Effect of ethanol and butanol on autotrophic growth of model homoacetogens

Sara Ramió-Pujol[1,2], Ramon Ganigué[3], Lluís Bañeras[1*], and Jesús Colprim[2]

[1]Group of Molecular Microbial Ecology, Institute of Aquatic Ecology (IEA), University of Girona, Campus Montilivi, E-17003 Girona, Catalonia, Spain

[2]LEQUIA, Institute of the Environment, University of Girona, Campus Montilivi, E-17003 Girona, Catalonia, Spain

[3]Centerfor Microbiology Ecology and Technology (CMET), Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

Corresponding author: LluísBañeras,

Campus Montilivi,

E-17003 Girona, Spain

Tel:+34972418177

Email address: lluis.banyeras@udg.edu

ABSTRACT

Research efforts aimed at increasing ethanol and butanol productivity from syngas are currently gaining attention. For most model carboxydotrophic bacteria, production rates, yields and maximum product titers have been studied in detail, but little is known on alcohol toxicity in these bacteria. The aim of this work was to investigate the inhibitory effects of ethanol and butanol on the growth of *Clostridium ljungdahlii* PETC, *Clostridium carboxidivorans* P7, and "Butyribacterium methylotrophicum DSM3468". Experiments to determine inhibitory effects due to product accumulation were carried out using a synthetic mixture of $CO:CO_2:H_2$ as a substrate. These conditions were chosen to mimic gaseous effluents of biomass and waste gasification plants. Inhibition effects were recorded as changes in growth parameters. No significant inhibition was observed for ethanol at concentrations below 15 g/L. The three species exhibited higher sensitivity to butanol. Half inhibition constants for butanol could be estimated for P7 (IC_{50}=4.12 g/L), DSM3468 (IC_{50}=1.79 g/L), and PETC (IC_{50}=9.75 g/L). In conclusion, at least for the tested strains, there is no urgent need to overcome alcohol inhibition in eventual industrial production of alcohols from syngas fermentation.

KEYWORDS

Alcohol toxicity, syngas, Clostridia, CO_2 utilisation, IC_{50}

The gasification of wastes coupled to synthesis gas (syngas) fermentation is a promising alternative approach to alcohol production from less valuable feedstocks (agricultural and forestry biomass, and municipal solid wastes) (McKendry 2002; Mohammadi *et al.* 2011). The conversion step in the process must be catalysed by specific autotrophic bacteria that convert carbon (basically CO and CO_2) into added-value products through the Wood-Ljungdahl pathway (Ragsdale and Pierce 2008). Alcohols and organic acids are excreted as end products, generally obeying at differences in the metabolic balance and thermodynamics of the cell during growth (Richter *et al.* 2016). Recently, it has been also reported that longer chain alcohols can be produced in mixed cultures by chain elongation and subsequent reduction of acids to alcohols (Ganigué *et al.* 2016).

Major end products of carboxydotrophs fermentation, i.e. acids and alcohols, show a potential inhibitory effect on bacterial growth and production (Baer, Blaschek and Smith 1987; Mohammadi *et al.* 2014; Ramió-Pujol *et al.* 2015a). Product toxicity has been extensively studied for acetone-butanol-ethanol (ABE) fermenters, and it has been reported that their metabolic activity significantly decreases when the concentration of a mix of solvents reaches levels of around 20 g/L (Jones and Woods 1986). However, fewer studies exist for homoacetogens, specially related to alcohol toxicity. It has been reported that *Clostridium carboxidivorans* and *Clostridium ljungdahlii* can grow at low concentration of ethanol (usually below 5 g/L) (Tanner, Miller and Yang 1993; Liou *et al.* 2005), but toxicity at industrially-relevant concentrations has not been studied in detail for most strains. Fernández-Naveira and co-workers reported ethanol and butanol toxicity on *C. carboxidivorans* P7 grown on 100% CO, and confirmed thatalcohol toxicity levels for this strain were similar to those reported for ABE fermenters (Fernández-Naveira *et al.* 2016). In this work we aimed at broadening the knowledge of alcohol toxicity and investigated the inhibitory effects of ethanol and butanol on the growth of two additional carboxydotrophic bacteria -*C. ljungdahlii* PETC (DSM13528^T) and "Butyribacterium methylotrophicum" (DSM3468) - using a mixture of ($CO:CO_2:H_2$) as a substrate. The tolerance of PETC to increasing concentrations of butanol was tested despite not being a butanol producer (Köpke *et al.* 2010;

Bengelsdorf, Straub and Dürre 2013). This was done because genetically modified strains designed for butanol production, engineered for active crotonase and butyryl-CoA dehydrogenase, have been obtained (Köpke *et al.* 2010). DSM3468 was chosen as a non-clostridial acetogen capable of producing C2 and C4 both alcohols and organic acids. The selected concentrations ranges were based on maximum concentrations reported in the literature for the studied strains: *Clostridium ljungdahlii* was able to produce up to 23.0 g of ethanol/L (Phillips *et al.* 1993), *Clostridium carboxidivorans* produced up to 3.3 g of ethanol/L and up to 1.9 g of butanol/L (Ramió-Pujol *et al.* 2015a; Fernández-Naveira *et al.* 2017), and "Butyribacterium methylotrophicum" presented lower production of ethanol but reach values of 2.7 g of butanol/L (Grethlein *et al.* 1991).

