/L butanol represented as X, 15 g/L butanol represented as *square*, and 20 g/L butanol represented as *filled circle*)

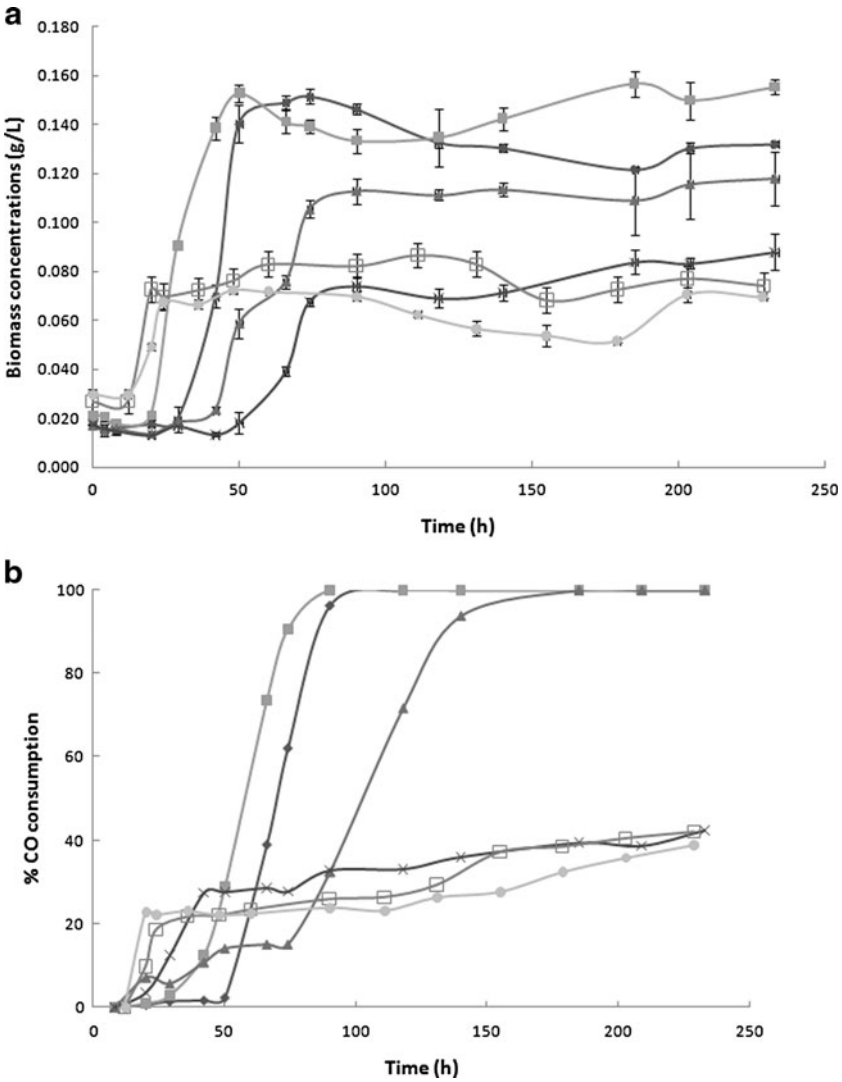


**Fig. 2** **a** Batch experiments with ethanol. Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as *filled diamond*, 1 g/L ethanol represented as *filled square*, 5 g/L ethanol represented as *filled triangle*, 20 g/L ethanol represented as X, 25 g/L ethanol represented as *square*, and 35 g/L ethanol represented as *filled circle*). **b** Batch experiments with ethanol. Percentage CO consumption (control bottles represented as *filled diamond*, 1 g/L ethanol represented as *filled square*, 5 g/L ethanol represented as *filled triangle*, 20 g/L ethanol represented as X, 25 g/L ethanol represented as *square*, and 35 g/L ethanol represented as *filled circle*)

reached a maximum biomass concentration of 0.053 g/L after 60 h of alcohols exposure. However, after that time of alcohols exposure, growth stopped and the biomass started slightly decaying.

The $IC_{50}$ for growth was 16.22 g/L after 111 h of alcohols exposure, which is similar, but slightly higher than in the assays with pure butanol, and can be explained by the lower toxicity of ethanol in the mixture of both alcohols.

