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Continuous syngas fermentation for the production of ethanol, n-propanol and n-butanol

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HIGHLIGHTS

- 6 g/L of ethanol was obtained in continuous syngas fermentor with cell recycle.
- Opportune contamination of the fermentor resulted in production of higher alcohols.
- Mixed culture was mainly made of *A. bacchi* strain CP15 and *C. propionicum*.
- Mixed culture formed a maximum of 8 g/L ethanol, 6 g/L propanol and 1 g/L butanol.
- Mixed culture presents an opportunity for higher alcohols production from syngas.

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ABSTRACT

Syngas fermentation to fuels is a technology on the verge of commercialization. Low cost of fermentation medium is important for process feasibility. The use of corn steep liquor (CSL) instead of yeast extract (YE) in *Alkalibaculum bacchi* strain CP15 bottle fermentations reduced the medium cost by 27% and produced 78% more ethanol. When continuous fermentation was performed in a 7-L fermentor, 6 g/L ethanol was obtained in the YE and YE-free media. When CSL medium was used in continuous fermentation, the maximum produced concentrations of ethanol, n-propanol and n-butanol were 8 g/L, 6 g/L and 1 g/L, respectively. n-Propanol and n-butanol were not typical products of strain CP15. A 16S rRNA gene-based survey revealed a mixed culture in the fermentor dominated by *A. bacchi* strain CP15 (56%) and *Clostridium propionicum* (34%). The mixed culture presents an opportunity for higher alcohols production from syngas.

1. Introduction

Syngas fermentation is part of the hybrid thermochemical–biochemical process, also called gasification–syngas fermentation. In this process, feedstocks such as biomass or municipal solid waste are gasified into syngas (CO, H_2 and CO_2), which is then converted into biofuels and chemicals using microbial catalysts (Wilkins and Atiyeh, 2011). Syngas can be converted into ethanol using acetogens such as *Clostridium ljungdahlii*, *Clostridium ragsdalei*, "*Clostridium carboxidivorans*" and "*Clostridium autoethanogenum*" (Phillips et al., 1993; Wilkins and Atiyeh, 2011; Ukpong et al., 2012).

Decreasing the medium cost and increasing ethanol titer and productivity are important to improve the economic feasibility of the production of biofuels and chemicals using syngas fermentation technology. Low cost nutrients such as cotton seed extract (CSE) and corn steep liquor (CSL), have been used instead of yeast extract (YE) in syngas fermentation for ethanol production (Kundiyana et al., 2010; Maddipati et al., 2011). CSL is rich in proteins, vitamins, minerals and amino acids (Lawford and Rousseau, 1997). The industrial cost of CSL was reported to be \$0.18/kg, which is about 2% of the industrial price of YE, \$9.20/kg (Maddipati et al., 2011). The use of CSL as a replacement of YE, vitamins and minerals in a 7-L fermentor with "*C. ragsdalei*" resulted in 40% more ethanol production compared to YE medium (Maddipati et al., 2011), indicating the potential of CSL as a low cost nutrient for syngas fermentation.

High ethanol titer and productivity can be achieved through higher cell concentration and improving the mass transfer of the substrate gases CO and H_2 to cells. The highest reported ethanol concentration was 48 g/L, achieved using *C. ljungdahlii* during continuous syngas fermentation with cell recycle and 4 g/L cell mass

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concentration (Phillips et al., 1993). However, only 6.5 g/L ethanol was produced during continuous syngas fermentation using *C. ljungdahlii* without cell recycle and 2.3 g/L cell mass concentration (Mohammadi et al., 2012). This showed the advantage of cell recycle to obtain high cell and ethanol concentrations during syngas fermentation. When "*C. ragsdalei*" was used in a two-stage continuous syngas fermentation with cell recycle, a maximum ethanol yield of 15 g ethanol/g cells was obtained (Kundiyana et al., 2011), which was comparable to the yield (12 g ethanol/g cells) with *C. ljungdahlii* (Phillips et al., 1993). The ability to produce high concentrations of ethanol depends on the microorganism, syngas composition and fermentor operating conditions. *Eubacterium limosum* KIST612 only produced 0.3 g/L ethanol in a continuous fermentation using pure CO and cell recycle with a 4 g/L cell mass concentration (Chang et al., 2001).

The H_2:CO ratios in previously reported syngas fermentations were mostly below 0.75, which results in lower carbon conversion efficiency to ethanol or acetic acid as shown in Table 1 (Wilkins and Atiyeh, 2011). H_2 serves as an electron source and CO can be used as either an electron source or converted by the microorganism to ethanol or acetic acid. The biochemical reaction proceed in the direction of favored thermodynamics (i.e., when Gibbs free energy, ΔG°, <0). All reactions in Table 1 are thermodynamically feasible. However, ΔG° decreases as the H_2:CO ratio increases, which makes the reaction less favorable thermodynamically. The H_2:CO ratios are affected by the gasification operating conditions and feedstock used. A H_2:CO ratio of 2 can be produced when steam and pure O_2 are used in the gasification process (Turn et al., 1998), which can result in a theoretical carbon to ethanol conversion efficiency of 100%. In addition, an H_2:CO ratio of 2 was reported from gasification of dairy biomass (cow manure) using air (Gordillo and Annamalai, 2010), indicating the potential of dairy biomass for biofuels production.

Recently, *Alkalibaculum bacchi* strains CP11^T, CP13 and CP15 were found to grow at an initial pH 8.0 and convert syngas into ethanol and acetic acid in YE medium (Allen et al., 2010; Liu et al., 2012). In bottle fermentations, strain CP15 was found to be the most promising *A. bacchi* strain for ethanol production because of its higher growth and ethanol production rate and yield compared to CP11^T and CP13 (Liu et al., 2012). However, further process development is required for strain CP15 to increase its potential use in large scale ethanol production. This includes reducing the fermentation medium cost and investigating characteristics of CP15 at larger scale than fermentation bottles. The YE medium cost was relatively expensive at $10.53/L, mostly due to the high cost of the [Tris (hydroxymethyl) methyl]-3-amino propanesulfonic acid (TAPS) buffer. Thus, the first object of the present study was to reduce the cost of CP15 fermentation medium by removal of costly TAPS buffer and replacing YE with CSL. The second objective was to scale up the fermentation from bottle to a 7-L fermentor in continuous mode with cell recycling.

