Bioresource Technology 152 (2014) 337–346

Contents lists available at ScienceDirect

Bioresource Technology

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

Mixed culture syngas fermentation and conversion of carboxylic acids into alcohols

Kan Liu [a], Hasan K. Atiyeh [a,*], Bradley S. Stevenson [b], Ralph S. Tanner [b], Mark R. Wilkins [a], Raymond L. Huhnke [a]

[a] Department of Biosystems and Agricultural Engineering, Oklahoma State University, Stillwater, OK 74078, USA
[b] Microbiology and Plant Biology, University of Oklahoma, 770 Van Vleet Oval, Norman, OK 73019, USA

HIGHLIGHTS

- Higher alcohols production using mixed culture from syngas was investigated.
- Ethanol, *n*-propanol, *n*-butanol were produced by a mixed culture.
- CP15 or mixed culture did convert carboxylic acids to respective alcohols.
- There was an increased productivity by the CP15 and *Clostridium propionicum* mixed culture.
- Mixed culture converted 50% more acids to alcohols than CP15 alone.

ARTICLE INFO

Article history:
Received 15 August 2013
Received in revised form 3 November 2013
Accepted 7 November 2013
Available online 14 November 2013

Keywords:
Mixed culture syngas fermentation
Drop-in biofuel
n-Propanol
n-Butanol
n-Hexanol

ABSTRACT

Higher alcohols such as *n*-butanol and *n*-hexanol have higher energy density than ethanol, are more compatible with current fuel infrastructure, and can be upgraded to jet and diesel fuels. Several organisms are known to convert syngas to ethanol, but very few can produce higher alcohols alone. As a potential solution, mixed culture fermentation between the syngas fermenting *Alkalibaculum bacchi* strain CP15 and propionic acid producer *Clostridium propionicum* was studied. The monoculture of CP15 produced only ethanol from syngas without initial addition of organic acids to the fermentation medium. However, the mixed culture produced ethanol, *n*-propanol and *n*-butanol from syngas. The addition of propionic acid, butyric acid and hexanoic acid to the mixed culture resulted in a 50% higher conversion efficiency of these acids to their respective alcohols compared to CP15 monoculture. These findings illustrate the great potential of mixed culture syngas fermentation in production of higher alcohols.

1. Introduction

Efforts to develop renewable energy are driven by the negative impacts of satiating an ever-increasing global consumption of energy with the burning of fossil fuels (Atsumi et al., 2008). The costs associated with transitioning from fossil to renewable transportation fuels will be minimized if major changes in existing infrastructures can be avoided. Ethanol is a renewable transportation fuel (i.e. biofuel) that can be directly blended with gasoline. Ethanol, however, is hygroscopic and has corrosive characteristics that translate into increased transportation costs because it must be transported mainly by trucks instead of existing gasoline pipelines (Tyner, 2010). Higher alcohols such as *n*-butanol and *n*-hexanol are candidates to replace ethanol due to their higher energy density and lower water solubility than ethanol. *n*-Butanol is less hygroscopic than ethanol and it has a 29% higher volumetric energy density than ethanol (ethanol 21 MJ/L vs *n*-butanol 27 MJ/L) (Mann et al., 2006; Atsumi and Liao, 2008). *n*-Propanol is an important chemical for ink, polymer and pharmaceutical industries (Demirer and Speece, 1998). *n*-Propanol has also been considered as a candidate for the replacement of gasoline (Simmons, 2011). *n*-Hexanol has low miscibility with water and less volatility than ethanol or *n*-butanol and can be blended with biodiesel or gasoline (Yeung and Thomson, 2013). Thus, these higher alcohols have more potential than ethanol as "drop-in" biofuels, and they also can be converted to jet fuels and chemicals (Harvey and Meylemans, 2011).

The hybrid gasification-syngas fermentation technology for the production of fuels and chemicals is on the verge of commercialization. In this process, syngas is produced by gasification of

* Corresponding author. Address: Department of Biosystems and Agricultural Engineering, 214 Ag Hall, Oklahoma State University, Stillwater, OK 74078, USA. Tel.: +1 405 744 8397; fax: +1 405 744 6059.
E-mail address: hasan.atiyeh@okstate.edu (H.K. Atiyeh).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.11.015

biomass or municipal solid waste followed by conversion of syngas components (mainly CO, H_2 and CO_2) to liquid fuels and chemicals (Wilkins and Atiyeh, 2011). Several reports have been published on the production of ethanol and higher alcohols such as *n*-butanol using syngas fermentation by *Clostridium carboxidivorans*, "*Clostridium ragsdalei*", and *Eubacterium limosum* ("*Butyribacterium methylotrophicum*") (Shen et al., 1999; Tanner, 2008; Maddipati et al., 2011; Ukpong et al., 2012; Ramachandriya et al., 2013). "*C. ragsdalei*" also converted acetone to isopropanol during syngas fermentation (Ramachandriya et al., 2011).

Previous studies of syngas fermentation for the production of liquid fuels have been focused on the use of monocultures. Mixed cultures of sulfate reducing bacteria, methanogenic archaea and homoacetogenic bacteria using H_2/CO_2 or CO have been reported to produce methane during anaerobic digestion of sludge (Esposito et al., 2003; Sipma et al., 2004). In another study, a mixed culture containing *Rhodospirillum rubrum*, *Methanobacterium formicicum* and *Methanosarcina barkeri* was reported to convert CO, CO_2 and H_2 to methane via synergy among the three bacteria (Klasson et al., 1990).

Alkalibaculum bacchi strain CP15 is capable of producing ethanol at a yield that is over 43% higher than *A. bacchi* strains CP11^T and CP13 (Liu et al., 2012). It was found that the cost of the syngas fermentation medium for strain CP15 can be reduced by over 27% by removing [N-*tris* (hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS buffer) as well as replacing the yeast extract (YE), minerals and vitamins with corn steep liquor (CSL) (Liu et al., 2014). 78% more ethanol was produced in 20 g/L CSL medium than in the YE medium using strain CP15, indicating the potential of CSL use as a cost-effective nutrient for large scale fermentation. Additionally, strain CP15 can grow to a high cell mass during continuous syngas fermentation with cell recycling (Liu et al., 2014). During this continuous syngas fermentation in CSL medium, *n*-propanol and *n*-butanol production was observed, which has not been reported previously for strain CP15 (Allen et al., 2010; Liu et al., 2012). These alcohols were produced from a serendipitous mixed culture formed at the late stages of a continuous fermentation as confirmed by 16S rRNA gene sequencing. The mixed culture consisted largely of *A. bacchi* CP15 (56%) and *Clostridium propionicum* (34%), with the remaining 10% made up of 4 other *Clostridium* species (Liu et al., 2014).

C. propionicum is known to consume amino acids and ferments lactate via the acrylate-CoA pathway, converting lactate into propionate and acetate (Cardon and Barker, 1946; Tholozan et al., 1992). *C. propionicum* was reported to not consume carbohydrates (Cardon and Barker, 1946; O'Brien et al., 1990). There have been no previous reports of *n*-propanol production during syngas fermentation via mixed culture. This study investigated the production of ethanol, *n*-propanol and *n*-butanol during syngas fermentation using the mixed culture in YE and CSL media in a 3 L fermentor. The ability of a monoculture of *A. bacchi* strain CP15 and a mixed culture of mainly *A. bacchi* CP15 and *C. propionicum* to convert carboxylic acids into their corresponding alcohols was also examined.

2. Methods

2.1. Microorganisms

The mixed culture was obtained from the fermentor at the end of the continuous syngas fermentation as reported previously (Liu et al., 2014). The mixed culture was transferred to yeast extract (YE) medium and maintained under syngas (40% CO, 30% CO_2 and 30% H_2) and used as the inoculum source for all experiments performed in the present study. The monoculture of *A. bacchi* strain CP15 and mixed culture were maintained on YE medium with an initial pH 8.0 under anaerobic condition at 37 °C. The YE medium preparation was described previously (Liu et al., 2012). Inocula of strain CP15 and the mixed culture were prepared by sub-culturing twice to reduce the lag phase of growth. The inocula of the mixed culture were from the same mother culture and were prepared in YE medium and incubated at 37 °C. Syngas made of 40% CO, 30% CO_2 and 30% H_2 was used. This was done to ensure consistency in the initial composition of the mixed culture used in all experiments. Fermentations with either strain CP15 or the mixed culture were inoculated with 10% (v/v) inocula.