Carbon monoxide consumption was monitored during all the experiment and is shown in Fig. 3b. In the control bottles, 50 % CO was consumed after 24 h and it was totally consumed after 60 h. In the case of 2 g/L, complete CO consumption was found after 130 h of alcohols exposure. In the bottles with 7 g/L, the maximum final consumption was 63 % after 60 h of alcohols exposure, and in the bottles with the highest concentrations of alcohols (15 and 25 g/L), only 30 and 22 % CO consumption were respectively observed, after 230 h of alcohols exposure.

### Comparison of inhibitory effects of alcohols

Figure 4 compares the inhibitory effect on growth rates of ethanol, butanol, and mixtures of both alcohols. It shows that pure ethanol is the least toxic to bacterial growth, followed by the mixture of alcohols and pure butanol with the highest inhibitory effects. A similar trend can be found for the inhibitory effects of the different alcohols on substrate consumption as well as $IC_{50}$ data.

## Discussion

Bioalcohols such as butanol can be produced through the conversion of lignocellulosic feedstocks into carbohydrates which are then fermented into biofuels. Alternatively, another recent approach consists in using CO-rich gases (i.e., syngas, waste gases) which can also be fermented to butanol and ethanol. Both the carbohydrate and the carbon monoxide routes use

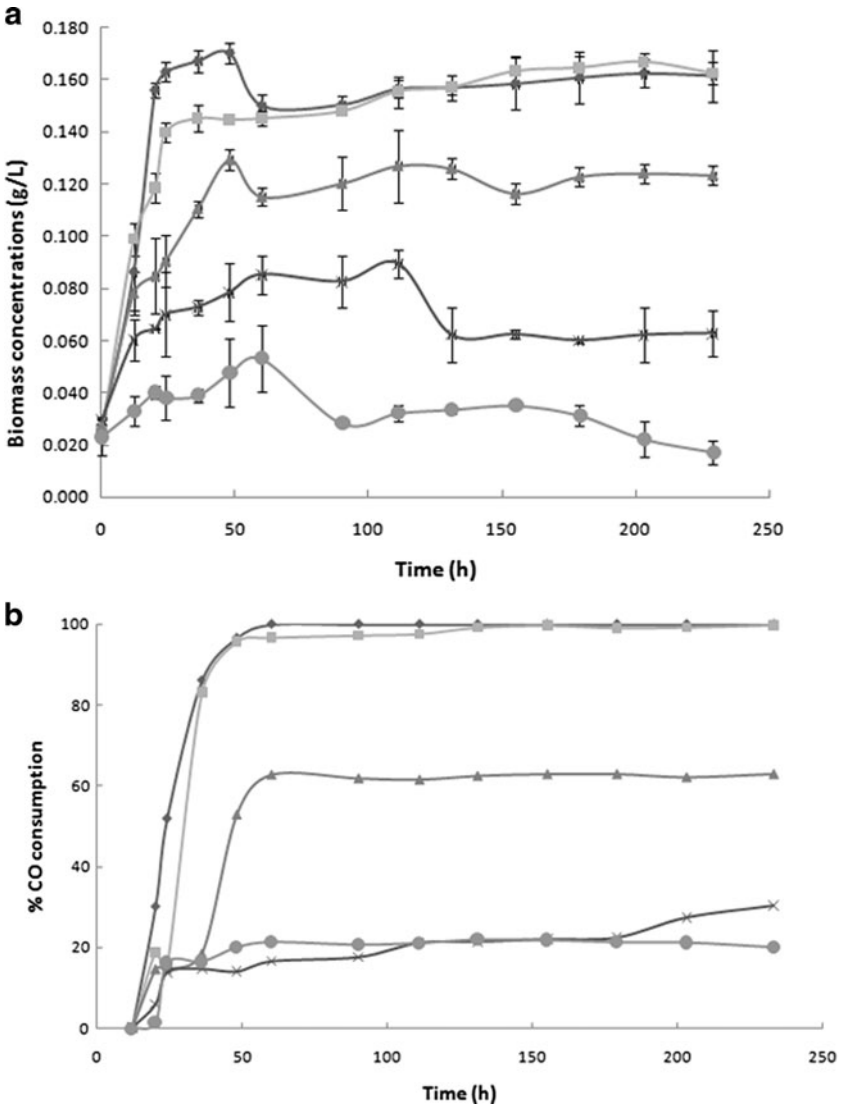


**Fig. 3** **a** Batch experiments with both butanol and ethanol (1:1, *w/w*). Measured biomass accumulation over time, expressed in g/L. Data are given as mean values ± standard deviation of the means (control bottles represented as *filled diamond*, 2 g/L represented as *filled square*, 7 g/L represented as *filled triangle*, 15 g/L represented as X, 25 g/L represented as *filled circle*). **b** Batch experiments with both butanol and ethanol (1:1, *w/w*). Percentage CO consumption (control bottles represented as *filled diamond*, 2 g/L represented as *filled square*, 7 g/L represented as *filled triangle*, 15 g/L represented as X, 25 g/L represented as *filled circle*)