2. Methods

2.1. Microorganisms

A. bacchi strain CP15 was maintained under anaerobic condition in a standard YE medium at initial pH 8.0 and 37 °C. The medium preparation and compositions were reported previously (Liu et al., 2012). Strain CP15 inoculum was prepared by sub-culturing twice to reduce the growth lag phase. Inoculum size used was 10% (v/v).

2.2. Effect of medium composition

Four fermentation media were formulated without the addition of TAPS buffer with an objective to reduce medium cost (Table 2). The composition of the YE medium with 3× minerals was similar to standard YE medium but with 3-fold more minerals, which was also similar to the amount of minerals added in the YE medium for "*C. ragsdalei*" in a previous study (Maddipati et al., 2011). In the two CSL media, 20 g/L or 50 g/L CSL replaced YE, vitamins and minerals in the standard YE medium. In addition, 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 CSL (Sigma–Aldrich, St. Louis, MO, USA) used in the present study was centrifuged at 16,000*g* for 10 min using Accuspin Micro centrifuge (Fisher Scientific, Pittsburgh, PA, USA) to remove the solids before preparing the fermentation medium. The solids were removed to allow measurement of cell mass concentration in the fermentation broth. The fermentation was done in 250-mL serum bottles (Wheaton, NJ, USA) each containing 100 mL of medium. The syngas mixture contained 20% CO, 15% CO_2, 5% H_2 and 60% N_2 by volume, which was similar to producer gas generated from the Oklahoma State University gasification facility using switchgrass (Ahmed et al., 2006). The syngas was fed in the fermentation bottles every 24 h at 239 kPa. Fermentation

Table 1
Carbon to ethanol and acetic acid conversion efficiencies from syngas with various H_2:CO ratios.

	Stoichiometry	H_2:CO	Carbon conversion efficiency (%)	Product yield from CO (%)	Gibbs free energy, ΔG° (kJ/mol)
(1)	$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$	0	33.3	16.7	−217.4
(2)	$3CO + 3H_2 \rightarrow C_2H_5OH + CO_2$	1	66.7	33.3	−157.2
(3)	$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O$	2	100.0	50.0	−137.1
(4)	$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$	0	50.0	25.0	−154.6
(5)	$2CO + 2H_2 \rightarrow CH_3COOH$	1	100.0	50.0	−114.5

Table 2
Compositions of four media formulations used in bottle fermentations.

Medium components (per L)[a]	YE (g/L)	CSL (g/L)	Minerals (mL/L)[b]	Trace metals (mL/L)[b]	Vitamins (mL/L)[b]
Standard YE medium	1	–	10	10	10
YE medium with 3× minerals	1	–	30	10	10
20 g/L CSL medium	–	20	–	10	–
50 g/L CSL medium	–	50	–	10	–

[a] TAPS absent from all media; other components added in all media include 5% $NaHCO_3$, 2.5 mL/L of 4% cysteine sulfide and 1 mL/L of 0.1% resazurin.
[b] Compositions of mineral, trace metal and vitamin stock solutions are provided in Tanner (2007).

bottles were incubated on an orbital shaker (Innova 2100, New Brunswick Scientific, Edison, NJ, USA) at 150 rpm and 37 °C. Liquid samples (2 mL) were withdrawn periodically from fermentation bottles under aseptic conditions to measure OD, pH and product concentrations. Gas samples were withdrawn from the headspace periodically to determine changes in gas composition during fermentation. All fermentations were done in triplicate.

2.3. Continuous syngas fermentation in a 7-L fermentor

Continuous syngas fermentation with cell recycle was operated in a 7-L Bioflo 415 fermentor with an in-place-sterilization system (New Brunswick Scientific Co., Edison, NJ, USA) as shown in Fig. 1. The working volume used was 3.3 L. Two six-blade Rushton impellers were mounted on the fermentor shaft separated by a distance equal to the impeller diameter as suggested by Bakker et al. (1994). Four baffles were used to avoid vortex formation and improve syngas mass transfer. Syngas was sparged into medium by a microsparger with a pore size of 10–15 μm. Two 0.2 μm filters (New Brunswick Scientific Co.) were place in the inlet and outlet gas lines. Fermentor temperature was controlled at 37 °C by a water heating jacket. A pH probe (Mettler Toledo, Columbus, OH, USA) and a dissolved oxygen (DO) probe (Mettler Toledo, Columbus, OH, USA) were used to monitor pH and DO, respectively. The DO probe was used to ensure no O_2 present in the fermentor during syngas fermentation.

Two 5-L Kimble bottles were used to feed fresh fermentation medium to the fermentor and to collect the product permeated from the cell recycle system. The cell recycle system was made of two 0.2 μm pore size hollow fiber membrane cartridges (Model CFP-2-E-5A, GE HealthCare, Piscataway, NJ, USA) and a pressure gauge at the inlet of the membrane cartridge. One membrane cartridge was in standby mode during operation. The flow rates of fresh medium and cell free permeate were controlled using peristaltic pumps (Model 7523-20, Cole-Parmer, Vernon Hills, IL, USA). The retentate from the cell recycle system containing concentrated cells was recycled back into the fermentor at a flow rate of 20 mL/min using a peristaltic pump. The syngas exiting the fermentor was cooled by passing it through a condenser kept at 5 °C using a refrigerated recirculator (1156D, VWR International, West Chester, PA, USA).

