Bioresource Technology 190 (2015) 114–121

Contents lists available at ScienceDirect

Bioresource Technology

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

Butanol and hexanol production in *Clostridium carboxidivorans* syngas fermentation: Medium development and culture techniques

John R. Phillips [a], Hasan K. Atiyeh [a,*], Ralph S. Tanner [b], Juan R. Torres [b], Jyotisna Saxena [b,1], Mark R. Wilkins [a], Raymond L. Huhnke [a]

[a] Biosystems and Agricultural Engineering Department, Oklahoma State University, Stillwater, OK, United States
[b] Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK, United States

HIGHLIGHTS

- *C. carboxidivorans*, a native organism, produces butanol and hexanol from CO.
- 1.0 g/L butanol, 0.9 g/L hexanol and 3.0 g/L ethanol produced in defined medium.
- Technique modification reduces substrate inhibition and mass transfer limitation.

ARTICLE INFO

Article history:
Received 22 February 2015
Received in revised form 13 April 2015
Accepted 15 April 2015
Available online 22 April 2015

Keywords:
Butanol
Hexanol
Syngas fermentation
Medium development
Culture techniques

ABSTRACT

Clostridium carboxidivorans was grown on model syngas ($CO:H_2:CO_2$ [70:20:10]) in a defined nutrient medium with concentrations of nitrogen, phosphate and trace metals formulated to enhance production of higher alcohols. *C. carboxidivorans* was successfully grown in a limited defined medium (no yeast extract, no MES buffer and minimal complex chemical inputs) using an improved fermentation protocol. Low partial pressure of CO in the headspace, coupled with restricted mass transfer for CO and H_2, was required for successful fermentation. In the absence of substrate inhibition (particularly from CO), growth limitation increased production of alcohols, especially butanol and hexanol. Concentrations of butanol (over 1.0 g/L), hexanol (up to 1.0 g/L) and ethanol (over 3.0 g/L) were achieved in bottle fermentations. Minimal medium and controlled supply of CO and H_2 should be used in characterizing candidate butanol and hexanol producing strains to select for commercial potential.

1. Introduction

Bacteria capable of autotrophic growth on CO and H_2 have been shown to produce ethanol, butanol, hexanol and other alcohols as byproducts of energy conservation for growth and cell function (Liou et al., 2005; Liu et al., 2014a; Phillips et al., 1994; Worden et al., 1991). *Clostridium carboxidivorans* derives energy and carbon for growth from CO and H_2, and produces alcohols by reduction of organic acids formed in the mechanism for energy conservation and synthesis of cell material (Liu et al., 2014b).

CO and H_2 are components of syngas derived from gasification of biomass, coal and wastes, or reforming of natural gas, and from manufacturing waste gases, particularly from steel production. Use of these feedstocks can reduce waste generation and contribute to meeting the demand for energy and chemical production without using food resources.

Ethanol is a major component in achieving the goals of renewable fuels proposed by the US Department of Energy (EIA, 2012). However, higher alcohols such as butanol and hexanol have been the focus of recent research initiatives. Butanol can be directly incorporated in current fuel infrastructure, and thus is viewed as a "drop-in" fuel (EERE, 2012). In acetone–butanol–ethanol (ABE) fermentation, molasses or corn starch was converted to a mixed solvent product by strains of *Clostridium* (Rogers et al., 2006). Syngas fermentation typically uses clostridial bacteria, and the initial investigation of the fermentation benefited from experience and theory of the ABE fermentation (Wilkins and Atiyeh, 2011; Worden et al., 1991).

Production of solvents in the ABE fermentation followed a shift from production of acetic and butyric acids that was induced by nutrient limitation for nitrogen or phosphate (Rogers et al.,

* Corresponding author at: 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).
[1] Present address: The School of Environment and Natural Resources, The Ohio State University, Columbus, OH, United States

http://dx.doi.org/10.1016/j.biortech.2015.04.043
0960-8524/

2006). In addition to enhancing formation of a specific product, medium design is driven by balancing the expense for nutrient supply with the requirement for an active syngas fermenting bacterial culture to achieve fermentation productivity (Gao et al., 2013). Design of production medium progresses from the isolation medium employed to promote growth (Saxena and Tanner, 2011; Tanner, 2007) to minimized inputs that support syngas fermentation activity in a controlled growth environment (Phillips et al., 2011).

Enzyme activities in the Wood–Ljungdahl pathway are subject to the availability of chemical nutrients like metals or cofactors provided in the medium. For example, the B vitamin pantothenate is a component of coenzyme A and can limit the rate of carbon fixation during syngas fermentation (Gaddy et al., 2007; Phillips et al., 1993). Production of ethanol at more than 20 g/L was achieved in syngas fermentation by supplying CO and H_2 balanced with the kinetic capability of *Clostridium ljungdahlii* in a minimal medium (Gaddy et al., 2007; Phillips et al., 1993).

The objective of the present study is to develop medium and culture methods for production of higher alcohols using syngas fermentation. Growth of *C. carboxidivorans* during syngas fermentation in defined medium without complex nutrients is reported in the present study. Accordingly, a minimized medium was sought to promote selectivity for butanol and hexanol in a production fermenter. Fermentation was monitored through successive modifications of the defined medium to assess the potential for commercial production of butanol and hexanol from syngas. The performance of *C. carboxidivorans* in batch culture using minimal defined medium was highly dependent on culture technique. A program of agitation, gas supply and pH adjustment was developed to sustain timely growth of *C. carboxidivorans* for culture maintenance and syngas fermentation.

2. Methods

C. carboxidivorans strain P7 (Liou et al., 2005) was used in all experiments. Cited costs are for laboratory grade chemicals purchased in quantities of 1 kg or less, with prices updated December 10, 2014 (Sigma–Aldrich, St. Louis, MO, or Fisher Scientific, Waltham, MA). Selected nutrient components were studied in serum bottle batch fermentation to allow replication of experiments in a timely manner.

2.1. Base defined medium (BDM) development

The base defined medium (BDM) was developed from rich medium used at the University of Oklahoma to maintain bacterial cultures and identify potential for alcohol production (Tanner, 2007). The developed medium contained no yeast extract and only three vitamins (biotin, pantothenic acid and PABA). Cu (1 × and 0×) and Mo (1 × and 10×) were varied in developing the BDM. The compositions of rich medium and BDM medium are shown in Table 1.