clostridia or acetogens in general as biocatalysts and present each their own advantages and drawbacks (Kennes et al. 2016). The lower biomass yield and slower bacterial growth rate is a typical drawback of the syngas approach, studied in this paper, reaching specific growth rates close to hardly 0.086 $h^{-1}$, to be compared to values of 0.23–0.48 $h^{-1}$ when clostridia are grown on sugars (Table 1). Thus, growth rates appear to be about 4–5 times higher on carbohydrates than on carbon monoxide. Similarly, our data show that the biomass yield on CO is 0.011$g_{biomass}/g_{CO}$, while it reaches 0.36–0.53 $g_{biomass}/g_{carbohydrate}$ when growing clostridia on sugars such as lactose (Napoli et al. 2012). Specific strategies are thus needed to overcome the low biomass production on such gaseous substrates in continuous bioreactors for B-E fermentation, such as cell recycling.

Another aspect to be taken into account, common to both the carbohydrate and the carbon monoxide (waste gas, syngas) approach, is the potential toxicity of end-metabolites, i.e., butanol and ethanol, on growth and substrate conversion itself. Toxicity studies have been performed and reported in the literature for clostridial strains converting sugars to solvents (ABE fermentation), but, to the best of our knowledge, no previous report is available on the effect of alcohols on clostridia fermenting CO-rich gases; although, in a recent patent application, it has been shown that recombinant strains of CO-fermenting clostridia might tolerate ethanol concentrations of up to around 50 g/L (Koepke et al. 2012).

The $IC_{50}$ value of butanol obtained for *C. carboxidivorans* grown on CO in the present work (14.50 g/L), and reported here for the first time, is rather close to the values available from studies with other *Clostridium* species grown on carbohydrates. Similar results in terms of butanol inhibition were obtained by Moreira et al. (1981) using sugars as carbon source in *C. acetobutylicum*. They found that 0.10–0.15 M (7.41–11.12 g/L) butanol caused 50 % inhibition of cell growth and sugar uptake rate by negatively affecting the

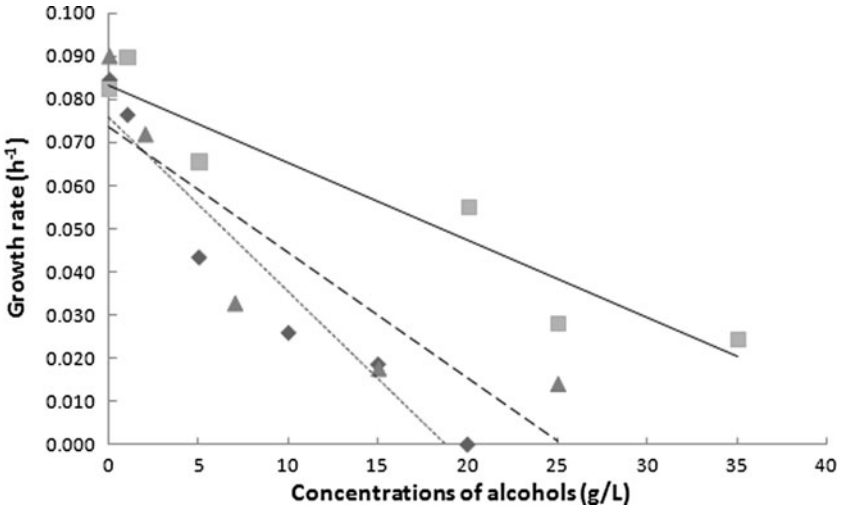


**Fig. 4** Comparison of growth rates in each experiment. Maximum specific growth rates (GR) of each treatment in the three experiments, expressed in $h^{-1}$. Butanol experiment represented as *filled diamond*, ethanol experiment represented as *filled square*, mixture of alcohols experiment represented as *filled triangle*. The lines represent the general trend of variation of the growth rates as a function of the concentration of alcohols (butanol experiment represented as *dotted line*, ethanol experiment represented as *solid line*, mixture of alcohols experiment represented as *dashed line*)

ATPase activity. A comparable effect was reported by Jones and Woods (1986), who found that 7–13 g/L butanol caused 50 % inhibition of cell growth.