2.2. Semi-continuous fermentation in a 3 L fermentor using mixed culture

A 3 L fermentor (Bioflo 110, New Brunswick Scientific Co., Edison, NJ, USA) with 2.5 L working volume was used in a semi-continuous fermentation (i.e., only continuous syngas feed). Two six-blade Rushton impellers separated by a distance equal to the impeller diameter were mounted on an agitator shaft as suggested by Bakker et al. (1994). Four baffles were used to avoid vortices.YE medium and 20 g/L CSL medium without TAPS buffer were used. The YE medium also contained YE, minerals, vitamins and trace metals as descried previously (Liu et al., 2012). The 20 g/L CSL was used to replace YE, vitamins and minerals in the YE medium. All media contained 5 g/L $NaHCO_3$ as a buffer, 2.5 mL/L of 4% cysteine sulfide solution as a reducing agent and 1 mL/L of 0.1% resazurin solution as a redox indicator. The compositions of the minerals, trace metals and vitamins stock solutions were reported previously (Tanner, 2007). The medium in the fermentor was sterilized at 121 °C for 30 min and allowed to cool to room temperature by purging 18 sccm (standard cubic centimeter per minute) N_2 at 150 rpm agitation for 3 h. The medium was then purged for 8 h with 18 sccm syngas (38% CO, 28.5% H_2, 28.5% CO_2 and 5% N_2 by volume, Stillwater Steel Co., Stillwater, OK, USA). Before inoculation, the medium was reduced by adding 2.5 mL/L 4% cysteine sulfide. The fermentor was inoculated with 10% (v/v) of the mixed culture. The inlet gas flow rate was controlled by a thermal mass flow controller (Porter, Hatfield, PA, USA). The pH of the medium during the fermentation was controlled above 6.1 by the addition of 7% $NaHCO_3$ based on preliminary results, which showed a substantial decrease in H_2 conversion at lower pH values. When foam in the fermentor was 1.3 cm above the level of liquid, 0.2 mL of 5% antifoam (Antifoam B emulsion, Sigma–Aldrich, St. Louis, MO, USA) was added. The fermentation temperature was controlled at 37 °C via a heating jacket (New Brunswick Scientific Co.). A condenser and a bubbler controlled at 5 °C by a refrigerated recirculator (1156D, VWR International, West Chester, PA, USA) were used to condense the vapor leaving the exhaust gas line. Liquid and gas samples were withdrawn from the reactor periodically to measure pH, cell mass and product concentrations, and gas composition in the exhaust gas line.

2.3. Conversion of carboxylic acids into alcohols in bottle fermentations

Fed-batch fermentations in 250 mL bottles (Wheaton, NJ, USA) with 100 mL working volume were used in the conversion of carboxylic acids into alcohols. The fermentation medium used was the YE medium. The carboxylic acids used included propionic acid, butyric acid, hexanoic acid and lactic acid. Each carboxylic acid was added separately to the medium at the beginning of fermentation at an initial concentration around 1.5 g/L. A control treatment contained all medium components but no carboxylic acid. The initial pH of the medium was adjusted to pH 7.5 by adding sterilized 2 N KOH, and each bottle was inoculated with 10% (v/v) of either strain CP15 or the mixed culture. The syngas used contained 40%

CO, 30% CO_2 and 30% H_2 by volume (Stillwater Steel Co.). The syngas was fed into all bottles every 24 h at 239 kPa, and the bottles were incubated at 37 °C and 150 rpm on an orbital shaker (Innova 2100, New Brunswick Scientific). In a related experiment, lactic acid was added to the YE medium, headspace in the bottle was pressurized with 100% N_2 at 121 kPa, and the medium was inoculated with the mixed culture. The purpose of this experiment was to determine if the mixed culture could produce *n*-propanol from lactic acid under N_2 headspace. Liquid and gas samples were withdrawn to measure pH, cell mass concentration, carboxylic acid and product concentrations, and gas compositions in the headspace. All fermentations were done in triplicate.

2.4. Analytical procedures

2.4.1. Cell mass, acid and solvent concentrations and gas analysis

Cell mass concentration measurements were made at 660 nm as reported previously (Liu et al., 2012). Each liquid sample (0.7 mL) was acidified using 0.7 mL 0.1 N HCl. Ethanol, *n*-propanol, *n*-butanol, *n*-hexanol, acetic acid, propionic acid, butyric acid and hexanoic acid concentrations were analyzed using gas chromatography (GC) (Agilent 7890N GC, Agilent Technologies, Wilmington, DE, USA) with a flame ionization detector (FID) and DB-FFAP capillary column (Agilent Technologies). H_2 was used as the carrier gas at an initial flow rate of 2.26 mL/min for 10 min. The inlet port temperature was kept at 225 °C with a split ratio 20:1. The initial oven temperature was set at 90 °C and held for 2 min, and then ramped at a rate of 40 °C/min to 250 °C with 1 min holding time. The FID temperature was set at 300 °C with H_2 and air flow rates of 30 mL/min and 400 mL/min, respectively.

A volume of 100 μL of gas from 250 mL bottle headspace or the 3 L fermentor's exhaust line was injected in an Agilent 6890N GC (Agilent Technologies) to determine gas composition in the headspace. Argon was used as the carrier gas with an initial gas flow rate of 2 mL/min and holding time of 2 min. The flow rate of the carrier gas was then increased to 4 mL/min at a ramping rate of 2.5 mL/min^2. The inlet port temperature was set at 200 °C with a split ratio 30:1. The initial oven temperature was 80 °C with a holding time of 5.5 min, and the TCD temperature was 230 °C.

The sugar content of the liquid samples in the CSL medium was measured using high pressure liquid chromatography (HPLC) (Agilent 1200 series) with a refractive index detector (RID). A Rezex™ RPM-Monosaccharide column (Phenomenex, Torrance, CA, USA) was used and operated at 80 °C with deionized water as the mobile phase pumped at 0.6 mL/min for 25 min per sample. Lactic acid concentration in the CSL medium in the 3 L fermentor or in bottle fermentations was determined using HPLC (Agilent 1200 series) with RID and BioRad HPX-87H column (BioRad Corporate, Hercules, CA, USA). The column temperature was 60 °C using 0.01 N H_2SO_4 as the mobile phase and pumped at 0.6 mL/min for 40 min per sample.

2.4.2. Statistical analysis and calculation of kinetic parameters

Duncan's multiple range test was analyzed by GLM procedure using SAS Release 9.3 (Cary, NC, USA) at 95% confidence level to determine pairwise statistical differences of ethanol concentration, cell mass concentration, gas utilizations and total alcohols production in bottle study among all treatments using either the strain CP15 alone or the mixed culture. Each TTEST was performed using SAS Release 9.3 to identify any significant differences ($\geqslant$95% confidence) in cell growth, carboxylic acid conversion, ethanol production, total alcohol production or gas utilization between strain CP15 and the mixed culture. The estimation of cell mass yield, gas utilization and ethanol yield were reported in previously (Liu et al., 2012).The percentage conversion of ethanol to *n*-propanol in % (mol/mol) was calculated using Eq. (1)

$$\text{EtOH converted to PrOH} = \frac{\text{PrOH produced from consumed EtOH, mol}}{\text{EtOH consumed, mol}} \times 100\% \quad (1)$$

Carboxylic acids (propionic acid, butyric acid, hexanoic acid or lactic acid) conversions to corresponding alcohols % (mol/mol) were calculated when the concentration of the alcohol reached a maximum using Eq. (2)

$$\text{Acid converted to alcohol} = \frac{\text{Maximum corresponding alcohol produced, mol}}{\text{Initial amount of carboxylic acid, mol}} \times 100\% \quad (2)$$

The carbon balance was calculated based on the percentage of total carbon produced divided by total carbon consumed. The total produced carbon included that from produced ethanol, *n*-propanol, *n*-butanol, *n*-hexanol, acetic acid, propionic acid, butyric acid and net CO_2. The net CO_2 was estimated as the difference between CO_2 provided in the feed gas and CO_2 remained in the headspace or left the fermentor. CO_2 content in the gas mixture was measured using the GC method discussed in the previous section. The total consumed carbon included that from consumed CO, sugars and lactic acid from CSL and the carboxylic acids added into the medium.