The effects of copper (Cu) and molybdenum (Mo) and were studied in 160-mL serum bottles with 20 mL of fresh medium prepared in an anaerobic chamber (Liou et al., 2005). The sealed bottles were autoclaved and cooled before the addition of 0.12 mL of a solution containing 2% (w/v) of each cysteine (Sigma–Aldrich, St. Louis, MO, USA) and $Na_2S{\cdot}9H_2O$. The headspace gas was replaced (Liou et al., 2005) with model syngas mix ($CO{:}H_2{:}CO_2$ [70:20:10]) and the pressure was set to 310 kPa (abs). The bottles were warmed to 37 °C and then inoculated with 10% (v/v) active culture. Experimental treatments, run in triplicate, were inoculated with cells grown in the experimental medium that had been inoculated with *C. carboxidivorans* maintained in yeast extract medium. The inoculated bottles were kept horizontal without shaking at 37 °C, and the pressure was returned to 310 kPa by the addition of pure CO on days 6 and 12 after inoculation. Liquid samples were taken on day 15 and analyzed for product concentrations by gas chromatography (GC), pH, and OD at 600 nm (Liou et al., 2005).

2.2. Removal of buffer and reduction of N and P from BDM medium

The composition of the BDM was modified to reduce cost, first by the elimination of the buffer 2-(N-morpholino)ethanesulfonic acid (MES) and associated base (KOH) that together account for over 90% of the BDM cost, and then reducing the concentration of ammonium and phosphate that comprise over 40% of the cost of the remaining medium. Nitrogen and phosphate limitation were identified as key factors in solvent production by *Clostridium* species used in commercial ABE fermentation (Rogers et al., 2006). A baseline for batch fermentation in shaken serum bottles was established using the BDM, then MES was removed and nitrogen was decreased. NH_4Cl (ammonium chloride) was replaced with a lesser amount of NH_3 (ammonia added as ammonium hydroxide, NH_4OH, an inexpensive bulk chemical). Fermentation was studied using 4% of nitrogen (designated N1) and 25% of phosphate (designated P1) compared to the BDM concentrations (N0P0 in similar designation, see media N1P0 and N1P1 in Table 1).

The syngas fermentations with modified BDM media were performed in 282-mL serum bottles (Wheaton, Millville, NJ, USA) with 30 mL of fresh medium boiled, purged with N_2 and dispensed into N_2 purged bottles. The bottles were sealed, autoclaved and cooled before an addition of 0.15 mL of a solution containing 4% (w/v) of each cysteine (Sigma–Aldrich) and $Na_2S{\cdot}9H_2O$. The gas in the headspace was purged with model syngas and the gas pressure was set to a target value of 238 kPa or less. The syngas was mixed from pure gases (H_2, CO and CO_2) using thermal conductivity mass flow controllers (Porter Instrument Division, Hatfield, PA, USA) to the target composition ($CO{:}H_2{:}CO_2$ [70:20:10]). The bottles were warmed to 37 °C and then inoculated with 5% (v/v) active culture. Experimental treatments were run in triplicate. Inoculated bottles were kept upright on a shaker (New Brunswick Scientific, Enfield, CT, USA) at 37 °C and shaken at 0–150 rpm, dependent on tolerance for the substrate gas. After the base addition for medium preparation, no effort was made to control pH.

2.3. Effect of trace metals

Molybdenum (Mo), zinc (Zn), tungsten (W) and nickel (Ni) reportedly affected solvent production (Andreesen and Makdessi, 2008; Saxena and Tanner, 2011; Strobl et al., 1992; White and Simon, 1992). Medium (designated as P7Mt in Table 1), with the low concentrations of nitrogen and phosphate (N1P1) and 20% of Mo and Zn concentrations from the BDM, was used to study trace metals. Concentrations of W, Ni and both W and Ni were doubled as variations in the trace metals experiment with P7Mt medium. The syngas fermentation was performed as in Section 2.2 in 282-mL serum bottles with the volume of fresh medium increased to 50 mL to accommodate more samples.

2.4. Development of cultivation protocol

The fermentation technique was modified to minimize the lag phase following inoculation and shorten overall fermentation time. The medium adopted was minimal defined medium (P7Mt medium in Table 1) prepared as in Section 2.3. The minimal defined medium with increased W and Ni (P7MtWNi) was used with three syngas compositions to study the microbial cultivation procedure. In the revised protocol, serum bottles containing 50 mL of medium were prepared with 140 kPa of ($CO{:}H_2{:}CO_2{:}N_2$ [20:5:15:60]) syngas (28 kPa CO) and inoculated with 20% v/v from a week old

Table 1
Composition and cost of media used in the present study.

Component	Formula	Rich medium[b]	BDM[c]	N1P0[d]	N1P1[d]	P7Mt[e]
		Concentration (mg/L)				
Yeast extract	Undefined	500	0	0	0	0
MES	$C_6H_{13}NO_4S{\cdot}H_2O$	10000	10000	0	0	0
Potassium hydroxide	KOH	1165	1165	0	0	0
Ammonia	NH_3	0	0	38.7	38.7	38.7
Minerals						
Ammonium chloride	NH_4Cl	2000	2000	0	0	0
Calcium chloride	$CaCl_2{\cdot}2H_2O$	80	80	80	80	80
Magnesium sulfate	$MgSO_4{\cdot}7H_2O$	400	400	400	400	400
Potassium chloride	KCl	200	200	200	200	200
Potassium phosphate	KH_2PO_4	200	200	200	50	50
Trace metals						
Cobalt chloride	$CoCl_2{\cdot}6H_2O$	2	2	2	2	2
Cupric chloride	$CuCl_2{\cdot}2H_2O$	0.2	0	0	0	0
Ferrous ammonium sulfate	$FeH_{20}N_2O_{14}S_2$	8	8	8	8	8
Manganese sulfate	$MnSO_4{\cdot}H_2O$	10	10	10	10	10
Nickel chloride	$NiCl_2{\cdot}6H_2O$	0.2	0.2	0.2	0.2	0.2
Nitrilotriacetic acid	$C_6H_9NO_6$	20	20	20	20	20
Sodium molybdate	$Na_2MoO_4{\cdot}2H_2O$	0.2	2	2	2	0.4
Sodium selenate	Na_2SeO_4	0.2	0.2	0.2	0.2	0.2
Sodium tungstate	$Na_2WO_4{\cdot}2H_2O$	0.2	0.2	0.2	0.2	0.2
Zinc sulfate	$ZnSO_4{\cdot}7H_2O$	2	2	2	2	0.4
Vitamins						
Biotin	$C_{10}H_{16}N_2O_3S$	0.02	0.02	0.02	0.02	0.02
Pantothenic acid	$Ca(C_9H_{16}NO_5)_2$	0.05	0.05	0.05	0.05	0.05
PABA	$C_7H_7NO_2$	0.05	0.05	0.05	0.05	0.05
Resazurin						
Resazurin	$C_{12}H_6NO_4Na$	1	1	1	1	1
Cysteine/Sulfide						
Cysteine	$C_3H_7NO_2S$	120	200	200	200	200
Sodium sulfide	$Na_2S{\cdot}9H_2O$	120	200	200	200	200
Total cost per liter[a]		$6.22	$6.13	$0.25	$0.23	$0.23