The mechanism of butanol toxicity seems to be related to its hydrophobic nature, as this alcohol is a lipophilic solvent which can disrupt the phospholipid and fatty acid composition of the cell membrane causing an increase in membrane fluidity (Bowles and Ellefson 1985). This increase in the membrane fluidity would cause destabilization of the membrane and disruption of membrane functions such as transport processes, substrate (glucose) uptake, and membrane ATPase activity (Bowles and Ellefson 1985). On the other hand, Gottwald and Gottschalk (1985) also found that butanol can inhibit the ability of *C. acetobutylicum* to maintain its internal pH and abolishes the membrane pH gradient. Butanol toxicity has also been related to the autolytic degradation of the solventogenic cells in *C. acetobutylicum* P262 (Van der Westhuizen et al. 1982), and it was suggested that concentrations of butanol near the inhibitory concentration were involved in the release of autolysin during the solventogenic phase (Barber et al. 1979).

The effect of ethanol was also studied in the present work and it was shown that concentrations of 35 g/L could reduce the growth of *C. carboxidivorans* by 50 % after 200 h of ethanol exposure. That value is similar to the values obtained in the fermentation of sugars, for which it was suggested that the addition of acetone and ethanol up to 40 g/L reduced growth by 50 % (Jones and Woods 1986), and total growth inhibition appeared at concentrations of about 50–60 g/L of ethanol (Leung and Wang 1981; Costa and Moreira 1983).

In the butanol toxicity experiment with *C. carboxidivorans*, the $IC_{50}$ (14.50 g/L) was much lower the value of $IC_{50}$ observed in the ethanol toxicity experiment (35 g/L). A similar trend was observed for the growth rate. In the assay on ethanol toxicity, in the bottle with 35 g/L of ethanol (the most concentrated one), a growth rate of 0.024 $h^{-1}$ was reached, which is a value close to the one obtained in the assays with 10 g/L of butanol. These results show that ethanol has a quite weaker inhibitory effect on *C. carboxidivorans* than butanol, as more ethanol is required to observe the same toxic effect as with butanol.

In both experiments with individual alcohols, the % CO consumed was monitored. This parameter shows that the bottles with ethanol reached higher percentages of CO consumption than the bottles with butanol. The assays with the highest butanol concentrations (20 g/L) did not even reach 20 % CO consumption, whereas in the bottles with the same concentration of ethanol, as much as 83 % CO was consumed. Besides, the bottles with the highest amount ethanol (35 g/L) still reached as much as 40 % CO consumption. There was basically no growth at all of *C. carboxidivorans* in the presence of a concentration of 20 g/L butanol; that way, this concentration would thus be near the concentration of full inhibition. As described above, the effect of alcohols on the level of CO consumption could be related to the fact that butanol disrupts the membrane fluidity, so the uptake of CO is a function of the characteristics of the membrane, which can get damaged as a result of butanol toxicity (Bowles and Ellefson 1985). Also, butanol at high concentrations could be involved in the release of autolysin, with an effect on the autolytic degradation of the cell (Van der Westhuizen et al. 1982; Barber et al. 1979).

Ethanol added at low concentration (up to 1 g/L) appeared not to cause any negative effect on the cells, and a somewhat

faster biomass accumulation than in control bottles was even observed. This might be related to the fact that ethanol may favor the uptake of cholesterol or saturated fatty acids into membranes (Goldstein 1986). Goldstein (1986) suggested that the bacteria he studied could be able to uptake a higher quantity of molecules such as fatty acids or other compounds, which could favor their growth.

In the last experiment, the effect of similar amounts of both alcohols in mixtures was analyzed. In that experiment, the $IC_{50}$ was found to reach a value of 16.22 g/L of the ethanol and butanol mixture. This value is higher than the value obtained in the butanol toxicity assay, and it can most probably be related to the lower inhibitory effect of ethanol in the mixture. The growth rates in that experiment were higher than in the butanol experiment, at all the concentrations assayed. However, compared with the ethanol toxicity experiment, the growth rate was lower in the mixture than in the ethanol experiment. That difference is related to the strong toxic effect of butanol in the mixture in comparison with ethanol.