3. Results and discussion

3.1. Semi-continuous fermentation in a 3 L fermentor using mixed culture

3.1.1. Cell growth and pH profiles

The inoculum of the mixed culture for the 3 L fermentor with YE and CSL media was from the same inoculum source. The maximum cell mass concentrations of the mixed culture were similar in both the YE and CSL media and reached 0.73 g/L (OD 1.9) (Fig. 1A). Cells entered stationary growth phase 48 h after inoculation in both media. Cell mass concentration remained constant in the CSL medium but started to decrease after 84 h in the YE medium, suggesting nutrient limitation. In addition, the growth rate of the mixed culture in the CSL medium was 56% higher than in the YE medium (Table 1), which could be due to the consumption of the 1 g/L lactic acid present in the CSL medium in the first 48 h by *C. propionicum* of the mixed culture.

C. propionicum can grow on lactic acid, converting it into propionic acid and acetic acid (Johns, 1952). Propionic acid (0.45 g/L) was produced during the first 24 h of mixed culture growth in CSL medium, likely due to fermentation of the lactic acid by *C. propionicum* (Fig. 1C). It should be noted that *A. bacchi* cannot consume lactic acid (Allen et al., 2010).

The initial pH of both YE and CSL media before inoculation was 8.0. However, before the addition of reducing agent into the fermentor and inoculation, the medium was purged 8 h with syngas (38% CO, 28.5% H_2, 28.5% CO_2 and 5% N_2 by volume), which resulted in the decrease in pH to 7.1 due to CO_2 from the syngas. This value was consistent with the calculated equilibrium medium pH 7.2 using a modified Henderson–Haselbach equation for $NaHCO_3/CO_2$ buffer system (Sowers and Noll, 1995). In the present study, TAPS buffer was not added to the medium. Therefore, the buffer capacity was mainly dependent on the $NaHCO_3/CO_2$ buffer system. During the fermentation, the pH of both media decreased to 6.1 in the first 40 h and remained at this level until 80 h with the pH controlled at above 6.1 by the addition of 7% $NaHCO_3$ (Fig. 1A). The decrease in pH during the first 80 h resulted mainly from acetic acid production (Fig. 1B). After 80 h, the pH increased to 6.4 in YE medium and 6.3 in CSL medium due to conversion of acetic acid to ethanol (Fig. 1B). However, it was observed that the pH of both media decreased to 6.1 after 100 h. This second decrease of pH was asso-

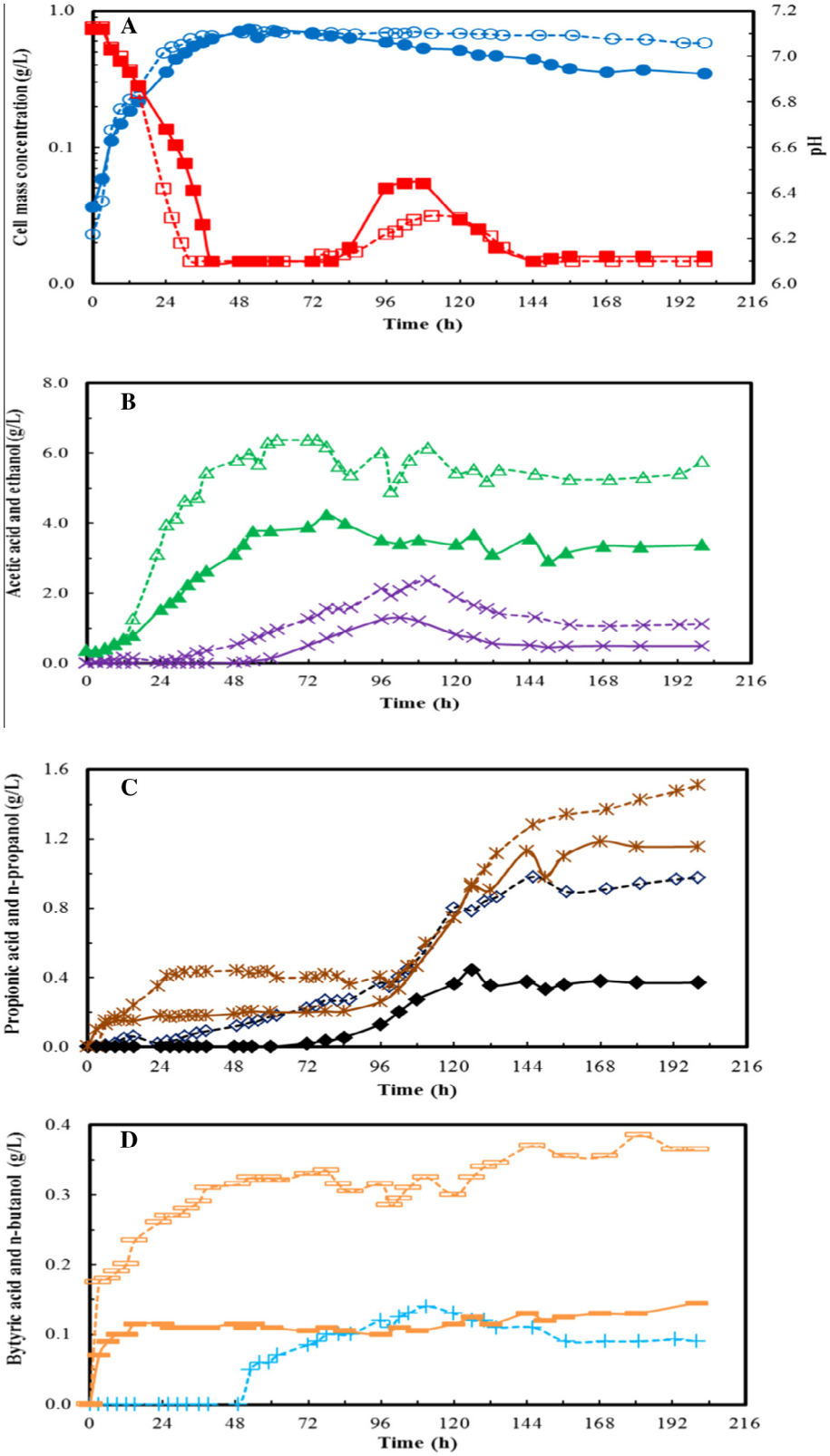

Fig. 1. Semi-continuous fermentation profiles in 3 L fermentor using the mixed culture in YE medium (solid line) and CSL medium (dash line); (A) pH (■), cell mass (●); (B) acetic acid (▲), ethanol (×); (C) *n*-propanol (♦), propionic acid (✳); (D) butanol (+), butyric acid (▬).

ciated with a rapid increase in the propionic acid concentration from 0.4 g/L to above 1 g/L in both media (Fig. 1C). This was the first time that propionic acid was produced during syngas fermentation, which was due to the mixed culture.

3.1.2. Product formation

Acetic acid concentrations in the YE and CSL media reached a maximum of 4.2 g/L and 6.4 g/L at 80 h, respectively (Fig. 1B). After reaching a maximum, acetic acid concentrations in the YE and CSL

Table 1
Kinetic parameters in the YE and CSL media in the 3 L fermentor with the mixed culture.

Kinetic parameters	3 L fermentor study	
Medium	YE medium	CSL medium
Specific growth rate, h^{-1}	0.16	0.25
Cell mass yield, g cells/mol CO	3.68	3.08
Ethanol yield from CO, %[a]	44.7	62.0
Maximum ethanol concentration, g/L[a]	1.30	2.36
Final ethanol concentration, g/L	0.50	1.13
Final *n*-propanol concentration, g/L	0.37	0.98
Ethanol conversion to *n*-propanol, % (mol/mol)[b]	15.7	24.5
Final *n*-butanol concentration, g/L	ND[c]	0.09
Total alcohols concentration, g/L	0.87	2.20
CO utilization, %[a]	40.4	50.3
H_2 utilization, %[a]	28.1	23.9

[a] Cell mass yield was calculated at maximum cell mass concentration; ethanol yield was calculated at maximum ethanol concentration at 102 h and 111 h in YE and CSL medium, respectively. CO and H_2 utilization were calculated at 200 h.
[b] Calculation was based on the time when ethanol started to decrease in YE medium and CSL medium after 102 h and 111 h, respectively, to the end of fermentation using Eq. (1).
[c] ND = not detectable.

media slowly decreased to 3.4 g/L and 4.9 g/L at around 100 h, respectively, due to acetic acid conversion to ethanol. The maximum ethanol concentrations measured in the YE and CSL media were 1.3 g/L at 102 h and 2.4 g/L ethanol at 111 h, respectively (Fig. 1B). Ethanol production in both media was due to the conversion of syngas by the mixed culture, since all of the monosaccharides (glucose, fructose, mannose and galactose) present in the 20 g/L CSL medium at a total concentration of 0.3 g/L were consumed during the first 111 h. Based on the maximum theoretical conversion of sugars to either acetic acid or ethanol, however, the sugars could only contribute about 3–6% to these products, indicating that syngas mainly contributed to product formation. Moreover, the production of ethanol by the mixed culture was non-growth related, which was observed in a previous study with strain CP15 monoculture (Liu et al., 2012).