Fermentation experiments were conducted in tubes containing 6 mL of modified ATCC 1754PETC medium (Ramió-Pujol *et al.* 2015b) adjusted to pH 5.8 and buffered using 2-(N-morpholino) ethanesulfonic acid (MES). Ethanol and butanol was added at final concentrations of: 2, 5, 8, and 15 g/L. Controls with no-added alcohols were also included. Early-exponential growing cultures of PETC and DSM3468 were inoculated (3.3 % ratio) into the test tubes containing increasing ethanol and butanol concentrations. The use of an inoculum in the early exponential phase also minimized the presence of residual alcohols in the inoculated broth. Tubes were thoroughly flushed with a high purity syngas mixture ($CO:H_2:N_2:CO_2$ [32:32:28:8]) (Praxair Technology Ltd, Spain) every 24 hours. Headspace overpressure was set at 100 kPa. Tubes were incubated horizontally to enhance gas-liquid mass transfer on a rotatory shaker at 100 rpm and 25 ºC. Growth was monitored on a daily basis by measuring the absorbance at 600 nm using a CE1021 spectrophotometer (CECIL, Cambridge, UK) and linear regression of natural logarithm transformed absorbance readings at time intervals from 21 to 69 hours, beginning of the exponential growth, were used to calculate growth rates (μ, h^{-1}). A control experiment in order to calculate the loss of alcohols due to its volatility showed only minimal losses (≤2%) of ethanol or butanol.

PETC and DSM3468 grew under all tested ethanol concentrations and inhibitory effects were minimal (Figure 1A and 1B). PETC biomass at the end of the growth phase was positively affected by the presence of ethanol, while DSM3468 remained unaffected. Significant reduction of PETC growth was observed at concentrations above 5 g butanol/L (Figure 1C). Butanol addition caused a significant decrease on maximum cell biomass of DSM3468 cultures at concentrations higher than 8 g/L (Figure

1D). None of the tested bacteria grew at a concentration of 15 g/L of butanol. In all tested ethanol and butanol concentrations, pH remained stable (5.8±0.2) through all the experiment.

The inhibitory effect of ethanol and butanol was investigated by evaluating the impact on the specific growth rate (μ) in the early exponential growth phase. Since both medium composition and incubation conditions varied from those used in a previous study (Fernández-Naveira *et al.* 2016), additional experiments with *C. carboxidivorans* P7 using the same experimental conditions defined here were also included for comparison. A variation of the Monod expression including a product inhibition term (Eq. 1) was used as an inhibition model to describe the potential effect of alcohols over growth rate, and allowed the estimation of kinetic inhibition parameters:

$$\mu = \mu_{max}[\Pi(1-(C_p/C_{pi})^{ni})] \quad (1)$$

In Equation 1, C_{pi} is defined as the critical concentration of the inhibitor above which growth is completely inhibited (μ=0). *ni* is a constant coefficient which shows the extent of the inhibition effect, that is the concentration range at which inhibition remains at undetectable levels. μ_{max} is the maximum specific growth rate (calculated at zero alcohol concentration with the given incubation conditions for each strain), and C_p is the concentration of the inhibitor (Zeng and Deckwer 1991; Yang and Tsao 1994; Lin *et al.* 2008). The kinetic parameters of Eq. 1 were estimated using SigmaPlot 11.0 (Systat Software, San Jose, California, USA). In addition, half inhibitory constants (IC_{50}) were defined as the concentration of alcohol at which $\mu=\mu_{max}/2$, and calculated using the parameters showing the best fit to the experimental data.