The pH in the medium was controlled using 5 N KOH and 4 N H_2SO_4. The inlet gas flow rate and compositions of CO, CO_2, H_2 and N_2 were controlled using four thermal mass flow controllers (Burkert, Charlotte, NC, USA). Three syngas compositions (Syngas I: 39% CO, 24% CO_2, 27% H_2, 10% N_2, H_2:CO molar ratio ≈ 0.7; Syngas II: 20% CO, 25% CO_2, 43% H_2, 12% N_2, H_2:CO molar ratio ≈ 2; Syngas III: 28% CO, 60% H_2, 12% N_2, H_2:CO molar ratio ≈ 2) were studied to evaluate the effect of different H_2:CO ratios on the fermentation process. The syngas flow rate was controlled at 200 sccm (standard cubic centimeter per minute) during the whole fermentation. The headspace pressure in the fermentor was 101 kPa. In addition, three medium formulations (standard YE medium, YE-free medium: no YE in standard YE medium, and 20 g/L CSL medium) were examined (Table 2). During fermentation, a foam probe was used to control the foam level not exceed 1.3 cm above the liquid medium level by the addition of 5% antifoam (Antifoam B emulsion, Sigma–Aldrich, St. Louis, MO, USA) with a peristaltic pump.

Before the beginning of the fermentation, the medium was sterilized in place at 121 °C for 30 min and cooled by purging 200 sccm N_2 at 150 rpm agitation. Then, the medium was purged for 8 h with Syngas I at 200 sccm. Before inoculation, the medium was reduced by the addition of 2.5 mL/L of 4% cysteine sulfide to scavenge any residual dissolved O_2 in the medium. The pH was adjusted to 8.0 with 5 N KOH. The fresh medium in the feeding tank and the

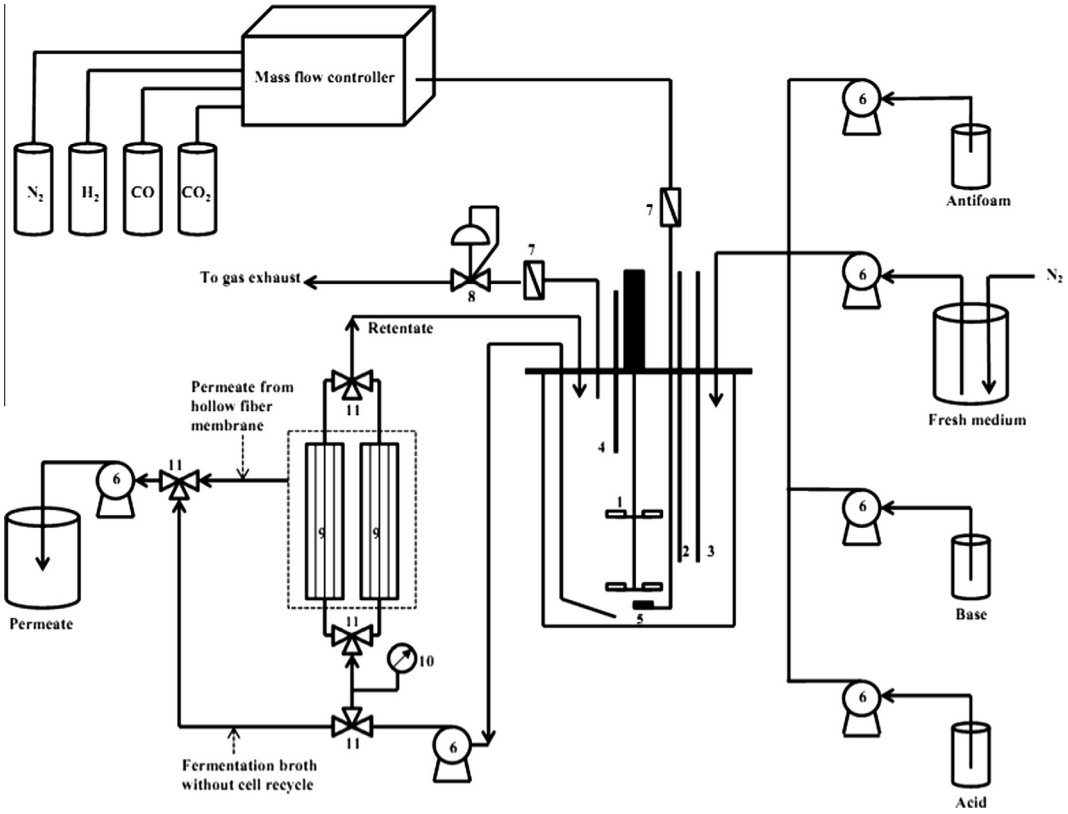

Fig. 1. Continuous syngas fermentation in 7-L Bioflo 415 fermentor setup; (1) six-blade Rushton impeller, (2) DO probe, (3) pH probe, (4) foam probe, (5) microsparger, (6) peristaltic pump, 7. 0.2 μm gas filter, (8) backpressure regulator, (9) hollow fiber membrane cartridge, (10) cell recycle system inlet pressure gauge, (11) two-way valve.

hollow fiber membrane cartridge in the cell recycle system were sterilized separately using an autoclave at 121 °C for 30 min. Before installation of the cell recycle system, the hollow fiber membrane cartridge was purged with N_2 for 10 min to remove O_2.