The consumption of ethanol after it reached a maximum was observed simultaneously with the production of *n*-propanol (Fig. 1B and C). The decrease in ethanol concentration was also observed in the continuous syngas fermentation in the 20 g/L CSL medium (Liu et al., 2014). The final concentrations of *n*-propanol in the YE and CSL media were 0.37 g/L and 0.98 g/L, respectively (Fig. 1C). The accumulation of *n*-propanol in the YE medium was associated with the increase in propionic acid concentration after 80 h. Production of *n*-propanol in the CSL medium started at the beginning of fermentation through the conversion of lactic acid to propionic acid by *C. propionicum*, followed by reduction of the propionic acid to *n*-propanol by strain CP15 in the mixed culture. Another source of *n*-propanol was the conversion of ethanol, whose concentrations decreased by 38.5% and 47.9% from their maxima in the YE and CSL media, respectively. There was 1.6 times more ethanol converted to *n*-propanol in the CSL medium and total alcohol concentration in the CSL medium was 2.5 times higher than in the YE medium (Table 1).

The production of *n*-propanol and propionic acid from ethanol with the mixed culture could be due to *C. propionicum* by a metabolic pathway similar to *Clostridium neopropionicum*: ethanol → acetaldehyde → acetyl-CoA → pyruvate → lactate → lactyl-CoA → propionyl-CoA → propionate (Tholozan et al., 1992). Additionally, strain CP15 could be reducing propionic acid to *n*-propanol during syngas fermentation, a property shared by *C. ljungdahlii* and "*C. ragsdalei*" (Perez et al., 2012). In this case, CO and H_2 would serve as reducing equivalents to generate NADH needed by strain CP15 in the mixed culture to reduce the propionic acid likely made by *C. propionicum*.

Table 2
Possible reactions for ethanol conversion to *n*-propanol and propionic acid and their standard Gibbs free energy (adapted from Wu and Hickey, 1996).

	Reactions	$\Delta_r G^{0\prime}$, kJ/mol
1	$CH_3CH_2OH + 3H_2 + HCO_3^- + H^+ \rightarrow CH_3CH_2CH_2OH + 3H_2O$	−78.9
2	$CH_3CH_2OH + HCO_3^- + 2H_2 + 1/2H^+ \rightarrow 1/2CH_3CH_2CH_2OH + 1/2CH_3CH_2COO^- + 2.5\ H_2O$	−72.8
3	$CH_3CH_2OH + HCO_3^- + H_2 \rightarrow CH_3CH_2COO^- + 2H_2O$	−66.7
4	$CH_3CH_2COO^- + 2H_2 + H^+ \rightarrow CH_3CH_2CH_2OH + H_2O$	−51.9
5	$CH_3CH_2OH + CO + 2H_2 \rightarrow CH_3CH_2CH_2OH + H_2O$	−94.2

Thermodynamically, several possible reactions exist for the conversion of ethanol to propionic acid and *n*-propanol from anaerobic mixed cultures (Wu and Hickey, 1996), which are listed in Table 2. Reactions 1 and 2 describe the conversion of ethanol to *n*-propanol and propionic acid. Reaction 1 may be less likely than reaction 2 as both *n*-propanol and propionic acid were produced in the present study. Reaction 3 describes the conversion of ethanol to propionic acid, which could due to *C. propionicum*. Reaction 4 describes the direct reduction of propionic acid to *n*-propanol which could due to *A. bacchi* CP15. Reaction 5 describes the direct conversion of ethanol to *n*-propanol using CO and H_2 as reactants. Reaction 5 has not been described for any biological system (Wu and Hickey, 1996), nor have syngas fermenting strains or *C. propionicum* been described as capable of directly converting CO and H_2 to *n*-propanol.

In addition to *n*-propanol, *n*-butanol (0.14 g/L) was produced by the mixed culture in the CSL medium (Fig. 1D). Butyric acid, a growth associated product, was formed in both YE and CSL media at concentrations of 0.15 g/L and 0.45 g/L, respectively. The production of butyric acid could be due to *C. propionicum* fermenting four carbon amino acids such as threonine into butyric acid (Cardon and Barker, 1946). Given the ability of strain CP15 to convert carboxylic acids to their respective alcohol, it is posited that *C. propionicum* produced butyric acid, which was further reduced to *n*-butanol by strain CP15 in the mixed culture. Details of this synergy between *C. propionicum* and strain CP15 are discussed in Section 3.2.

Although there might be changes in the composition of the mixed culture during fermentation, the trends in cell mass, pH, acetic acid, propionic acid, butyric acid, propanol and ethanol profiles in both YE and CSL media were similar (Fig. 1). This indicates a similar function and stability of the mixed culture.

3.1.3. Gas utilization and carbon balance

The cumulative CO consumption by the mixed culture in CSL medium was 1.4 mol, which was 27% higher than in YE medium. The cumulative consumption of H_2, however, did not differ considerably between the CSL and YE medium. The percentages of CO and H_2 utilized by the mixed culture in the YE medium at the end of fermentation were 40% and 28%, respectively (Table 1). The CO and H_2 utilized by the mixed culture in the CSL medium were 50% and 24%, respectively. The carbon balance in both media showed that there were 86% and 104% carbon recovery in the YE and CSL (including the sugars and lactic acid in CSL) media, respectively.

3.2. Conversion of carboxylic acids into alcohols

3.2.1. Effect of carboxylic acids on cell growth

Cell growth was observed in all treatments with the carboxylic acids used with either strain CP15 or the mixed culture (Fig. 2). However, the maximum cell mass concentration in each treatment varied depending on the individual carboxylic acid added (Table 3). The maximum cell mass concentration in each treatment was over