Growth rate of PETC and P7 decreased almost linearly with increasing ethanol concentrations, although complete inhibition was not observed within the concentration range used here (Figure 2). Growth rate of PETC decreased by a 35.3% (0.024 h^{-1} to 0.016 h^{-1}) at the highest concentrations of ethanol tested. Lesser effects were observed for DSM3468 in which growth rate was not negatively influenced by ethanol (0.013 h^{-1} to 0.014 h^{-1} at 15 g/L). Consequently, C_{pi} for the three strains are over 15 g ethanol/L (Table 1). The narrow concentration range assayed in the present work and the low inhibition effect of ethanol prevented the estimation of reliable IC_{50} values for the three strains. Low ethanol inhibition effects have been previously proven, at least for *C. carboxidivorans* P7 (Fernández-Naveira *et al.* 2016). Taking into

account previously published data, estimations of C_{pi} values for ethanol would be higher than 35 g/L, far above the maximum obtained concentrations using syngas fermentation (Fernández-Naveira *et al.* 2016). The *ni* values for ethanol could be estimated for P7, being 0.659 and 0.926 for the data set obtained here and the one published in Fernandez-Naveira's et al work, respectively (Fernández-Naveira *et al.* 2016).

PETC, DSM3468, and P7 showed no growth at 15 g butanol/L. C_{pi} values were 17.44, 14.98, and 13.92 g butanol/L for PETC, DSM3468, and P7, while IC_{50} values were estimated as 9.75, 1.79, and 4.12 g butanol/L, respectively. PETC, which is not a natural butanol producer, showed a higher tolerance to butanol compared to DSM3468 and P7. The *ni* values estimated for butanol were >1 for PETC and <1 for DSM3468 and P7. This indicates a significant effect of alcohol at rather low concentrations for the latter strain and suggests significant differences in detoxification capabilities of the three strains.

Values of IC_{50} calculated in this study for P7 differed to those calculated using CO as the sole carbon source in the medium (IC_{50}: 6.36 g butanol/L, (Fernández-Naveira *et al.* 2016)). Besides, the addition of hydrogen in the medium (incubation conditions in the present study) seemed to decrease the maximum concentrations of butanol at which growth of P7 can proceed (C_{pi}:20.4 g butanol/L in CO fed cultures compared to 13.9 g butanol/L in CO:CO_2:H_2 fed cultures). Recently, Pomaranski and co-workers showed that "B. methyolotrophicum" ATCC33266 (DSM3468) incubated at 37 ºC and under an atmosphere of 100% CO can grow even in the presence of 30 g butanol/L (Pomaranski and Tiquia-Arashiro 2016). This contrasts with the observations of the present work, and is likely due to a higher sensitivity of DSM3468 to incubation conditions. The results found suggest that incubation conditions (most likely reducing agent sources) may have a significant effect on alcohol toxicity of these two strains although more experiments are still needed to know the extent of this effect, especially during solventogenesis.

The *Cpi* values of ethanol and butanol reported in this study for the three homoacetogens are in line with the threshold toxicity of ABE fermenters. Huffer et al. reported that *Clostridium beijerinckii* can grow in the presence of 45 g ethanol/L, but only tolerates butanol at concentrations up to 12 g /L (Huffer *et al.* 2011). One of the species reported with a significant tolerance to ethanol is *Zymomonas mobilis*, which can grow at concentrations up to 65 g/L (Huffer *et al.* 2011). However, its tolerance to butanol is much lower, < 12 g/L of butanol. Despite higher ethanol tolerance compared to butanol, common in bacteria due to inherent properties and effects of the two alcohols on cellular membranes, bacterial strains

showing a reversed effect also have been described, e.g. *Thermoanaerobacterium saccharolyticum*, revealing specific detoxification activities for butanol (Huffer *et al.* 2011).

The results of the present work point out that homoacetogens have tolerances to ethanol and butanol which are comparable to those found in ABE fermenters. In view of the inhibition ranges calculated here and the actual alcohol production titers from syngas fermentation, we may conclude that alcohol toxicity is not an immediate handicap for increasing alcohol production in homoacetogenic strains. However, the effects of medium composition and incubation conditions in alcohol toxicity may need further investigation.

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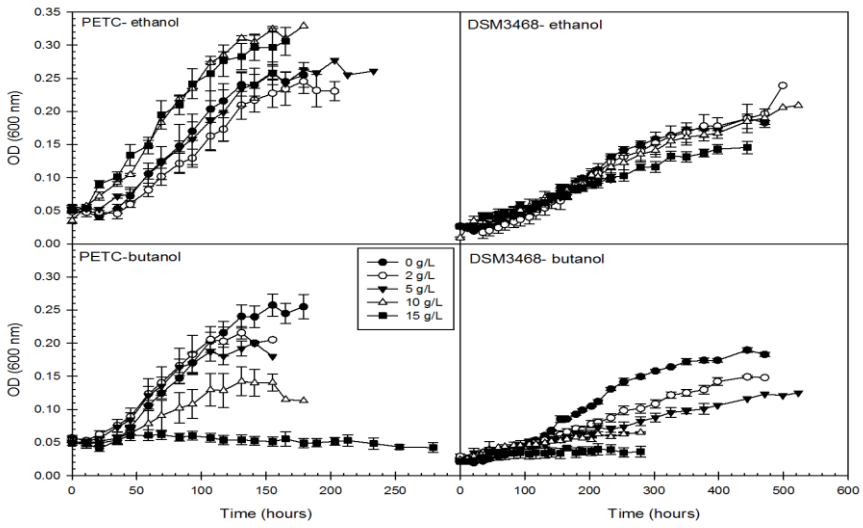