2.4. 16S rRNA analysis of continuous fermentation culture

A cell pellet (40 mL) culture from the 7-L fermentor at the end of the continuous fermentation was used to obtain genomic DNA with the PowerBiofilm™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). After suspending the cell pellet in 350 μL of solution "BF1", DNA isolation was conducted following the manufacturer's protocol. Cell lysis was achieved by homogenization at full speed for 1 min using a Mini-BeadBeater-8 (BioSpec Products, Bartlesville, OK, USA). Nearly full length bacterial 16S rRNA gene fragments were amplified from 50 ng of DNA in a PCR containing 1× *Taq* buffer with KCl (Fermentas, Waltham, MA, USA), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM of the forward (27F) and reverse (1391R) primers and 0.5 U of *Taq* DNA Polymerase (Fermentas) in a final volume of 25 μL. Thermal cycling was carried out in a Techne TC-512 thermal cycler (Techne, Burlington, NJ, USA) using the following conditions: initial denaturation for 3 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C; and a final extension of 10 min at 72 °C. Amplified 16S rRNA genes were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corp., Carlsbad, CA, USA). Vector-specific primers M13F and M13R were used to amplify cloned regions from 96 transformants, purified with ExoSAP-IT® (Affimetrix, Santa Clara, CA, USA) according to the manufacturer's protocol and sequenced on an ABI model 3730 capillary sequencer using the 27F primer. The resulting sequences were trimmed for quality using SeqMan Pro (version 10.1.2; DNAStar, Madison, WI, USA) and aligned with the NAST alignment tool against the Greengenes multiple sequence alignment (DeSantis et al., 2006a). The taxonomic identity for each sequence was determined with the Greengenes (DeSantis et al., 2006b) and SILVA (Pruesse et al., 2007) classifiers, as well as pairwise sequence comparison using the EzTaxon Server 2.1 (Chun et al., 2007).

2.5. Analytical procedures

2.5.1. Cell mass and product concentrations and gas analysis

The cell mass concentration was determined at 660 nm as described previously (Liu et al., 2012). The pH of liquid samples from the 250-mL bottle study was measured using a pH meter. Each liquid sample of 0.7 mL was acidified using 0.7 mL of 0.1 N HCl before solvent analysis. The solvent concentrations were measured using gas chromatography (GC) (Agilent 7890 N GC, Agilent Technologies, Wilmington, DE, USA) with a flame ionization detector (FID) and DB-FFAP capillary column (Agilent Technologies, Wilmington, DE, USA). H_2 was used as 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 of 20:1. The initial oven temperature was set at 90 °C and held for 2 min. Then it was 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. For gas analysis, a volume of 100 μL of gas from the 250-mL bottle headspace or the 7-L fermentor's exhaust line was injected in an Agilent 6890 N GC (Agilent Technologies) to determine gas composition. Argon was used as carrier gas with an initial gas flow rate of 2 mL/min and holding time of 2 min. The flow rate of 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 of 30:1. The initial oven temperature was set at 80 °C with a holding time of 5.5 min. The TCD temperature was set at 230 °C. When CSL medium was used, the sugar contents in the liquid samples were measured using an 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.

2.5.2. Statistical analysis and estimation 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 maximum cell concentration, final ethanol concentration, CO and H_2 utilization among four media in the bottle study. The calculations of ethanol yields, CO and H_2 utilization, if applicable, were described previously (Liu et al., 2012). Specific growth rate, specific gas uptake rate, dilution rate and productivity were calculated below (Shuler and Fikret, 2002):

$$\ln(X/X_0) = \mu \cdot t \tag{1}$$

$$q_x = \mathrm{GUR}/(X \cdot V) \tag{2}$$

$$D = F/V \tag{3}$$

$$\text{Productivity} = D \cdot P \tag{4}$$

where X is the cell mass concentration (g/L), X_0 is the initial cell mass concentration (g/L), μ is specific growth rate (h^{-1}), t is time (h), q_x is specific gas uptake rate (mmol gas/g cells h), GUR is CO or H_2 uptake rate (mmol/h), V is the fermentation working volume (L), D is dilution rate (h^{-1}), F is fresh medium and cell recycle system permeate flow rate via peristaltic pump (L/h) and P is ethanol concentration (g/L).

3. Results and discussion

3.1. Effect of medium composition

The maximum cell mass concentration, final ethanol concentration, CO and H_2 utilization, and cost of the four media formulations are compared in Table 3. The results showed that 20 g/L and 50 g/L CSL media produced about twice as much ethanol as the media with YE ($p < 0.05$). There was no statistical difference between the amounts of ethanol produced in standard YE medium and YE medium with 3× minerals. Also, the difference in ethanol production between the 20 g/L CSL and 50 g/L CSL media was insignificant ($p > 0.05$). Comparable maximum cell mass concentrations were obtained in the standard YE medium and YE medium with 3× minerals. However, 10% more cell mass was obtained in the 50 g/L CSL medium than in the 20 g/L CSL medium, which could be due to presence of more nutrients in the 50 g/L CSL medium.

The maximum cell mass concentration in both YE media was 50% and 38% higher than in the 20 g/L and 50 g/L CSL media, respectively. This could be due to the additional minerals and vitamins added in the YE medium. Only trace metal solution was added to the CSL medium. Ethanol was produced mostly from the conversion of syngas and not the carbohydrates in the CSL. The theoretical amounts of ethanol that could be produced from consumed monosaccharides in CSL were 0.04 g/L (1.6% of total ethanol produced) and 0.1 g/L (3.6% of total ethanol produced) in 20 g/L and 50 g/L CSL medium, respectively. The reason why more ethanol was produced in CSL medium compared to YE medium is not due to the consumed monosaccharides. The reason could be related to presence of micronutrients in CSL medium that are not available or present in lower concentrations in YE medium, which warrant further investigation.

Table 3
Maximum cell mass and final ethanol concentrations, CO and H_2 utilization and cost of the four media used during syngas fermentation by *A. bacchi* CP15 in bottle fermentations.

Medium[a]	Standard YE medium	YE medium with 3× mineral	20 g/L CSL medium	50 g/L CSL medium
Max. cell concentration (g/L)	0.33 ± 0.00^{A}	0.33 ± 0.00^{A}	0.22 ± 0.00^{C}	0.24 ± 0.02^{B}
Final ethanol (g/L)	0.84 ± 0.11^{B}	1.24 ± 0.28^{B}	2.21 ± 0.25^{A}	2.65 ± 0.40^{A}
CO utilization (%)	$42.00 \pm 0.53^{B,C}$	$45.59 \pm 0.37^{A,B}$	39.26 ± 0.21^{C}	$43.15 \pm 3.68^{B,C}$
H_2 utilization (%)	44.86 ± 1.23^{A}	47.25 ± 1.03^{A}	28.83 ± 0.86^{B}	30.44 ± 2.02^{B}
Medium cost[b] ($/L)	0.41	0.61	0.30	0.31

[A,B,C]The same letter in the same row from Duncan's multiple range test indicates there was no statistical difference at 95% level ($p > 0.05$).
[a] TAPS absent from all media.
[b] Based on industrial cost of YE and CSL (Maddipati et al., 2011) and cost of nutrients from Sigma–Aldrich in May, 2013 (St. Louis, MO, USA); Standard YE medium with TAPS cost $10.53/L.