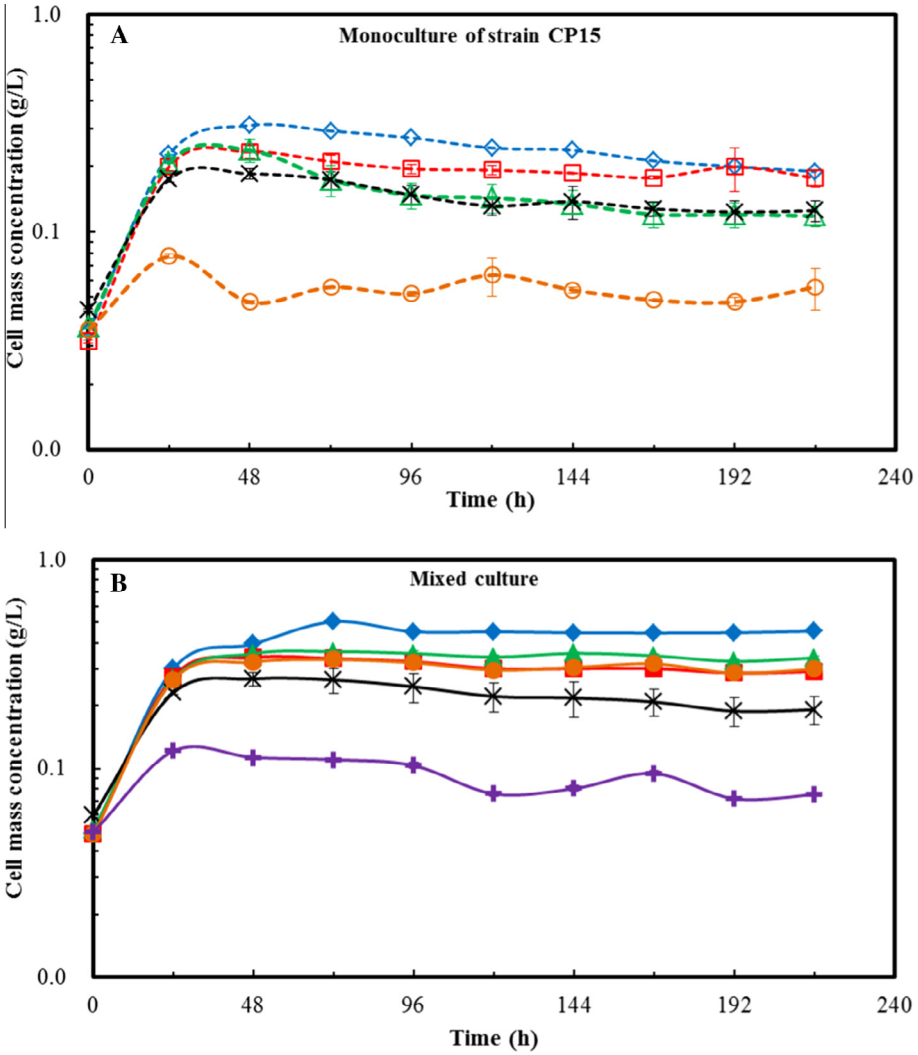

Fig. 2. Cell mass concentration profiles during syngas bottle fermentations with the addition of various carboxylic acids (A) monoculture of strain CP15 (open symbol and dash line) and (B) mixed culture (solid symbol and solid line); treatment with no acid (♦), propionic acid (■), butyric acid (▲), hexanoic acid (×), lactic acid (●), lactic acid with N_2 headspace (+).

Table 3
Syngas fermentation parameters during the conversion of carboxylic acids into alcohols in 250 mL bottle fermentors.

Treatments[1]	No acid		Propionic acid		Butyric acid		Hexanoic acid		Lactic acid	
	CP15	Mixed culture	CP15	Mixed culture	CP15	Mixed culture	CP15	Mixed culture	CP15	Mixed culture
Max. cell conc., g/L	$0.3 \pm 0.0^{A,Y}$	0.5 ± 0.1^{a}	$0.2 \pm 0.0^{B,Y}$	0.3 ± 0.0^{b}	$0.2 \pm 0.0^{B,Y}$	0.4 ± 0.0^{b}	$0.2 \pm 0.0^{C,Y}$	0.3 ± 0.0^{c}	$0.1 \pm 0.0^{D,Y}$	0.3 ± 0.00^{d}
% Of growth inhibition[2]	–[3]	–	24.3 ± 4.9^{C}	32.6 ± 2.2^{b}	23.4 ± 9.3^{C}	29.7 ± 0.4^{b}	40.1 ± 3.1^{B}	46.8 ± 4.0^{a}	$75.0 \pm 0.6^{A,Y}$	34.1 ± 1.3^{b}
Final ethanol, g/L	$0.4 \pm 0.0^{A,Y}$	0.2 ± 0.0^{b}	0.2 ± 0.0^{C}	0.2 ± 0.0^{b}	$0.3 \pm 0.0^{B,Y}$	0.6 ± 0.0^{a}	0.3 ± 0.0^{B}	0.5 ± 0.2^{a}	–	$0.2 \pm 0.0^{b,Y}$
Ethanol yield from CO, %	$21.2 \pm 1.1^{A,B,Y}$	10.5 ± 2.1^{c}	$11.8 \pm 0.7^{C,Y}$	8.6 ± 0.7^{c}	19.0 ± 1.7^{B}	20.7 ± 1.2^{b}	$23.1 \pm 2.2^{A,Y}$	34.7 ± 6.1^{a}	–	11.7 ± 1.2^{c}
n-Propanol, g/L	–	–	0.4 ± 0.0^{Y}	1.0 ± 0.2	–	0.9 ± 0.1	–	0.1 ± 0.0	–	0.4 ± 0.0
n-Butanol, g/L	–	–	–	–	0.5 ± 0.1^{Y}	0.8 ± 0.0	–	–	–	–
Hexanol, g/L	–	–	–	–	–	–	0.8 ± 0.1^{Y}	1.0 ± 0.0	–	–
Acid converted to alcohol, %	–	–	36.8 ± 2.0^{Y}	83.4 ± 2.8	38.6 ± 5.3^{Y}	74.7 ± 5.6	63.6 ± 6.0^{Y}	90.7 ± 5.0	–	–
Total alcohols, g/L	$0.4 \pm 0.0^{D,Y}$	0.2 ± 0.0^{e}	$0.6 \pm 0.0^{C,Y}$	1.2 ± 0.1^{c}	$0.8 \pm 0.1^{B,Y}$	2.3 ± 0.1^{a}	$1.0 \pm 0.1^{A,Y}$	1.6 ± 0.2^{b}	–	0.6 ± 0.0^{d}
CO utilization, %	30.6 ± 1.1^{A}	30.5 ± 0.2^{c}	$22.9 \pm 1.0^{B,Y}$	36.4 ± 1.0^{b}	$21.7 \pm 1.2^{B,Y}$	43.5 ± 1.3^{a}	17.2 ± 1.5^{C}	21.5 ± 4.8^{d}	$4.8 \pm 1.0^{D,Y}$	30.5 ± 1.2^{c}
H_2 utilization, %	28.1 ± 3.1^{A}	32.1 ± 1.1^{a}	20.9 ± 3.1^{B}	25.6 ± 1.5^{b}	$16.7 \pm 3.8^{B,Y}$	27.6 ± 2.6^{b}	9.0 ± 3.1^{C}	6.0 ± 1.8^{c}	$4.6 \pm 2.8^{C,Y}$	24.6 ± 1.9^{b}

[A,B,C,D]Duncan's test group for the monoculture of strain CP15; the same letter in the same row indicates there was no statistical difference among treatments ($p > 0.05$).
[a,b,c,d,e]Duncan's test group for the mixed culture; the same letter in the same row indicates there was no statistical difference among treatments ($p > 0.05$).
[Y]There was statistical difference between the monoculture of strain CP15 and mixed culture using the *T*-Test ($p < 0.05$).
[1] Product concentrations, yields or gas conversion efficiencies were calculated at the end of experiment of 216 h.
[2] Equal to (1 − (maximum cell concentration with added acid/maximum cell mass concentration with no acid)) × 100%.
[3] Not applicable or products were not detectable.

50% higher with the mixed culture than with strain CP15 monoculture ($p < 0.05$).

Growth, measured as maximum cell mass concentration, was inhibited in each treatment with acid compared to cultures with no acid added. Except in the treatment with lactic acid, there were no statistical differences in the percentage of growth inhibition between strain CP15 monoculture and mixed culture with the other acids ($p > 0.05$) (Table 3). The order of growth inhibition among the acids used with strain CP15 alone was lactic acid > hexanoic acid > butyric acid ≈ propionic acid > no acid (Table 3). The highest inhibition of growth observed was 75% for strain CP15, when lactic acid was added to the medium (Table 3). Lactic acid is able to cross bacterial cell membranes in an undissociated form, reducing cell internal pH and disrupting transmembrane proton motive force for ATP formation (Herrero et al., 1985). This inhibition has been observed for other bacteria. For example, growth of *Clostridium thermocellum* was 50% inhibited when grown on cellobiose with an initial lactic acid concentration of 2.7 g/L at pH 7.4 (undissociated concentration of lactic acid equals 7.8×10^{-4} g/L) with N_2 headspace (Herrero et al., 1985). In the present study, the growth cessation of strain CP15 monoculture in the medium with lactic acid was at pH 6.6 with 4.0×10^{-3} g/L undissociated lactic acid, which was fivefold higher than with *C. thermocellum*. This may account for the high level of inhibition for strain CP15 caused by lactic acid.

In the lactic acid treatment under syngas headspace, growth inhibition of the mixed culture less than half of that for strain CP15. This was because 1.2 g/L of lactic acid was consumed (likely by *C. propionicum*) during the first 24 h of growth, which have reduced the inhibition of strain CP15 in the mixed culture. The inhibition order in the treatments with acid using the mixed culture was hexanoic acid > propionic acid ≈ butyric acid ≈ lactic acid > no acid.