[a] Cost was based on laboratory grade chemicals on December 10, 2014.
[b] Rich medium was used to grow stock culture.
[c] Base defined medium (BDM) was developed and used for baseline.
[d] N1P1 and N1P0 are media with no MES and reduced N and P.
[e] P7Mt is N1P1medium, modified with reduced Mo and Zn supplemented with W and Ni.

culture. The bottles were not shaken at 37 °C for 60 h, then shaken upright at 125 rpm. The headspace gas was replaced with syngas ($CO:H_2:CO_2$ [40:30:30]) to 100 kPa (40 kPa CO) at about 84 h, and replaced again to 140 kPa (56 kPa CO) at 110 h. The headspace gas was again replaced to 140 kPa at 144 h with agitation increased to 150 rpm and the addition of NH_4OH (0.4 mL of 1.5 M NH_4OH into 60 mL fermentation) to raise pH of fermentation broth from 4.50 to near 4.75. The bottle was used as inoculum in the weekly transfer after another 0.4 mL addition of NH_4OH solution at 168 h, then gas was replaced with ($CO:H_2:CO_2$ [40:30:30]) syngas to 224 kPa (90 kPa CO) and shaking was reduced to 125 rpm. The remaining culture was suitable for inoculation of fermentation experiments during the second week.

2.5. Analytical procedures

Gas pressure in the headspace was monitored, and gas composition determined was by GC analysis (Agilent Technologies, Wilmington, DE, USA) as described previously (Liu et al., 2014a). Cell concentration determined spectroscopically as optical density (1.0 OD unit is approximately 0.4 g dry cell weight per liter, g/L) at 660 nm using Cole Parmer UV-2100 spectrophotometer (Vernon Hills, IL, USA). Liquid product concentrations were determined after centrifugation of samples 10 min at 13,000 rpm to remove cells. The pH was measured using a pH meter from 1.5 mL samples. Supernatant was mixed with an equal amount of 0.085 M HCl to ensure all acetate was converted to the volatile free acetic acid form before solvent analysis. The liquid product concentrations determined by GC with a DB-FFAP capillary column (Agilent Technologies, Wilmington, DE, USA) and quantitation using a flame ionization detector (FID) as described previously (Liu et al., 2014a).

3. Results and discussion

3.1. Rich medium

C. carboxidivorans growth (rich medium in Table 1) with 0.5 g/L yeast extract achieved up to 0.231 h^{-1} specific growth rate (3 h doubling time). However, the rich medium is expensive, which prohibits its use as a production medium (Table 1).

3.2. BDM (base defined medium) development

3.2.1. Medium development of BDM without shaking

Ethanol, butanol and hexanol were produced in the defined BDM medium (Saxena and Tanner, 2008). Trace metals, particularly Mo, were required for growth and product formation, and either elimination of Cu (treatment −Cu), or a tenfold increase in Mo (treatment + 10 × Mo) enhanced product formation (Table 2). However, when Cu was removed and Mo was increased tenfold (treatment −Cu/+10 × Mo), the production of ethanol, butanol and hexanol was increased at the expense of the corresponding acid. These fermentations were performed without shaking and with CO up to 70% and pressures up to 307 kPa (absolute). Concentrations of ethanol at 2.1 g/L (45 mM), butanol at 1.1 g/L

Table 2
Summary of products from fermentation medium development with *C. carboxidivorans*.

Treatment	Ethanol (g/L)	Acetic acid (g/L)	Butanol (g/L)	Butyric acid (g/L)	Hexanol (g/L)	Hexanoic acid (g/L)
+Trace metals[a]	1.12	1.05	0.93	0.12	0.09	0.13
−Trace metals[a]	0.16	0.13	0.03	0.00	0.00	0.00
−Cu[a]	1.53	2.02	0.90	0.55	0.74	0.13
−Mo[a]	0.40	0.16	0.08	0.00	0.00	0.00
+10 × Mo[a]	1.35	2.05	0.87	0.64	0.88	0.11
−Cu/+10 × Mo[a,b]	2.05	0.73	1.09	0.32	0.94	0.36
BDM[b,c]	3.25	0.25	0.83	0.01	0.24	0.01
N1P0[c] (low N)	2.20	0.56	0.37	0.05	0.07	0.06
N1P1[c] (low N and P)	3.01	0.50	0.76	0.06	0.16	0.04
P7Mt[d]	1.48	0.55	0.41	0.08	0.13	0.04
P7MtW[e]	1.64	0.53	0.37	0.07	0.11	0.04
P7MtNi[f]	1.28	0.60	0.20	0.04	0.06	0.03
P7MtWNi[g]	1.42	0.58	0.28	0.06	0.08	0.04
P7MtWNi[h] P21 40CO	1.58	1.20	0.36	0.23	0.07	0.05
P7MtWNi[i] P21 70CO	3.01	0.38	1.00	0.14	0.31	0.06

[a] ($CO:H_2:CO_2$ [70:20:10]) syngas at 307 kPa, not shaken.
[b] Medium designated "−Cu/+10 × Mo" was chosen as the base defined medium (BDM).
[c] Medium composition shown in Table 1 and ($CO:H_2:CO_2$ [70:20:10]) syngas.
[d] Medium composition shown in Table 1, N1P1 medium with less Mo (20%) and Zn (20%) and ($CO:H_2:CO_2$ [70:20:10]) syngas.
[e] P7Mt medium with doubled W and ($CO:H_2:CO_2$ [70:20:10]) syngas.
[f] P7Mt medium with doubled Ni and ($CO:H_2:CO_2$ [70:20:10]) syngas.
[g] P7Mt medium with doubling both W and Ni and ($CO:H_2:CO_2$ [70:20:10]) syngas.
[h] P7MtWNi medium with new protocol and ($CO:H_2:CO_2$ [40:30:30]) syngas.
[i] P7MtWNi medium with new protocol and ($CO:H_2:CO_2$ [70:20:10]) syngas.