Both CO and H_2 utilizations by CP15 were between 42% and 47% in the standard YE medium and the YE medium with 3× minerals ($p > 0.05$) (Table 3). There were no significant differences in the gas utilizations by CP15 in the two CSL media ($p > 0.05$). However, strain CP15 utilized over 45% more H_2 in YE media compared to CSL media.

All media used in the present study did not contain TAPS buffer. Compared to a previous study using standard YE medium with TAPS buffer (Liu et al., 2012), the media cost in the present study decreased by over 94% (i.e., from $10.53/L to below $ 0.61/L) as shown in Table 3. The costs of the CSL media were only 3% of the cost of the standard YE medium with TAPS and 73% of the YE medium without TAPS. The results showed that TAPS can be removed from the medium without negative effect on syngas fermentation. In addition, 20 g/L CSL can replace YE as a less expensive medium component in syngas fermentation using strain CP15 with a potential use in large scale ethanol production.

3.2. Continuous fermentation in a 7-L fermentor

3.2.1. Fermentation in yeast extract medium

The fermentation was started using the standard YE medium without TAPS (Table 2) in a liquid batch mode, in which syngas I (39% CO, 24% CO_2, 27% H_2, 10% N_2, H_2:CO molar ratio ≈ 0.7) was fed continuously. Cells started to grow after about 3 h of lag phase. During growth, acetic acid was produced as a growth-associated product (Fig. 2A). The specific CO and H_2 uptake rates increased as cells were growing (Fig. 2B). The agitation speed was set at 150 rpm. When cells were in the exponential growth phase and a cell mass concentration of 0.2 g/L (OD 0.5) was achieved at 13 h, fresh medium was fed continuously in the fermentor at a dilution rate of 0.011 h^{-1} (i.e., 8% of calculated specific growth rate 0.13 h^{-1}) and 100% cell recycle was initiated. During the fermentation period from 13 h to 120 h, the pH of the fermentation medium was controlled at 6.5 because ethanol production started at pH 6.5 with CP15 (Liu et al., 2012) and pH values below 6.5 did not support cell growth (Allen et al., 2010). When the dilution rate was set to 0.011 h^{-1}, there was a gradual increase in cell mass concentration from 0.2 g/L to 0.5 g/L at 72 h. The cell mass concentration did not change from 72 h to 120 h (Fig. 2A). The agitation speed was increased to 300 rpm at 25 h as cell concentration was increasing to supply more syngas. Ethanol production started at 40 h when the pH decreased to 6.5 and ethanol concentration increased to 0.6 g/L at 120 h. Acetic acid production started as cells started to grow and increased to 3.4 g/L at 40 h. Then, acetic acid concentration gradually decreased to 1.5 g/L at 120 h as ethanol was produced. The specific CO and H_2 uptake rates increased to 43 mmol CO/(g cells h) and 35 mmol H_2/(g cells h), respectively, in the first 25 h of fermentation (Fig. 2B). Then, the specific CO and H_2 uptake rates decreased to 27 mmol CO/(g cells h) and 19 mmol H_2/(g cells h) at 120 h, respectively, due to the increase in cell mass concentration.

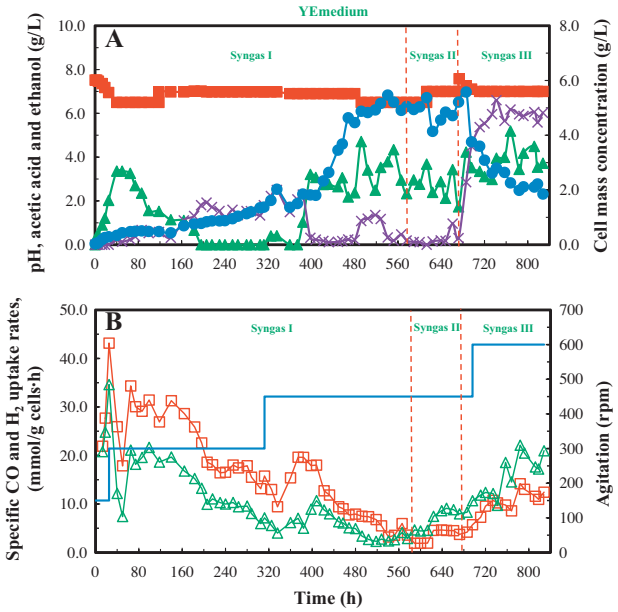

Fig. 2. (A) Growth and products profiles. (B) Specific gas uptake profiles during continuous syngas fermentation in YE medium with cell recycle; pH (■), cell mass concentration (●), acetic acid (▲), ethanol (×), specific CO uptake rate (□), specific H_2 uptake rate (Δ), and agitation (solid line and no symbol).

Even with 100% cell recycle, the accumulation of cell mass in the fermentor was slow at the dilution rate of 0.011 h^{-1} between 13 h and 120 h, which could be due to low cell growth at pH 6.5, which was reported previously (Allen et al., 2010). Thus, the pH was increased to 7.0 and controlled at this level after 120 h. This resulted in an increase in cell mass concentration from 0.5 g/L to 0.9 g/L between 120 h and 216 h. During the same period, ethanol concentration increased from 0.6 g/L to 1.7 g/L. This showed that ethanol can be produced during cell growth, and pH 7.0 was better than pH 6.5 for both cell growth and ethanol production. The acetic acid concentration dropped to 0 at 216 h due to its conversion to ethanol as previously reported (Liu et al., 2012). During the fermentation period from 120 h to 216 h, the specific CO and H_2 uptake rates decreased by 33% and 41%, respectively, due to the increase in cell mass concentration (Fig. 2B).