The cell mass concentration of the mixed culture in the lactic acid treatment with N_2 headspace was 38% lower than with syngas headspace (Fig. 2B). The growth of the mixed culture with a N_2 atmosphere was likely due to the growth of *C. propionicum* that consumed 1.2 g/L lactic acid and produced 0.48 g/L propionic acid and 0.6 g/L acetic acid (carbon recovery in products was 99.1%).

3.2.2. Product formation

Ethanol was produced in all treatments with acids except lactic acid under N_2 headspace (Fig. 3). Strain CP15 did not consume ethanol in any of the treatments (Fig. 3A). The mixed culture, however, clearly consumed ethanol after 120 h in the treatments containing propionic acid, butyric acid and lactic acid (Fig. 3B). However, no ethanol was consumed by the mixed culture in the hexanoic acid treatment or the treatment without acid.

Acetic acid was also produced during the syngas fermentation using strain CP15 and the mixed culture (Fig. 4), with the greatest amount produced in the treatment without acid. The mixed culture produced a maximum of 4.7 g/L acetic acid, which was 38% higher than with CP15 alone. In treatment with no acid added to the mixed culture, acetic acid concentration did not decrease after

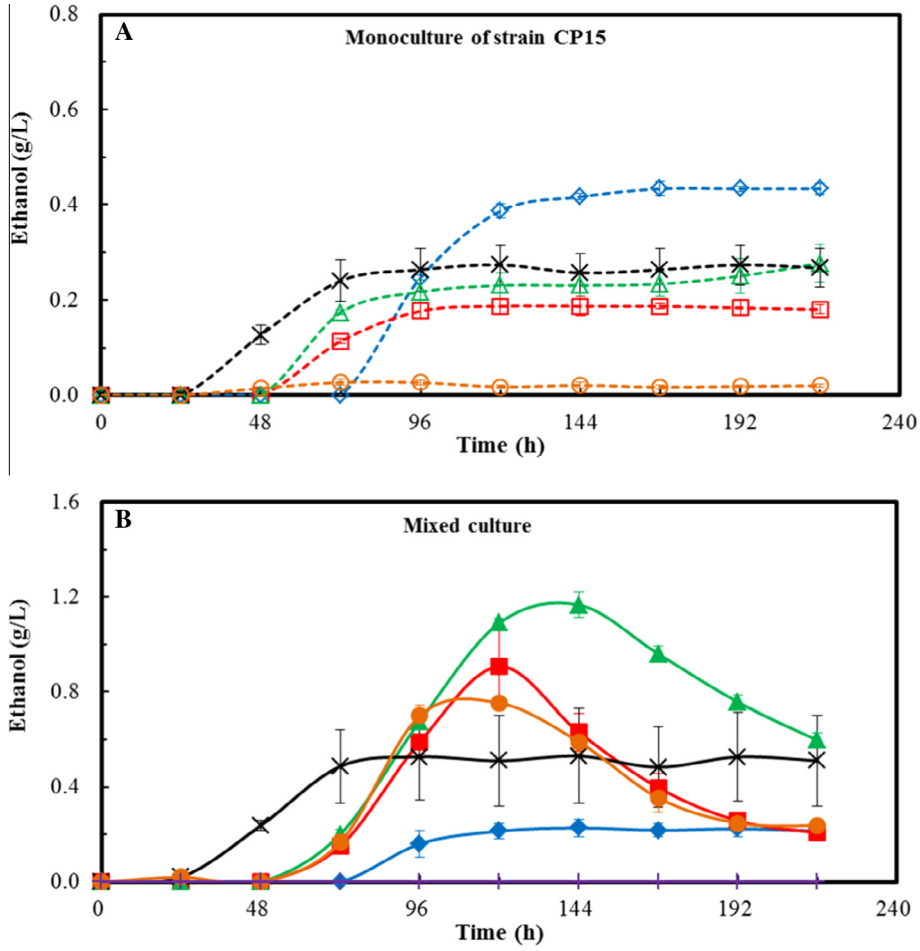

Fig. 3. Ethanol profiles during syngas bottle fermentations with the addition of various carboxylic acids (A) monoculture of CP15 (open symbol and dash line) and (B) mixed culture (solid symbol and solid line); treatment with no acid (♦), propionic acid (■),butyric acid (▲), hexanoic acid (×), lactic acid (●), lactic acid with N_2 headspace (+).

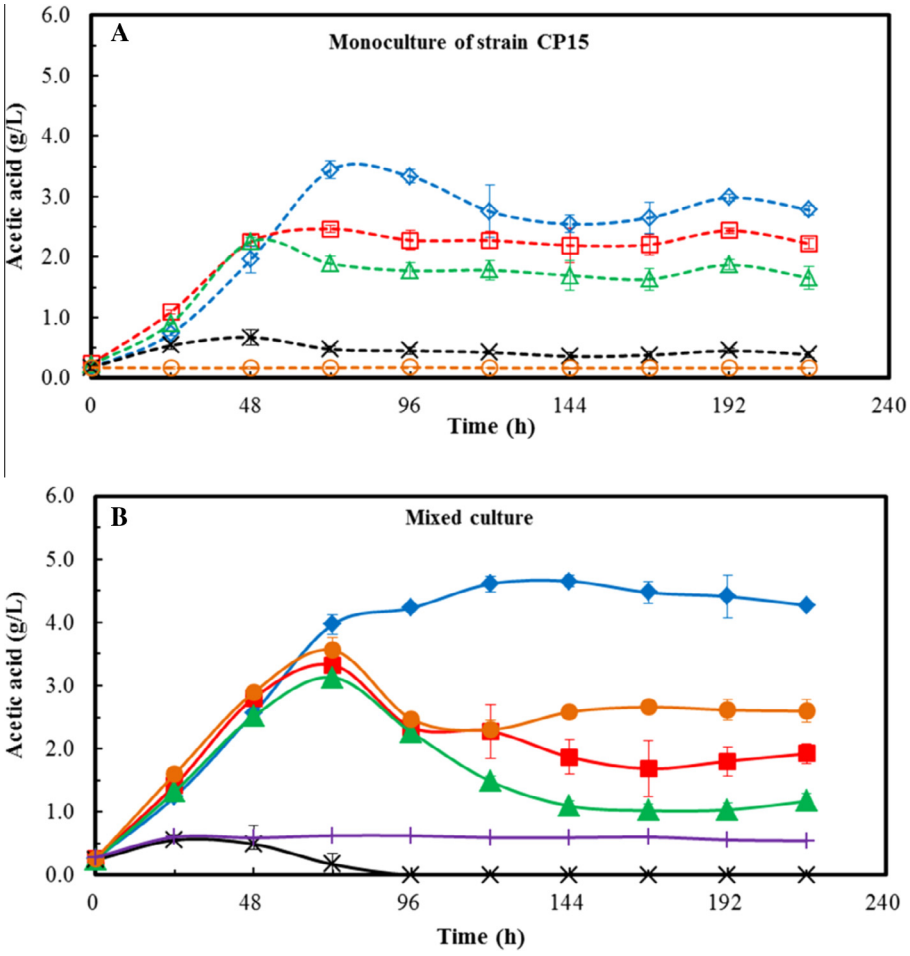

Fig. 4. Acetic acid profiles during syngas bottle fermentations with the addition of various carboxylic acids (A) monoculture of CP15 (open symbol and dash line) and (B) mixed culture (solid symbol and solid line); treatment with no acid (♦), propionic acid (■), butyric acid (▲), hexanoic acid (×), lactic acid (●), lactic acid with N_2 headspace (+).

reaching a maximum (Fig. 4B). However, acetic acid concentration decreased from 3.4 g/L to 2.8 g/L in treatments with no acid added to strain CP15 (Fig. 4A). This indicated that the mixed culture generated less NADH to reduce acetic acid to ethanol than strain CP15 alone, resulting in less ethanol production. However, acetic acid conversion to ethanol by the mixed culture was observed in the treatments that contained propionic acid, butyric acid, hexanoic acid or lactic acid (Fig. 4B). The mixed culture was more efficient in the conversion of acetic acid to ethanol in media with acid amendment compared to strain CP15 alone, suggesting an interaction between CP15 and *C. propionicum* in the mixed culture that is not yet understood. Characterizing the changes in the abundance of CP15 and *C. propionicum* in the mixed culture during the course of fermentation can explain the interaction between the two strains, which warrant further investigation.