(15 mM) and hexanol at 0.9 g/L (9 mM) were achieved after 360 h (Table 2). This trace metals modification resulted in reproducible, rather than sporadic, formation of hexanol from CO by *C. carboxidivorans*. The modified medium with no Cu and tenfold Mo (designated as −Cu/10 × Mo) was chosen as the base defined medium (BDM) for subsequent fermentations.

3.2.2. Medium development of BDM with shaking

Syngas fermentations in shaken serum bottles with frequent sampling of gas and liquid were pursued with the goals of increasing production of alcohols and shortening the fermentation time through further medium development. Growth of *C. carboxidivorans* was slow in defined medium without yeast extract and typically achieved specific growth rates over 0.02 h^{-1} (35 h doubling time). A lag in start of the fermentation was noted with ($CO:H_2:CO_2$ [70:20:10]) syngas at initial gas pressures ranging from 140 kPa (abs) up to 238 kPa. An effective strategy for initial cultivation was to maintain the freshly inoculated bottles unshaken for the first 48 h of fermentation as for the fourth passage of *C. carboxidivorans* in the P7 BDM (P7P4 bottles) in Fig. 1B.

Another strategy to establish cell growth was to limit initial gas pressure in the headspace to lower than 140 kPa. Cell growth and uptake of CO and H_2 were increased by increased agitation only after growth and gas consumption were established. The pH dropped slowly from the initial value of about 5.9 to near 4.8 as an acetate buffer formed supplanting the MES buffer. Cell concentration around 1.0 OD was supported by the BDM (Fig. 1A).

The P7P4 bottles were not shaken for 60 h after inoculation and established an active fermentation that consumed gas and grew cells when placed on the shaker at 50 rpm. In contrast, the seventh transfer in the BDM (P7P7 in Fig. 1B) was shaken at 50 rpm immediately after inoculation, and did not consume gas or grow cells until agitation was stopped for 24 h before the sampling at 232 h. Agitation was then reset to 75 rpm, and the fermentation proceeded to match the P7P4 results with 3.0 g/L ethanol, 0.8 g/L butanol and 0.25 g/L hexanol, with a total fermentation time of 600 h (Fig. 1C, E, F).

3.3. Removal of buffer and reduction of N and P

The Good's buffer MES accounted for 94% of the cost of the BDM, while the cost of NH_4Cl for nitrogen contributed 2.3%. MES was removed from the medium and NH_4Cl was replaced by a lower concentration of NH_4OH in medium preparation for comparison to the BDM fermentations. NH_3 or NH_4OH dissociates at pH below 10 to NH_4^+ and OH^- and acts as a strong base to raise pH in concert with the bicarbonate/CO_2 (pK_a 6.3) and acetate/acetic acid (pK_a 4.75) buffer systems present in the syngas fermentation.

NH_4^+ is a suitable nutrient source of nitrogen for production of cell mass. Incorporation of NH_4^+ into cell mass as protein (generally R-NH_3^+) results in no change of acidity, so that NH_4OH added to the syngas fermentation generates a permanent acetate buffer. Without MES buffer, fermentation pH is stabilized by the concentration of CO_2 dissociated to bicarbonate (HCO_3^-) and subsequently by acetic acid (CH_3COOH) neutralized to acetate (CH_3COO^-). Without the MES, medium pH decreased quickly below 5.0 and eventually below 4.0 during fermentation. Low pH, below 5.0, thermodynamically favors reduction of organic acids to alcohol, while pH below 4.0 can inhibit cell function (Kundiyana et al., 2011; Worden et al., 1991). NH_4OH replaced NH_4Cl as the chosen source of N and, combined with a much lower concentration of NH_4^+ used, reduced the cost of NH_4^+ by 96% (although cysteine at a much higher cost adds 1.65 mM nitrogen, about 72% of that derived from NH_4^+).

Commercial ABE fermentation used nitrogen and phosphate limitation to induce solvent formation (Rogers et al., 2006). In the present study, initial concentrations of N and P were chosen based on a cell composition model to impose nutrient limitations on the syngas fermentation with *C. carboxidivorans*. The reduced nitrogen concentration was set at 4.08 mM (N1) compared to 86.1 mM in the base defined medium (N0). The reduced phosphate concentration was set at 0.37 mM (P1) compared to 1.48 mM in the defined base medium (P0). Growth and product profiles of *C. carboxidivorans* were compared in bottles with reduced N and standard P (N1P0) and bottles with reduced N and P (N1P1) as shown in Fig. 2.

Growth proceeded slowly with a lag over the first 200 h after inoculation (Fig. 2A). At 215 h, growth was noted and shaking at 50 rpm was started for all bottles (Fig. 2B). Growth continued