The dilution rate was increased to 0.017 h^{-1} between 216 h and 288 h to provide more nutrients and grow more cells. The average ethanol concentration between 216 h and 288 h was 1.5 g/L, which was comparable to the ethanol concentration produced at 0.011 h^{-1} (Table 4). However, no acetic acid was detected during this period. Cell concentration increased to 1.2 g/L at 288 h. The increase in dilution rate by 50% in this period did not affect ethanol concentration. However, ethanol productivity increased by 60% in the period between 216 h and 288 h compared to the period from

120 h to 216 h. To further increase ethanol productivity, the dilution rate was increased from 0.017 h^{-1} to 0.022 h^{-1} between 288 h and 384 h. This resulted in a further increase in the cell mass concentration by 58%. Ethanol concentration and productivity increased to 2.0 g/L and 42.1 mg/L h, respectively. The concentration of acetic acid was only 0.4 g/L. During the fermentation period from 216 h to 288 h, the average specific CO and H_2 uptake rates were 17 mmol CO/(g cells h) and 10 mmol H_2/(g cells h), respectively. The agitation speed was increased from 300 rpm to 450 rpm at 312 h to improve the mass transfer of CO and H_2.There was a slight increase in cell mass, ethanol, acetic acid concentrations and specific CO uptake rate between 288 h and 384 h. However, specific H_2 uptake rate decreased slightly (Fig. 2B).

When the dilution rate was increased to 0.033 h^{-1} between 384 h and 576 h, a rapid increase in the cell mass concentration to 5.5 g/L (equivalent to an optical density, OD, of 14) was measured in the fermentor (Fig. 2A). This was the maximum cell mass concentration achieved. During this rapid cell growth, 5 g/L acetic acid was produced. However, the high cell mass concentration did not improve ethanol production. Instead, ethanol concentration decreased to 0.2 g/L from 384 h to 480 h. This decrease could be due to ethanol utilization by strain CP15 when CO and H_2 mass transfer was limited. In addition, high cell mass concentration required a high amount of carbon for maintenance, requiring ATP through acetic acid production instead of making ethanol. Ethanol was also reported as a substrate for strain CP15 growth (Allen et al., 2010), which explains the decrease of ethanol concentration. Another possible reason for the low ethanol concentration could be due to the increase in dilution rate that washed out ethanol.

Continuous fermentation can wash out cells and products. However, the cells were retained in the fermentor with the cell retention system. Ethanol was not retained in the fermentor as its production rate was lower than its removal rate from the reactor. However, acetic acid accumulated in the fermentor because its production rate was higher than its removal rate. Even though decreasing the pH from 7.0 to 6.5 in the fermentor helped to stimulate ethanol production from 0.2 g/L to 1.4 g/L between 480 h and 518 h, ethanol concentration then decreased back to 0.2 g/L between 518 h and 576 h with the high cell mass concentration. The specific CO and H_2 molar uptake rates dropped from 20 mmol to 4 mmol CO/(g cells h) and from 5 mmol to 3 mmol H_2/(g cells h) between 384 h and 576 h, which was mainly due to the large increase in cell mass concentration from 1.9 g/L to 5.1 g/L. In addition, the increase in cell mass concentration did not result in an improvement in specific CO and H_2 uptake rates (Fig. 2B), which is an indication of CO and H_2 mass transfer limitations. If mass transfer limitations were not present in the fermentor with cell mass concentration increase, the specific CO and H_2 uptake rates should have increased or remained constant.

The syngas fed to the fermentor between 576 h and 672 h was switched from Syngas I (39% CO, 24% CO_2, 27% H_2, 10% N_2, H_2:CO molar ratio ≈ 0.7) to Syngas II (20% CO, 25% CO_2, 43% H_2, 12% N_2, H_2:CO molar ratio ≈ 2) to investigate if the product profiles change with the higher H_2:CO ratio (Fig. 2A). During this period, the pH was increased to 7.0 at 624 h, which increased cell mass concentration but had only a small effect on ethanol formation. Ethanol concentration was not changed, which could be due to either washout or utilization by strain CP15 as discussed earlier. There was a minor change in the specific CO uptake rate from 3.6 mmol to 3.7 mmol/(g cells h). The specific H_2 uptake rate increased from 2.9 mmol to 8.0 mmol/(g cells h) as shown in Fig. 2B. It is important to note that the specific H_2 uptake rate exceeded the specific CO uptake rate when Syngas II with a H_2:CO ratio of 2 was used due to higher H_2 content. When Syngas I with H_2:CO ratio of 0.7 was used, the specific H_2 uptake rate was lower than the specific CO uptake rate (Fig. 2B).

To examine if the high cell mass concentration in the fermentor caused ethanol production to drop, cell recycle was stopped from 672 h to 742 h and dilution rate was reduced to 0.011 h^{-1} to prevent a fast cell washout. In addition, Syngas III (28% CO, 60% H_2, 12% N_2, H_2:CO molar ratio ≈ 2) with a similar H_2:CO ratio as in Syngas II was sparged into the fermentor to provide cells with more CO and H_2. This resulted in a decrease in cell mass concentration in the fermentor from 5.2 g/L to 2.8 g/L at 742 h (Fig. 2A). However, ethanol concentration increased to 6.6 g/L. During the same fermentation period, the specific CO and H_2 uptake rates increased from 3.7 mmol to 10.2 mmol CO/(g cells h) and from 8.0 mmol to 9.7 mmol H_2/(g cells h), due to the decrease in the cell mass concentration from 5.2 g/L to 2.8 g/L (Fig. 2). The agitation speed was increased to 600 rpm at 696 h to provide more gas to cells as their uptake rates were increasing.

The cell recycle system was restarted after 742 h because of the large decrease in cell mass concentration within 70 h of operation and to avoid complete cell washout. From 742 h to 828 h, the cell mass concentration decreased by 34% and acetic acid and ethanol concentrations decreased by 6% and 9%, respectively (Fig. 2A).