Also, differences were observed regarding the % CO consumed. A rather similar effect in terms of CO consumption was observed between the butanol experiment and the assay with mixtures of both alcohols, again as a result of the more significant inhibitory effect of butanol compared to ethanol. The bottles with the highest concentrations of alcohols only consumed 20–30 % CO in both cases, whereas in the bottles with ethanol only, the % CO consumption was always higher and exceeded at least 40 % in all cases.

It can be concluded that: (a) The alcohol with lowest inhibitory effect has the highest $IC_{50}$, meaning that butanol had the highest toxic effect on CO fermentation by *C. carboxidivorans*, followed by the 1:1 mixture of alcohols and finally ethanol; (b) small quantities of ethanol (around up to 1 g/L) have no toxic effect and seem even to exhibit a slightly positive effect on biomass growth and accumulation compared to the control cultures; (c) the alcohols produce a negative effect on the growth rate, on biomass accumulation as well as on the CO consumption rate in all the experiments (except at low ethanol concentrations).

**Acknowledgments** This research was financially supported by the Spanish Ministry of Economy and Competitiveness (MINECO) as well as European FEDER funds, through project CTM2013-45581-R.

**Compliance with ethical standards**

**Funding** This study was financed by the Spanish Ministry of Economy and Competitiveness (MINECO) and through European FEDER funds.

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal rights** This article does not contain any studies with human participants or animals performed by any of the authors.

## References

Abdehagh N, Tezel FH, Thibault J (2014) Separation techniques in butanol production: challenges and developments. Biomass Bioenerg 60:222–246. doi:10.1016/j.biombioe.2013.10.003

Abubackar HN, Veiga MC, Kennes C (2011) Biological conversion of carbon monoxide-rich syngas or waste gases to bioethanol. Biofuels Bioprod Biorefin 5:93–114. doi:10.1002/bbb.256

Abubackar HN, Veiga MC, Kennes C (2015) Carbon monoxide fermentation to ethanol by *Clostridium autoethanogenum* in a bioreactor with no accumulation of acetic acid. Bioresour Technol 186:122–127. doi:10.1016/j.biortech.2015.02.113

Balat M, Balat H (2009) Recent trends in global production and utilization of bio-ethanol fuel. Appl Energ 86(11):2273–2282. doi:10.1016/j.apenergy.2009.03.015

Barber JM, Robb FT, Webster JR, Woods DR (1979) Bacteriocin production by *Clostridium acetobutylicum* in an industrial fermentation process. Appl Environ Microbiol 37:433–437

Bellido C, Pinto ML, Coca M, González-Benito G, García-Cubero MT (2014) Acetone-butanol-ethanol (ABE) production by *Clostridium beijerinckii* from wheat straw hydrolysates: efficient use of penta and hexa carbohydrates. Bioresour Technol 167:198–205. doi:10.1016/j.biortech.2014.06.020

Bowles LK, Ellefson WL (1985) Effects of butanol on *Clostridium acetobutylicum*. Appl Environ Microbiol 50:1165–1170

Costa JM, Moreira AR (1983) Growth kinetics of the acetone-butanol fermentation. In: Blanch HW, Papoutsakis ET, Stephanopoulos G (eds) Foundations of biochemical engineering, kinetics and thermodynamics in biological systems, ACS symposium series 207. American Chemical Society, Washington DC, pp. 501–504

Dürre P (2007) Biobutanol: an attractive biofuel. Biotechnol J 2:1525–1534. doi:10.1002/biot.200700168

Fernández-Naveira Á, Abubackar HN, Veiga MC, Kennes C (2016) Efficient butanol-ethanol (B-E) production from carbon monoxide fermentation in *Clostridium carboxidivorans*. Appl Microbiol Biotechnol. doi:10.1007/s00253-015-7238-1

Goldstein DB (1986) Effect of alcohol on cellular membranes. Ann Emerg Med 15(9):1013–1018. doi:10.1016/S0196-0644(86)80120-2

Gottwald M, Gottschalk G (1985) The internal pH of *Clostridium acetobutylicum* and its effect on the shift from acid to solvent formation. Arch Microbiol 143:42–46