The profiles of carboxylic acid consumption and production of their respective alcohols are shown in Fig. 5 for strain CP15 and the mixed culture. There was a lag phase of 48 h before either strain CP15 alone or the mixed culture began to convert propionic acid to *n*-propanol. The production of propionic acid after 120 h was observed for the mixed culture (Fig. 5A), which was due to the conversion of ethanol as shown in Fig. 3B. The increase in carboxylic acid concentration was not observed with the butyric acid, hexanoic acid or lactic acid with the mixed culture (Fig. 5). Strain CP15 alone did not consume lactic acid (Fig. 5B). The mixed culture, however, directly consumed the lactic acid in the treatments with syngas or N_2 headspace. There was also a lag of about 48 h in the conversion of butyric acid to *n*-butanol by strain CP15 alone and the mixed culture (Fig. 5C). Both cultures, however, converted hexanoic acid to *n*-hexanol immediately after inoculation (Fig. 5D).

The mixed culture converted 83.4% of propionic acid to *n*-propanol (including n-propanol from ethanol), 74.7% of butyric acid to *n*-butanol and 90.7% of hexanoic acid to hexanol, which were 2.3 times, 1.9 times and 1.4 times, respectively, higher than with strain CP15 alone ($p < 0.05$) (Table 3). In addition, with similar initial carboxylic acid concentrations in the medium, there were 2.0 times, 2.9 times, and 1.6 times higher total alcohol production from propionic acid, butyric acid, hexanoic acid treatments, respectively, with the mixed culture compared to strain CP15 alone.

The mixed culture completely used the added lactic acid with syngas in the headspace but only utilized 52% of available lactic acid when N_2 was in the headspace (Fig. 5B). Also, it was observed that 23% of lactic acid on a molar basis was converted to *n*-propanol by the mixed culture when syngas was used in the headspace (Fig. 5B). These results with the mixed culture conversion of lactic acid to propionic acid supports the results obtained in the CSL medium in the 3 L fermentor, in which lactic acid in the CSL was converted to propionic acid.

In addition, the ability of strain CP15 to convert propionic acid to n-propanol supports the hypothesis that *n*-propanol produced in the 3 L fermentor was the result of a synergistic interactions. *C. propionicum* likely converted ethanol to propionic acid, which was converted by strain CP15 to *n*-propanol. The *n*-butanol produced in the CSL medium in the 3 L fermentor could be attributed

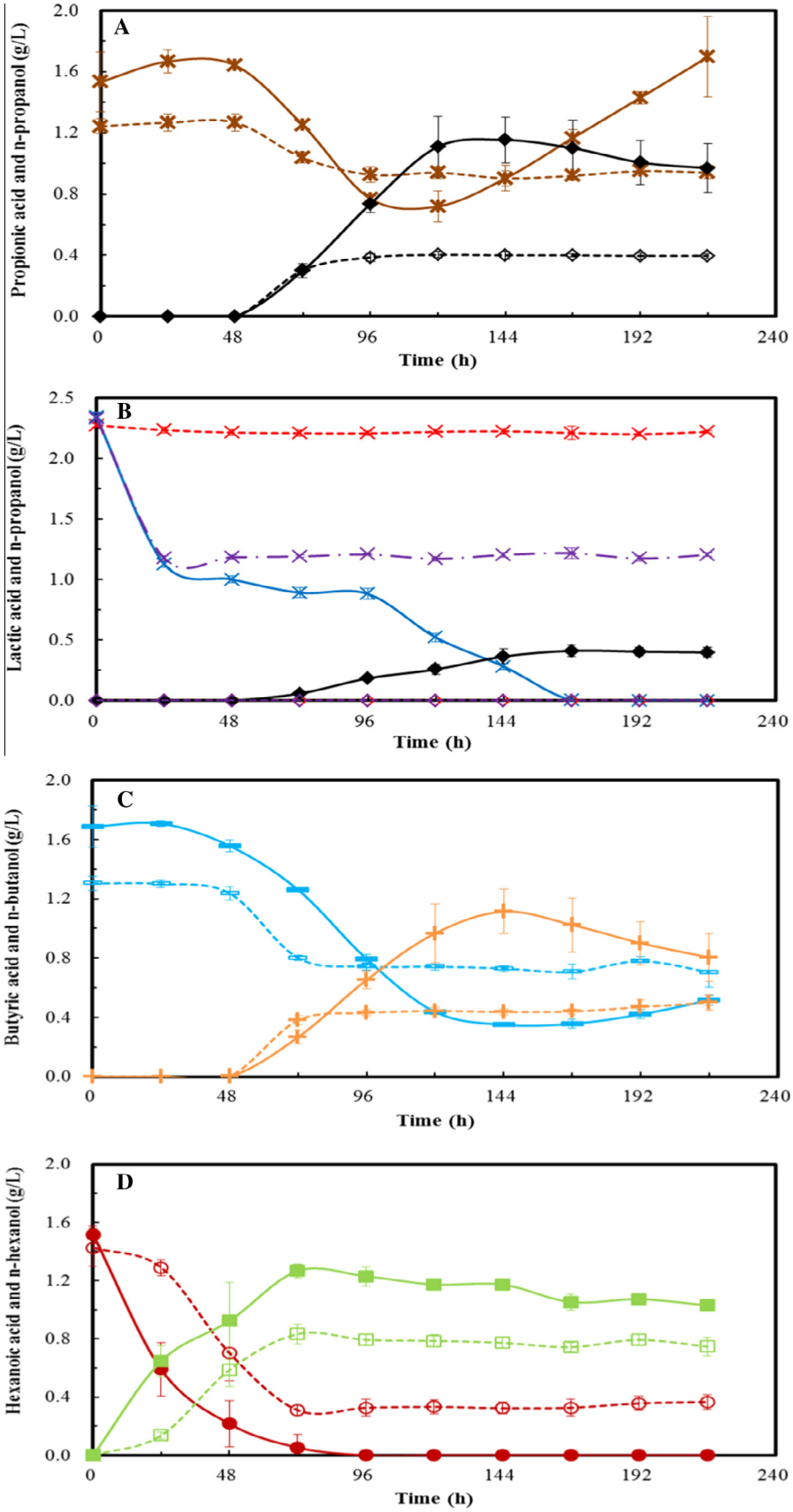

Fig. 5. Carboxylic acids and their respective alcohols profiles during syngas bottle fermentations using the monoculture of CP15 (open symbol and dash line) and mixed culture (solid symbol and solid line) for treatments (A) propionic acid (B) lactic acid (C) butyric acid (D) hexanoic acid; *n*-propanol (♦), propionic acid (∗), lactic acid (×), lactic acid under nitrogen headspace (× and dash dot line), butanol (+), butyric acid (▬), hexanoic acid (●), hexanol (■).

to the conversion of butyric acid by strain CP15. The mixed culture did not produce alcohol in the lactic acid treatment with a N_2 headspace but it did produce *n*-propanol with a syngas headspace by CP15 due to presence of H_2 and CO as reductants (Fig. 5B). This result, further supports the assertion that *n*-propanol produced in the 3 L fermentor was mainly because of synergistic interactions within the mixed culture.

The conversion efficiencies of propionic acid to *n*-propanol in the present study and published reports for *C. ljungdahlii* ERI-2 and *C. ragsdalei* (Perez et al., 2012) were as follows: *C. ljungdahlii* ERI-2 (92.7%) > mixed culture (83.4%, present study) > "*C. ragsdalei*" (72.3%) > strain CP15 monoculture (36.8%, present study). The conversion efficiencies of butyric acid to *n*-butanol were as follow: mixed culture (74.7%, present study) > *C. ljungdahlii* ERI-2 (68.2%) > strain CP15 monoculture (38.6%, present study) > "*C. ragsdalei*" (21.0%). For hexanoic acid conversion to *n*-hexanol, the conversion efficiencies were as follows: mixed culture (90.7%, present study) > strain CP15 monoculture (63.6%, present study) > *C. ljungdahlii* ERI-2 (46.0%).