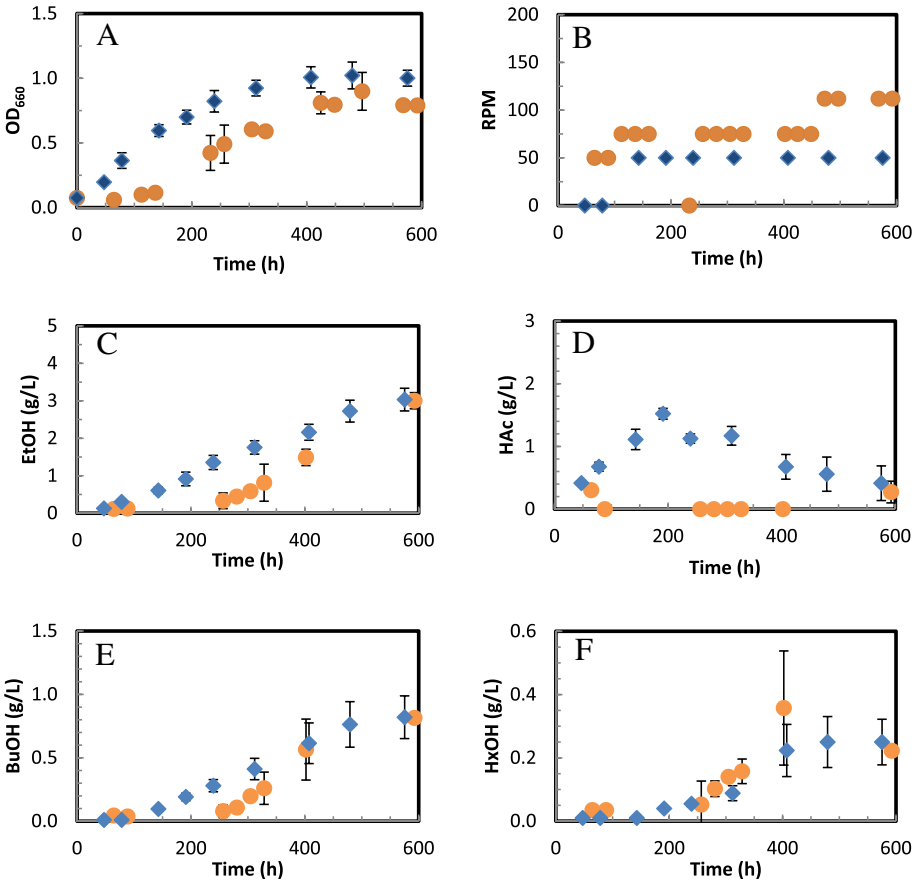

Fig. 1. (A) Growth (B) agitation speed (C) ethanol (D) acetic acid (E) butanol and (F) hexanol profiles during syngas fermentation by *C. carboxidivorans* for averaged triplicate bottles in the fourth and seventh passages in BDM, the base defined medium, bottles ♦ P7P4 and ● P7P7. Error bars represent ± one standard deviation.

and shaking was increased to 75 rpm after 411 h to increase mass transfer. At 503 h, the depleted gas was replaced with fresh syngas mix at the initial pressure of 102 kPa in all bottles. Ethanol, butanol and hexanol production began as the initial gas charge was depleted and continued after gas was replenished (Fig. 2C, E, F). Performance in all bottles was similar until the gas was replaced with fresh syngas ($CO:H_2:CO_2$ [70:20:10]), but the performance diverged after the introduction of CO at 70 kPa partial pressure with the shaker set at 75 rpm. The pH of all bottles dipped below 4.0 before the gas was replaced at 503 h, but moved above 4.0 as acetic acid was reduced to ethanol (Fig. 2C and D) with the addition of fresh CO and H_2 in the headspace. *C. carboxidivorans* culture became inhibited at low pH and high supply of CO from the fresh syngas and high agitation. A limitation for N may be indicated at 0.90 OD (implying 16% N in cell dry weight versus 14% expected), but the growth was likely restricted by the gas supply technique used and the low pH established. Butanol and hexanol accumulated and, as for ethanol compared to acetic acid, a higher ratio of alcohol to acid was achieved in media BDM, N1P0 and N1P1 as cell growth slowed (Table 2).

A limitation for phosphate was observed when cell growth stopped as OD reached 0.20 in two bottles, representing a maintenance transfer of *C. carboxidivorans* in the N1P1 medium, because a phosphate solution was not added in the medium preparation. The initial phosphate concentration in these bottles was only 0.019 mM or 5% of the intended level. Growth resumed when the phosphate solution was added after 765 h of the fermentation and OD reached 0.60 before the liquid volume was exhausted from sampling. The apparent growth limitation caused by low phosphate concentration sets a growth requirement for phosphorus at 1 OD unit from 0.095 mM P, or phosphorus is about 0.3% of the cell dry weight compared to 3% expected in the model cell composition. Using this requirement for P, the growth limit for P in the N1P1 medium would be about 4.00 OD and the N1P1 medium is not phosphate limited. Although ethanol was favored under the phosphate limitation, accumulation of butanol and hexanol was not increased and phosphate limitation did not increase butanol and hexanol in syngas fermentation.

3.4. Effect of trace metals

The adjusted trace metals concentrations in the medium were intended to increase accumulation of butanol and hexanol. Metal atoms are often active site centers for substrate binding or electron transfer in the Wood–Ljungdahl pathway (Ragsdale, 2006, 2008). Nickel (Ni) is part of the active center of carbon monoxide dehydrogenase (CODH), the enzyme that receives electrons from CO as one of the first reactions in the Wood–Ljungdahl pathway. Aldehyde oxidoreductase (AOR) and formate dehydrogenase are critical tungsten dependent enzymes in alcohol production. Tungsten (W) is a trace contaminant in molybdate with a molar ratio of about 1:10,000 inferred from activity of W specific AOR (10 μM molybdenum is equivalent to 1 nM W) (Andreesen and Makdessi, 2008). Mo is an analog of W and is suspected to bind in W active sites in enzymes, particularly in the AOR involved in reduction of organic acids to alcohols. Iron (Fe) is required for many electron transfer centers in enzymes and cofactors, with 2–8 atoms of Fe required per enzyme (Ragsdale, 2006, 2008). The requirement for Fe in acetogenic cell function should exceed that of other metals by orders of magnitude. In the BDM, Mo and zinc