Table 4
Fermentation parameters during continuous syngas fermentation in 7-L fermentor at various operating conditions.

Medium	YE[a]								YE-free[a]	CSL[a]
Time range (h)	13–120	120–216	216–288	288–384	384–480	480–576	576–672	742–828	828–924	924–1224
Syngas	I	I	I	I	I	I	II	III	III	III
Agitation (rpm)	150–300	300	300	450	450	450	450	600	600	600
pH	6.5	7.0	7.0	7.0	7.0	6.5	6.5–7.0	7.0	7.0	7.0–7.5
Dilution rate (h^{-1})	0.011	0.011	0.017	0.022	0.033	0.033	0.017–0.033	0.011	0.011	0.011–0.017
Avg. cell mass conc. (g/L)	0.40	0.73	1.01	1.59	3.10	5.05	4.85	2.25	1.82	1.73
Avg. acetic acid conc. (g/L)	2.22	0.48	0.00	0.26	2.76	3.44	2.74	4.06	3.73	1.09
Avg. ethanol conc. (g/L)	0.28	1.36	1.46	1.96	0.20	0.75	0.25	5.99	6.39	3.42
Avg. ethanol productivity (mg/L h)	3.04	15.01	24.13	42.06	6.61	24.73	8.37	65.91	70.30	39.97
Avg. ethanol yield from CO (%)	2.98	11.50	18.54	23.78	2.61	10.83	6.01	34.65	39.35	30.57
Avg. CO utilization (%)	19.28	28.35	28.47	41.22	59.22	48.57	57.57	61.48	56.97	43.90
Avg. H_2 utilization (%)	18.30	23.81	22.81	22.21	53.62	37.18	53.52	44.02	37.88	30.62
Avg. specific CO uptake rate (mmol/g cells h)	28.84	24.18	16.97	15.14	11.82	5.43	3.46	11.35	12.73	10.87
Avg. specific H_2 uptake rate (mmol/g cells h)	19.89	14.32	9.60	5.84	7.35	2.89	6.95	17.65	17.14	16.25
Max. n-propanol (g/L)	ND	ND	ND	ND	ND	ND	ND	0.16	0.21	6.01
Max. n-butanol (g/L)	ND	ND	ND	ND	ND	ND	ND	0.09	0.09	1.11

ND, not detectable.
[a] YE = yeast extract medium; YE-free = yeast extract free medium; CSL = 20 g/L corn steep liquor medium.

The specific CO and H_2 uptake rates increased by 22% and 116%, respectively, when the agitation speed was increased from 450 rpm to 600 rpm (Fig. 2B). This indicated that the mass transfer limitation was alleviated by reducing cell mass concentration and increasing agitation. The average molar uptake ratios of H_2:CO using Syngas I, Syngas II and Syngas III were 0.6, 2.1 and 1.6, respectively. These ratios were close to the H_2:CO ratios supplied in Syngas I (0.7) and in Syngas II and Syngas III (2.0). In addition, cells utilized more H_2 than CO when the H_2:CO ratio was above 1. This indicates that the H_2:CO ratios in syngas affect the cells' preference to uptake H_2 or CO as the electron and carbon source. However, the molar ratio of ethanol to acetate produced with Syngas I (H_2:CO ratio ≈ 0.7) was between 0.1 and 9.8. This wide range was due to changes in the agitation speed, dilution rate, pH and cell mass concentration during the fermentation (Fig. 2 and Table 4). The molar ratios of ethanol to acetate produced with Syngas II and Syngas III (H_2:CO ratio ≈ 2.0) were 0.1 and 1.9, respectively. This showed that there was no direct correlation between the H_2:CO ratio and molar ratio of ethanol to acetate produced at the conditions investigated.

3.2.2. Fermentation in yeast extract free medium

In the second stage of the continuous fermentation from 840 h to 924 h, YE-free medium was used to evaluate if removing YE would have a positive effect on ethanol production. A previous report showed that eliminating YE from *C. ljungdahlii* syngas fermentation medium increased ethanol concentration (Phillips et al., 1993). However, there was only 13% increase in ethanol production upon removing YE with strain CP15 during the fermentation from 840 h to 924 h (Fig. 3A). In addition, cell mass concentration decreased by 15% and acetic acid concentration decreased by 40% at 924 h. The specific CO uptake rate in YE-free medium with Syngas III (28% CO, 60% H_2, 12% N_2, H_2:CO molar ratio ≈ 2) increased by 3%. However, the specific H_2 uptake rate in YE-free medium with Syngas III decreased by 18% (Fig. 3B).

3.2.3. Fermentation in CSL medium

In the last stage of the continuous fermentation from 936 h to 1224 h, the medium fed to the fermentor was 20 g/L CSL medium.

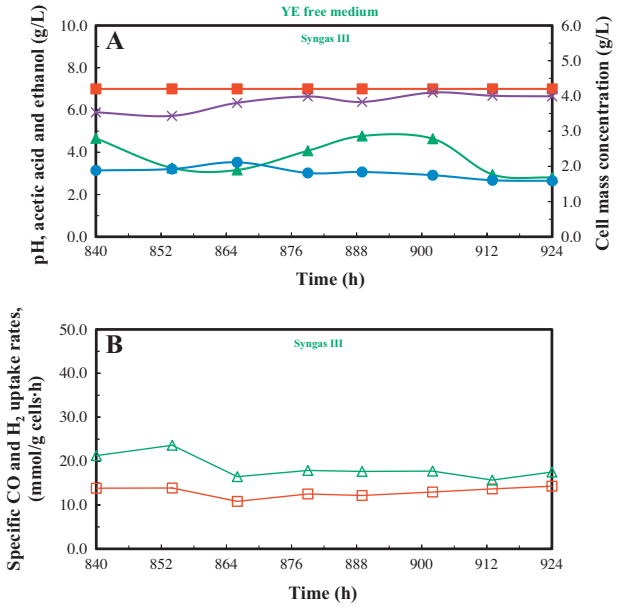

Fig. 3. (A) Growth and products profiles. (B) Specific gas uptake profiles during continuous syngas fermentation in YE-free medium with cell recycle; pH (■), cell mass concentration (●), acetic acid (▲), ethanol (×), specific CO uptake rate (□), specific H_2 uptake rate (Δ), agitation speed was 600 rpm.