Gowen CM, Fong SS (2011) Applications of systems biology towards microbial fuel production. Trends Microbiol 10:516–524. doi:10.1016/j.tim.2011.07.005

Jones DT, Woods DR (1986) Acetone–butanol fermentation revisited. Microbiol Rev 50:484–524

Kennes C, Veiga MC (2013) Air pollution prevention and control: bioreactors and bioenergy. J. Wiley & Sons, Chichester, United Kingdom, 549 pp

Kennes D, Abubackar HN, Diaz M, Veiga MC, Kennes C (2016) Bioethanol production from biomass: carbohydrate *vs* syngas fermentation. J Chem Technol Biotechnol 91:304–317. doi:10.1002/jctb.4842

Koepke M, Liew F, Simpson SD (2012) Recombinant microorganisms with increased tolerance to ethanol. Patent application WO2012105853 A1

Leboulanger C, Rimet F, de Lacotte MH, Berard A (2001) Effects of atrazine and nicosulfuron on freshwater microalgae. Environ Int 26:131–135. doi:10.1016/S0160-4120(00)00100-8

Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS (2008) Fermentative butanol production by clostridia. Biotechnol Bioeng 101:209–228. doi:10.1002/bit.22003

Leung JCY, Wang DIC (1981) Production of acetone and butanol by *Clostridium acetobutylicum* in continuous culture using free cells and immobilized cells. Proc 2nd World Congr Chem Eng 1:348–352

Liou JSC, Balkwill DL, Drake GR, Tanner RS (2005) *Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of the acetogen *Clostridium scatologenes*strain SL1 as *Clostridium drakei* sp. nov. Int J Syst Evol Microbiol 55:2085–2091. doi:10.1099/ijs.0.63482-0

Liu S, Qureshi N (2009) How microbes tolerate ethanol and butanol. New Biotechnol 26:117–121. doi:10.1016/j.nbt.2009.06.984

Moreira AR, Ulmer DC, Linden JC (1981) Butanol toxicity in the butylic fermentation. Biotechnol Bioeng Symp 11:567–579

Napoli F, Olivieri G, Russo ME, Marzocchella A, Salatino P (2012) Continuous lactose fermentation by *Clostridium acetobutylicum*—assessment of energetics and product yields of the acidogenesis. Enzyme Microb Technol 50:165–172. doi:10.1016/j.enzmictec.2011.11.003

Papoutsakis ET (2008) Engineering solventogenic clostridia. Curr Opin Biotechnol 19:420–429. doi:10.1016/j.copbio.2008.08.003

Qureshi N, Meagher MM, Huang J, Hutkins RW (2001) Acetone butanol ethanol (ABE) recovery by pervaporation using silicalite–silicone composite membrane from fed-batch reactor of *Clostridium acetobutylicum*. J Membr Sci 187:93–102. doi:10.1016/S0376-7388(00)00667-0

Srivastava AK, Volesky B (1990) Updated model of the batch acetone-butanol fermentation. Biotechnol Lett 12:693–698. doi:10.1007/BF01088196

Tanner RS (2007) Cultivation of bacteria and fungi. In: Hurst CJ, Crawford RL, Garland JL, Lipson DA, Mills AL, Stetzenbach LD (eds) Manual of environmental microbiology. ASM Press, Washington D.C., pp. 69–78

Ukpong MN, Atiyeh HK, De Lorme JM, Liu K, Zhu X, Tanner RS, Wilkins MR, Stevenson BS (2012) Physiological response of *Clostridium carboxidivorans* during conversion of synthesis gas to solvents in a gas-fed bioreactor. Biotechnol Bioeng 109:2720–2728. doi:10.1002/bit.24549

Van der Westhuizen A, Jones DT, Woods DR (1982) Autolytic activity and butanol tolerance of *Clostridium acetobutylicum*. Appl Environ Microbiol 44:1277–1282

Wallner T, Miers SA, McConnell S (2009) A comparison of ethanol and butanol as oxygenates using a direct-injection, spark-ignition engine. J Eng Gas Turbines Power 131:032802–1–032802-9. doi:10.1115/1.3043810

Woods DR (1995) The genetic engineering of microbial solvent production. Trends Biotechnol 13:259–264. doi:10.1016/S0167-7799(00)88960-X