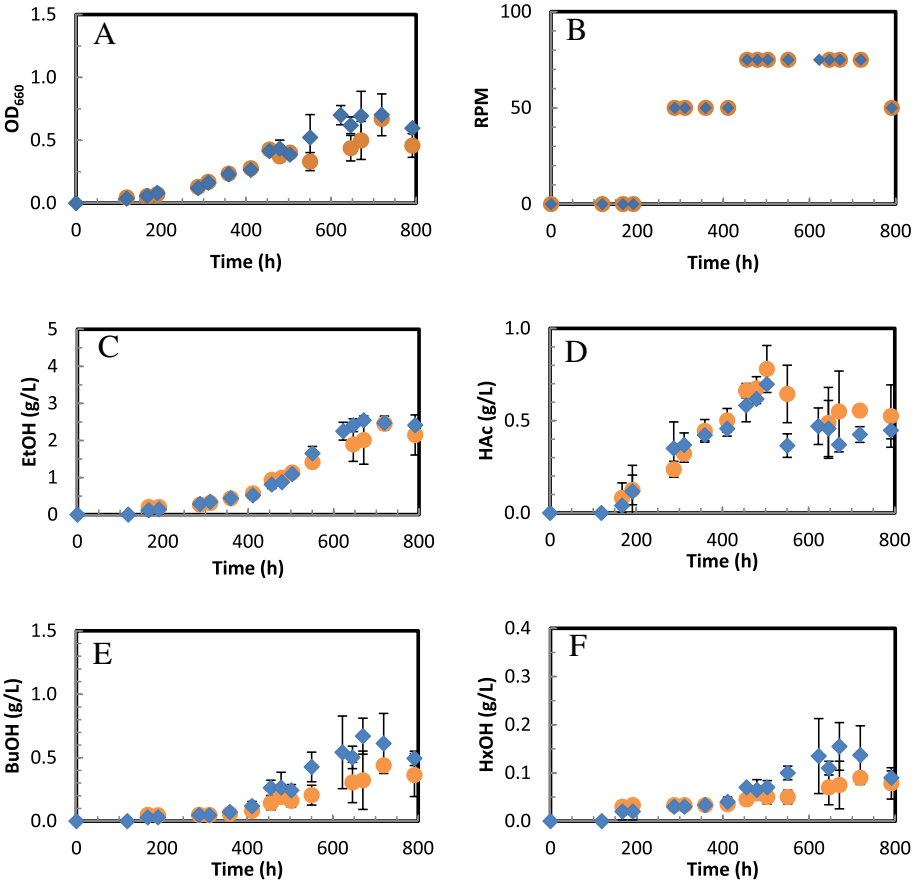

Fig. 2. (A) Growth (B) agitation speed (C) ethanol (D) acetic acid (E) butanol and (F) hexanol profiles during syngas fermentation by *C. carboxidivorans* for averaged triplicate bottles in reduced N and P medium, bottles ♦ P7N1P1 (reduced N and P) and ● P7N1P0 (reduced N with P as in BDM). Error bars represent ± one standard deviation.

(Zn) were deemed to be in excess supply as the molar concentrations were similar to that of Fe.

All bottles were kept upright on the shaker but not shaken for the first 90 h of fermentation to initiate growth and culture activity. After cell growth was noted at 90 h, the shaker was started at 30 rpm to increase gas transfer. A lag phase in the first 400 h, characterized by slow cell growth and CO uptake, was seen in all trace metal treatments. Small variations in product concentrations were observed in the trace metals treatments (Table 2). However, the P7Mt fermentations had long lag phase and low productivity. This is attributed to the inhibition of *C. carboxidivorans* by the substrate gas with the fermentation protocol used.

3.5. Development of cultivation protocol

The fermentation technique, particularly the supply of CO and H_2, is most important in determining the results of fermentation. A revised procedure was developed for maintenance transfers of *C. carboxidivorans* in the minimal defined P7MtWNi medium with three syngas compositions to improve the microbial cultivation protocol. Two lines of *C. carboxidivorans* were maintained through 39 weekly transfers using this procedure. The average specific growth rate observed in the first week was 0.01–0.03 h^{-1}. The duration of these fermentations was 336 h, by design, and consistently produced ethanol, butanol and hexanol.

The revised maintenance protocol was used with increased sampling in the 21st passage in P7MtWNi medium (P7MtWNi P21C) to compare fermentation by *C. carboxidivorans* under the ($CO:H_2:CO_2$ [40:30:30]) syngas used for maintenance to the ($CO:H_2:CO_2$ [70:20:10]) syngas used in the original studies. The average results for triplicate bottles are shown in Fig. 3. The average specific growth rate observed in the first week was 0.02–0.03 h^{-1} for both treatments. Cell growth and production of alcohols were higher using the ($CO:H_2:CO_2$ [70:20:10]) syngas. The differences in growth and production increased after the gas was replaced at 135 h and shaking was increased from 125 rpm to 150 rpm at about 142 h. The culture with lower cell concentration under ($CO:H_2:CO_2$ [40:30:30]) syngas slowed from inhibition imposed by high CO delivered to fewer cells. The more robust culture grown under ($CO:H_2:CO_2$ [70:20:10]) syngas used additional reductant from CO and H_2 to convert acids to alcohols, as shown by the decreasing concentration of acetic acid (Fig. 3D). Product concentrations were similar to the best observed for BDM medium in 282-mL serum bottles, with ethanol at 3.0 g/L, butanol at 1.0 g/L and hexanol at 0.3 g/L (Table 2). About 47% more ethanol was produced in P7MtWNi P21 70CO medium in the 282-mL serum bottles compared to treatment −Cu/+10 × Mo in the 160-mL serum bottles. However, three times more hexanol and 9% more butanol were produced in the −Cu/+10 × Mo treatment. This was due to the difference in the syngas fermentation conditions used in the two treatments.

The results showed that appropriate medium formulation and fermentation technique can enhance butanol and hexanol production in syngas fermentation. No nutrient limit was identified for any of the studied medium components (N, Zn, Mo, W or Ni) except for P as phosphate. Phosphate limitation increased the ratio of ethanol to acetic acid in syngas fermentation, but was not shown to increase the concentration of butanol or hexanol. The

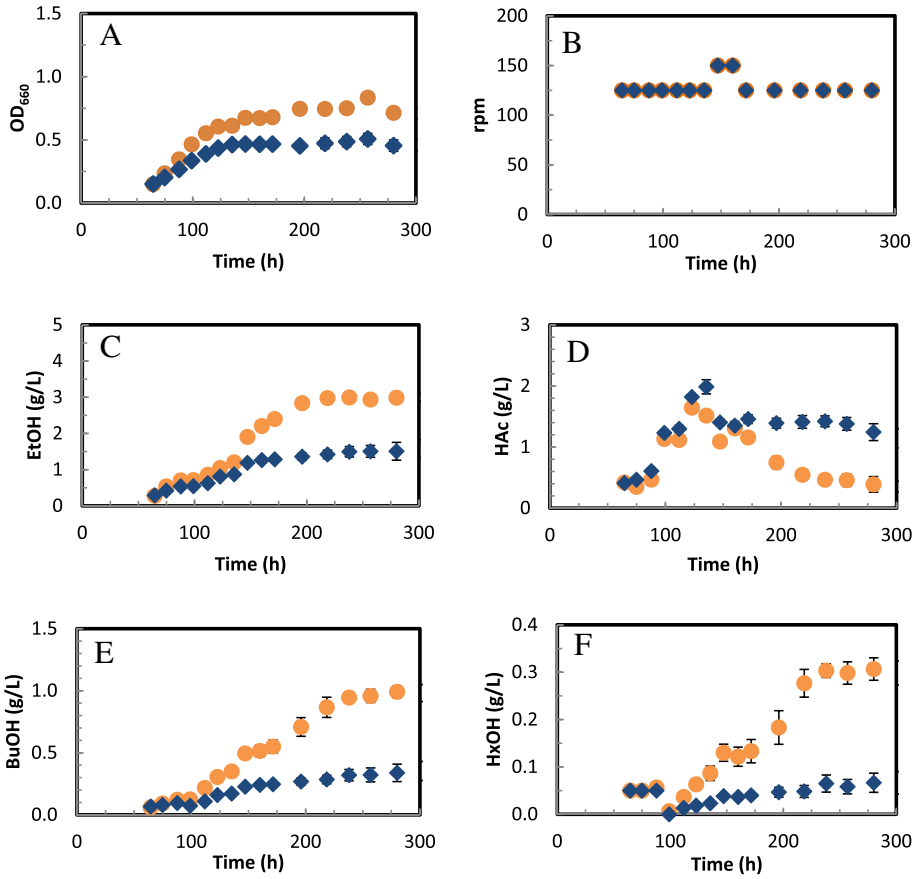

Fig. 3. (A) Growth (B) agitation speed (C) ethanol (D) acetic acid (E) butanol and (F) hexanol profiles during syngas fermentation by *C. carboxidivorans* for averaged triplicate bottles in minimal defined medium P7MtWNi using the revised protocol and ● ($CO:H_2:CO_2$ [70:20:10]) syngas or ♦ ($CO:H_2:CO_2$ [40:30:30]) syngas. Error bars represent ± one standard deviation.

accumulation of alcohols occurred as the fermentation approached a growth limit, as pH dropped below 4.75 and cell activity slowed to a stationary phase.