Figure 1: Time variation of optical density at 600 nm (mean values and SD, n=3) of *C. ljungdahlii* PETC and "B. methylotrophicum" DSM3468 at different initial concentrations of ethanol and butanol. Note differences in the time axis for the two strains.

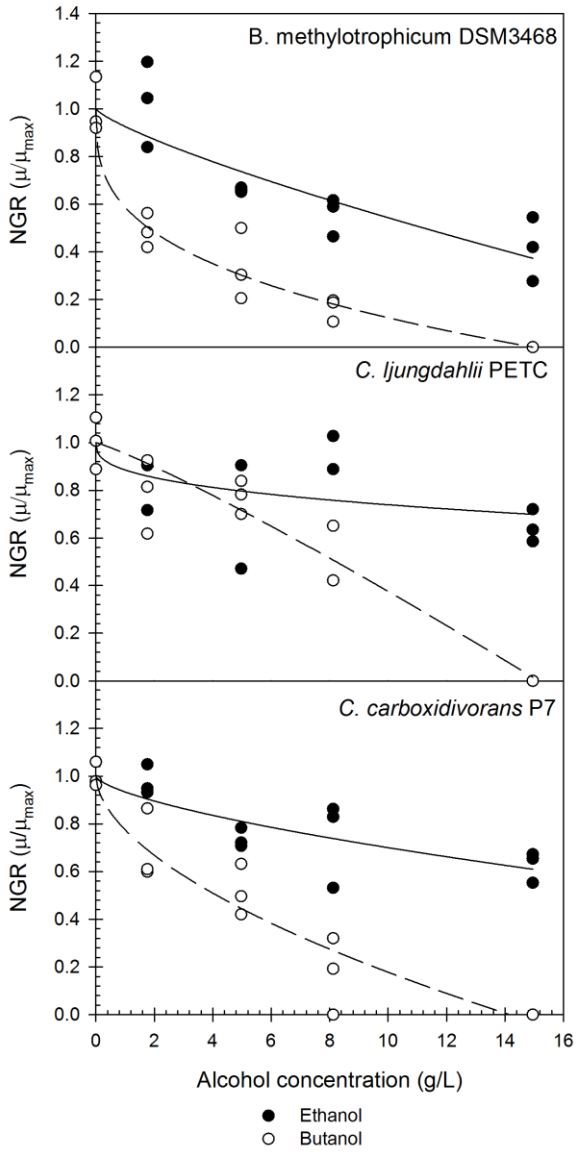

Figure 2: Changes of normalized growth rate (μ/μ_{max}) according to initial ethanol (filled dots) and butanol (empty dots) concentration for *C. carboxidivorans*P7, *C. ljungdahlii* PETC, and "B. methylotrophicum" DSM3468. Straight lines show best fit to the Monod based inhibition model (Eq. 1, see text for details).

Table 1. Estimated cultures growth parameters with standard errors in brackets for ethanol and butanol incubation experiments of *C.ljungdahlii* PETC, *C. carboxidivorans* P7 and "B. methylotrophicum DSM3468".

		Ethanol			**Butanol**		
	μ_{max} (h^{-1})	**IC_{50} (g/L)**	**C_{pi}[b] (g/L)**	**ni**	**IC_{50} (g/L)**	**C_{pi}[b] (g/L)**	**ni**
P7[a]	0.084 (±0.005)	**na**	>35.00	0.926 (±0.508)	6.36	20.44 (±1.91)	0.594 (±0.115)
P7	0.037 (±0.004)	**na**	>15.00	0.659 (±0.368)	4.12	13.92 (±2.22)	0.569 (±0.167)
PETC	0.024 (±0.002)	**na**	>15.00	**ns**	9.75	17.44 (±2.19)	1.192 (±0.416)
DSM3468	0.013 (±0.004)	**na**	>15.00	0.789 (±0.207)	1.79	14.98 (±1.96)	0.327 (±0.048)

a: Recalculated data from (Fernández-Naveira *et al.* 2016); **b**: critical concentration; **ni**: constant coefficient; **μ_{max}:** maximum specific growth rate calculated at zero alcohol concentration with given incubation conditions for each strain; **ns**: estimated parameters not relevant, r^2 below 0.600; **na**: not applicable.