3.2.3. Gas utilization and carbon balance

The mixed culture consumed significantly more CO ($p < 0.05$) in the treatments that contained propionic acid, butyric acid and lactic acid than strain CP15 monoculture (Table 3). The mixed culture also utilized more H_2 in the treatments that contained butyric acid and lactic acid. CP15 utilized more CO and H_2 ($p < 0.05$) in the treatments without acid than treatments where acids were added. The carbon recoveries in the products were generally 100 ± 10% for strain CP15 and the mixed culture, indicating that no other major products were generated during the conversion of carboxylic acids to their respective alcohols.

4. Conclusions

Over twofold more alcohol was produced in CSL medium than in YE medium during the semi-continuous fermentation in the 3 L fermentor with the mixed culture. Bottle fermentations suggested that *n*-propanol and *n*-butanol production were the result of synergistic interactions between strain CP15 and *C. propionicum*, resulting in over 60% more alcohol production than with strain CP15 alone. In addition, the mixed culture converted 50% more carboxylic acids into their corresponding alcohols than the CP15 monoculture. These results show the advantage of using the mixed culture for higher alcohols production from syngas.

Acknowledgements

This research was supported by USDA-NIFA Special Research Grant Award USDA-NIFA 2010-34447-20772, Oklahoma NSF EPSCoR Grant EPS-0814361 and Oklahoma Agricultural Experiment Station.

References

Allen, T.D., Caldwell, M.E., Lawson, P.A., Huhnke, R.L., Tanner, R.S., 2010. *Alkalibaculum bacchi* gen. nov., sp. nov., a CO-oxidizing, ethanol-producing acetogen isolated from livestock-impacted soil. Int. J. Syst. Evol. Microbiol. 60, 2483–2489.

Atsumi, S., Hanai, T., Liao, J.C., 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451, 86–89.

Atsumi, S., Liao, J.C., 2008. Metabolic engineering for advanced biofuels production from *Escherichia coli*. Curr. Opin. Biotechnol. 19, 414–419.

Bakker, A., Smith, J., Myers, K., 1994. How to disperse gases in liquids. Chem. Eng., 101.

Cardon, B., Barker, H., 1946. Two new amino-acid-fermenting bacteria, *Clostridium propionicum* and *Diplococcus glycinophilus*. J. Bacteriol. 52, 629–634.

Demirer, G., Speece, R., 1998. Anaerobic biotransformation of four 3-carbon compounds (acrolein, acrylic acid, allyl alcohol and *n*-propanol) in UASB reactors. Water Res. 32, 747–759.

Esposito, G., Weijma, J., Pirozzi, F., Lens, P., 2003. Effect of the sludge retention time on H_2 utilization in a sulphate reducing gas-lift reactor. Process Biochem. 39, 491–498.

Harvey, B.G., Meylemans, H.A., 2011. The role of butanol in the development of sustainable fuel technologies. J. Chem. Technol. Biotechnol. 86, 2–9.

Herrero, A.A., Gomez, R.F., Snedecor, B., Tolman, C.J., Roberts, M.F., 1985. Growth inhibition of *Clostridium thermocellum* by carboxylic acids: a mechanism based on uncoupling by weak acids. Appl. Microbiol. Biotechnol. 22, 53–62.

Johns, A., 1952. The mechanism of propionic acid formation by *Clostridium propionicum*. J. Gen. Microbiol. 6, 123–127.

Klasson, K., Cowger, J., Ko, C., Vega, J., Clausen, E., Gaddy, J., 1990. Methane production from synthesis gas using a mixed culture of *R. rubrum*, *M. barkeri*, and *M. formicicum*. Appl. Biochem. Biotechnol. 24, 317–328.

Liu, K., Atiyeh, H.K., Stevenson, B.S., Tanner, R.S., Wilkins, M.R., Huhnke, R.L., 2014. Continuous sysngas fermentation for the production of ethanol, *n*-propanol and *n*-butanol. Bioresour. Technol. 151, 69–77.

Liu, K., Atiyeh, H.K., Tanner, R.S., Wilkins, M.R., Huhnke, R.L., 2012. Fermentative production of ethanol from syngas using novel moderately alkaliphilic strains of *Alkalibaculum bacchi*. Bioresour. Technol. 104, 336–341.

Maddipati, P., Atiyeh, H.K., Bellmer, D.D., Huhnke, R.L., 2011. Ethanol production from syngas by *Clostridium* strain P11 using corn steep liquor as a nutrient replacement to yeast extract. Bioresour. Technol. 102, 6494–6501.

Mann, J., Yao, N., Bocarsly, A.B., 2006. Characterization and analysis of new catalysts for a direct ethanol fuel cell. Langmuir 22, 10432–10436.

O'Brien, D.J., Panzer, C.C., Eisele, W.P., 1990. Biological production of acrylic acid from cheese whey by resting cells of *Clostridium propionicum*. Biotechnol. Prog. 6, 237–242.

Perez, J.M., Richter, H., Loftus, S.E., Angenent, L.T., 2012. Biocatalytic reduction of short-chain carboxylic acids into their corresponding alcohols with syngas fermentation. Biotechnol. Bioeng. 110, 1066–1077.

Ramachandriya, K.D., Kundiyana, D.K., Wilkins, M.R., Terrill, J.B., Atiyeh, H.K., Huhnke, R.L., 2013. Carbon dioxide conversion to fuels and chemicals using a hybrid green process. Appl. Energy 112, 289–299.

Ramachandriya, K.D., Wilkins, M.R., Delorme, M.J.M., Zhu, X., Kundiyana, D.K., Atiyeh, H.K., Huhnke, R.L., 2011. Reduction of acetone to isopropanol using producer gas fermenting microbes. Biotechnol. Bioeng. 108, 2330–2338.

Shen, G.J., Shieh, J.S., Grethlein, A.J., Jain, M.K., Zeikus, J.G., 1999. Biochemical basis for carbon monoxide tolerance and butanol production by *Butyribacterium methylotrophicum*. Appl. Microbiol. Biotechnol. 51, 827–832.

Simmons, B.A., 2011. Opportunities and challenges in advanced biofuel production: the importance of synthetic biology and combustion science. Biofuels 2, 5–7.

Sipma, J., Meulepas, R., Parshina, S., Stams, A., Lettinga, G., Lens, P., 2004. Effect of carbon monoxide, hydrogen and sulfate on thermophilic (55 C) hydrogenogenic carbon monoxide conversion in two anaerobic bioreactor sludges. Appl. Microbiol. Biotechnol. 64, 421–428.

Sowers, K.R., Noll, K.M., 1995. Techniques for anaerboic growth. In: Robba, F.T., Sowers, K.R., Schreier, H.J. (Eds.), Archaea: a laboratory manual, 1st ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, p. 36.

Tanner, R.S., 2007. Cultivation of bacteria and fungi. In: Hurst, C.J., Crawford, A.L., Mills, A.L., Garland, J.L., Stetzenbach, L.D., Lipson, D.A. (Eds.), Manual Environ. Microbiol.. ASM Press, Washington, DC, pp. 69–78.

Tanner, R.S., 2008. Production of ethanol from synthesis gas. In: Wall, J.D., Harwood, C.S., Demain, A. (Eds.), Bioenergy. ASM Press, Washington, DC (pp. 147–151).

Tholozan, J., Touzel, J., Samain, E., Grivet, J., Prensier, G., Albagnac, G., 1992. *Clostridium neopropionicum* sp. nov., a strict anaerobic bacterium fermenting ethanol to propionate through acrylate pathway. Arch. Microbiol. 157, 249–257.

Tyner, W.E., 2010. Policy update: why the push for drop-in biofuels? Biofuels 1, 813–814.

Ukpong, M.N., Atiyeh, H.K., De Lorme, M.J.M., Liu, K., Zhu, X., Tanner, R.S., Wilkins, M.R., Stevenson, B.S., 2012. Physiological response of *Clostridium carboxidivorans* during conversion of synthesis gas to solvents in a gas-fed bioreactor. Biotechnol. Bioeng. 109, 2720–2728.

Wilkins, M.R., Atiyeh, H.K., 2011. Microbial production of ethanol from carbon monoxide. Curr. Opin. Biotechnol. 22, 326–330.

Wu, M.M., Hickey, R.F., 1996. *n*-Propanol production during ethanol degradation using anaerobic granules. Water Res. 30, 1686–1694.

Yeung, C., Thomson, M., 2013. Experimental and kinetic modeling study of 1-hexanol combustion in an opposed-flow diffusion flame. Proc. Combust. Inst. 34, 795–802.