MES buffer was unnecessary in production medium for syngas fermentation, as shown previously (Gao et al., 2013; Phillips et al., 2011). The current study demonstrated the sharp reduction of the nitrogen and mineral inputs that account for a large portion of the defined medium cost after MES is removed (Table 1). Ammonia (NH_4OH) was shown to be suitable for both the source of nitrogen for cell growth and as a base used to form an acetate buffer to stabilize fermentation pH. Medium cost was reduced from an estimated $6.22–$0.23 per liter of medium and several remaining components are known to be in excess or unnecessary demonstrating potential for further cost reduction.

The medium development study suffered from long periods of slow culture activity caused by high CO supply. A mixed syngas containing 70% CO at 307 kPa total pressure was chosen for the initial experiments, as CO was expected to promote cell growth and alcohol production, and the same mixture ($CO:H_2:CO_2$ [70:20:10]) was used throughout these experiments to maintain consistency in procedure. However, high CO concentration inhibits H_2 uptake by the hydrogenase enzyme (Ragsdale and Ljungdahl, 1984), and can cause shifts in the oxidation state of enzymes sufficient to inhibit cell function. In the present study using the revised protocol, lower CO partial pressure from an initial total pressure of only 100 kPa, coupled with lower shaker speed to apply less mass transfer, lessened CO inhibition after inoculation.

An increasing sensitivity to substrate inhibition, particularly from high CO concentrations, was evident as the medium moved away from the initial stock culture with a rich yeast extract formula. Complex chemical components, like carbohydrates or particular proteins or amino acids included in the nutrient medium, promote cell growth via direct incorporation into cell mass. Yeast extract contains proteins, amino acids, sugars and metals that can be incorporated directly into cell mass, and growth rates of 0.23 h^{-1} (a 3 h doubling time) were achieved in complex or rich medium used for stock culture. Energy from CO or H_2 (or carbohydrates) is required for synthesis of cell mass either from the complex chemicals or especially from CO, CO_2 and H_2 where complex chemicals are synthesized from these inorganic molecules. Specific growth rates (μ) varied in all of the media used from 0 to over 0.02 h^{-1} over any sampling period. If exponential growth is assumed, an initial cell concentration (X_0) grows to cell concentration (X) over time (t) according to $X/X_0 = \exp(\mu t)$. The use of a 5% (v/v) inoculum requires a twentyfold increase of cell concentration between inoculation and transfer as inoculum to sustain cell concentration in serial transfers. Weekly transfer of maintenance cultures (t = 168 h) should provide a ready supply of active inoculum; however, to achieve twentyfold increase of cell concentration will require μ to be at least 0.0178 h^{-1} sustained over 168 h. The specific growth rate in any of the defined medium used in the present study simply does not meet the cell production to sustain a weekly transfer, and 5% (v/v) inoculum caused the onset of substrate inhibition in the diluted culture produced by inoculation. However, if μ of 0.0100 h^{-1} can be sustained, then using 20% (v/v) inoculum will support the sixfold increase in cell concentration needed to sustain a serial transfer of culture grown on syngas in defined minimal medium. Further, the sixfold change in cell

concentration will require a similar change in gas supply and stabilize the transient conditions established in batch fermentation. Thus, a larger inoculum allows more reliable initial fermentation and a faster course of experiments as performed in the revised protocol.

In addition, the supply of the syngas components CO and H_2 was managed in the revised protocol through control of syngas pressure and mass transfer; and allowed consistent production of alcohols, particularly butanol and hexanol in addition to ethanol (Table 2). Production of acids indicates potential for alcohols, as the alcohols are formed by a four electron reduction of acid through the pathway reactions.

C. carboxidivorans grown in minimal defined medium produced up to 1.09 g/L butanol and 0.94 g/L hexanol from ($CO:H_2:CO_2$ [70:20:10]) syngas (Table 2). The butanol and hexanol produced in the current study by *C. carboxidivorans* were over twofold and eightfold, respectively, higher than in fermentation with ($H_2:CO_2:N_2$ [60:20:20]) (Ramachandriya et al., 2013). In the present study, *C. carboxidivorans* produced twofold more hexanol than in sugar fermentation by *Escherichia coli* modified with clostridial genes of the carboxylate platform (Dekishima et al., 2011; Machado et al., 2012). *C. carboxidivorans* produced similar amount of butanol and 74% more hexanol than reported for reduction of butyric and hexanoic acids in yeast fermentation broth fed to syngas fermentation using *C. ljungdahlii* (Richter et al., 2013).

The results in the present study showed that successful fermentation of syngas to butanol and hexanol can be achieved in a low cost defined medium. In addition, an improved protocol is critical to establish growth and consumption of CO and H_2, and complete the course of fermentation quickly. The use of minimal defined medium, such as the P7MtWNi formula, for serial transfer in maintenance of cultures in conjunction with a larger inoculum of 15–20% (v/v) can stabilize the fermentation conditions. The increase of shaker speed from a low initial speed can minimize lag after inoculation and direct substrate supply to sustain solvent productivity in batch fermentations. Intermediate chemicals like butanol and hexanol that are formed from acetyl-CoA have a high probability for successful development of control strategies for commercial production. Minimal medium and controlled supply of CO and H_2 should be used in isolation of candidate strains to select for commercial potential.

4. Conclusions

C. carboxidivorans consistently produced butanol and hexanol in a low cost defined medium. High CO partial pressure with mild agitation inhibits inoculum activity, but growth establishes mass transfer limitation. A programmed cultivation procedure was established to transfer *C. carboxidivorans* weekly in minimal defined medium. 20% v/v inoculum allowed a sixfold increase in cell concentration between weekly transfers. Initial CO partial pressure about 30 kPa without agitation was used with scheduled increase in CO partial pressure to 90 kPa, and agitation increased to 125 rpm and then 150 rpm. Modification of cultivation technique reduced substrate inhibition and mass transfer limitation.

Acknowledgements

This research was supported by Oklahoma NSF EPSCoR Grant No. EPS-0814361, and USDA-NIFA Project No. OKL02758 and Oklahoma Agricultural Experiment Station.

References

Andreesen, J.R., Makdessi, K., 2008. Tungsten, the surprisingly positively acting heavy metal element for prokaryotes. Ann. N. Y. Acad. Sci. 1125, 215–229.
Dekishima, Y., Lan, E.I., Shen, C.R., Cho, K.M., Liao, J.C., 2011. Extending carbon chain length of 1-butanol pathway for 1-hexanol synthesis from glucose by engineered *Escherichia coli*. J. Am. Chem. Soc. 133, 11399–11401.
EERE, 2012. Biomass Multi-Year Program Plan. <http://www1.eere.energy.gov/biomass/pdfs/mypp_april_2012.pdf> (11/15/2012).
EIA, 2012. Biofuels Issues and Trends, U.S. Energy Information Administration. <http://www.eia.gov/biofuels/issuestrends/> (11/6/2012).
Gaddy, J.L., Arora, D.K., Ko, C.-W., Phillips, J.R., Basu, R., Wikstrom, C.V., Clausen, E.C., 2007. Methods for Increasing the Production of Ethanol from Microbial Fermentation. US Patent 7285402.
Gao, J., Atiyeh, H.K., Phillips, J.R., Wilkins, M.R., Huhnke, R.L., 2013. Development of low cost medium for ethanol production from syngas by *Clostridium ragsdalei*. Bioresour. Technol. 147, 508–515.
Kundiyana, D.K., Wilkins, M.R., Maddipati, P., Huhnke, R.L., 2011. Effect of temperature, pH and buffer presence on ethanol production from synthesis gas by "*Clostridium ragsdalei*". Bioresour. Technol. 102, 5794–5799.
Liou, J.S.-C., Balkwill, D.L., Drake, G.R., Tanner, R.S., 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.
Liu, K., Atiyeh, H.K., Stevenson, B.S., Tanner, R.S., Wilkins, M.R., Huhnke, R.L., 2014a. Continuous syngas fermentation for the production of ethanol, n-propanol and n-butanol. Bioresour. Technol. 151, 69–77.
Liu, K., Atiyeh, H.K., Stevenson, B.S., Tanner, R.S., Wilkins, M.R., Huhnke, R.L., 2014b. Mixed culture syngas fermentation and conversion of carboxylic acids into alcohols. Bioresour. Technol. 152, 337–346.
Machado, H.B., Dekishima, Y., Luo, H., Lan, E.I., Liao, J.C., 2012. A selection platform for carbon chain elongation using the CoA-dependent pathway to produce linear higher alcohols. Metab. Eng. 14, 504–511.
Phillips, J.R., Klasson, K.T., Clausen, E.C., Gaddy, J.L., 1993. Biological production of ethanol from coal synthesis gas – medium development studies. Appl. Biochem. Biotechnol. 39, 559–571.
Phillips, J.R., Clausen, E.C., Gaddy, J.L., 1994. Synthesis gas as substrate for the biological production of fuels and chemicals. Appl. Biochem. Biotechnol. 45–6, 145–157.
Phillips, J.R., Remondet, N.M., Atiyeh, H.K., Wilkins, M.R., Huhnke, R.L., 2011. Designing syngas fermentation medium for fuels and bulk chemicals production. In: American Society of Agricultural and Biological Engineers Annual International Meeting 2011, August 7, 2011 – August 10, 2011, American Society of Agricultural and Biological Engineers, Louisville, KY, United States, pp. 4706–4718.
Ragsdale, S.W., 2006. Metals and their scaffolds to promote difficult enzymatic reactions. Chem. Rev. 106, 3317–3337.
Ragsdale, S.W., 2008. Enzymology of the Wood–Ljungdahl pathway of acetogenesis. Ann. N. Y. Acad. Sci. 1125, 129–136.
Ragsdale, S.W., Ljungdahl, L.G., 1984. Hydrogenase from *Acetobacterium woodii*. Arch. Microbiol. 139, 361–365.
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.
Richter, H., Loftus, S.E., Angenent, L.T., 2013. Integrating syngas fermentation with the carboxylate platform and yeast fermentation to reduce medium cost and improve biofuel productivity. Environ. Technol. 34, 1983–1994.
Rogers, P., Chen, J.-S., Zidwick, M.J., 2006. Organic acid and solvent production. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes. Springer, New York, pp. 511–755.
Saxena, J., Tanner, R.S., 2008. Production of hexanol and hexanoic acid by the acetogen *Clostridium carboxidivorans* strain P7. Abst. Ann. Meet. Am. Soc. Microbiol., I-022, 320.
Saxena, J., Tanner, R.S., 2011. Effect of trace metals on ethanol production from synthesis gas by the ethanologenic acetogen, *Clostridium ragsdalei*. J. Ind. Microbiol. Biotechnol. 38, 513–521.
Strobl, G., Feicht, R., White, H., Lottspeich, F., Simon, H., 1992. The tungsten-containing aldehyde oxidoreductase from C*lostridium thermoaceticum* and its complex with a viologen-accepting NADPH oxidoreductase. Biol. Chem. Hoppe-Seyler 373, 123–132.
Tanner, R.S., 2007. Cultivation of bacteria and fungi. In: Hurst, C.J., Crawford, R.L., Garland, J.L., Lipson, D.A., Mills, A.L., Stetzenbach, L.D. (Eds.), Manual of Environmental Microbiology. ASM Press, Washington, D.C., pp. 69–78.
White, H., Simon, H., 1992. The role of tungstate and or molybdate in the formation of aldehyde oxidoreductase in *Clostridium thermoaceticum* and Other acetogens – immunilogical distances of such enzymes. Arch. Microbiol. 158, 81–84.
Wilkins, M.R., Atiyeh, H.K., 2011. Microbial production of ethanol from carbon monoxide. Curr. Opin. Biotechnol. 22, 326–330.
Worden, R.M., Grethlein, A.J., Jain, M.K., Datta, R., 1991. Production of butanol and ethanol from synthesis gas via fermentation. Fuel 70, 615–619.