During this period, there was a slight increase in ethanol concentration from 6.5 g/L to 7.7 g/L from 936 h to 985 h (Fig. 4). However, ethanol concentration quickly decreased to below 2.0 g/L at 1071 h and was stable at a level of 1.7 g/L until the end of fermentation. The specific CO and H_2 uptake rates during this period decreased by 43% and 8%, respectively.

With the CSL medium, n-propanol and n-butanol were produced. The n-propanol and n-butanol concentrations increased to a maximum of 6 g/L and 1 g/L, respectively (Fig. 4C). In addition, the maximum concentrations of propionic acid and butyric acid were 3.0 g/L and 0.5 g/L, respectively. The decrease in ethanol concentration and production of both n-propanol and n-butanol in CSL medium previously were not observed with strain CP15 during syngas fermentations (Allen et al., 2010; Liu et al., 2012). In addition, strain CP15 was not reported to produce n-propanol or n-butanol. This indicated that the fermentation broth was contaminated with other microorganisms.

Thus, the microbial assemblage in the fermentation broth was characterized by surveying 16S rRNA gene sequences present and determining the identity of contaminants in the fermentor. A total of 61 sequenced clones were analyzed, which indicated that 34 (56%) were from *A. bacchi* strain CP15 and 21 (34%) were classified as *Clostridium propionicum*. The six remaining clones (10%) were characterized as *Clostridium amylolyticum* (2 clones), *Clostridium putrefaciens* (2 clones), *Clostridium carnis* and *Clostridium celerecrescens* (1 clone each). During this continuous fermentation, there was a power failure between 792 h and 812 h, which resulted in no gas and liquid flowing in and out of the fermentor, no agitation and no liquid flowing in the cell recycle system. In addition, n-propanol and n-butanol were first detected between 742 h and 828 h

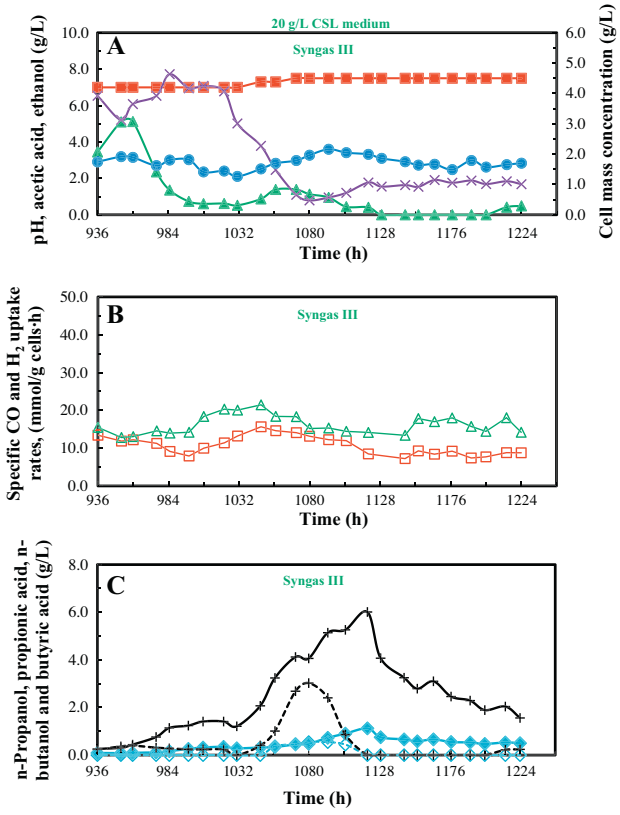

Fig. 4. (A) Growth and products profiles. (B) Specific gas uptake profiles (C) higher alcohols and corresponding acids profiles during continuous syngas fermentation in 20 g/L CSL medium with cell recycle; pH (■), cell mass concentration (●), acetic acid (▲), ethanol (×), specific CO uptake rate (□), specific H_2 uptake rate (Δ), n-propanol (+ and solid line), propionic acid (+ and dash line), n-butanol (♦ and solid line), butyric acid (◇ and dash line), agitation speed was 600 rpm.

(Table 4), which indicates this could be when contamination occurred. However, the exact reason of contamination was unknown.

The product profiles at various dilution rates in YE medium with Syngas I and pH 7.0 are shown in Fig. 5. Cell mass concentration increased with the increase in dilution rate, which is expected because of the cell recycle system and addition of more nutrients in the fermentor. Acetic acid concentration increased when the dilution rate was above 0.017 h^{-1}. Ethanol production also increased with increasing the dilution rate and reached its highest concentration, 2.1 g/L, at dilution rate 0.022 h^{-1}, at which ethanol productivity was 42 mg/g cells h (Fig. 5A). However, ethanol concentration and productivity quickly decreased when the dilution rate was increased to 0.033 h^{-1}.

CO and H_2 utilization improved with the increase in the dilution rate due to production of more cells (Fig. 5B). The specific CO uptake rate decreased with the increase of dilution rate. However, H_2 uptake rates decreased until dilution rate was increased to 0.022 h^{-1}, and then it remained constant at dilution rate 0.033 h^{-1}. It was predicted from the cell growth kinetic model that *E. limosum* KIST 612 required 5.2 mmol CO/g cells h for maintenance in fermentation with pure CO (Chang et al., 2001). In the present study, the average specific uptake rates of CO between 480 h and 672 h in the YE medium with Syngas I and Syngas II at a cell mass concentration of 5 g/L were 5.4 mmol CO/g cells h and 3.5 mmol CO/g cells h, respectively (Table 4). These specific uptake rates of CO by strain CP15 were close to the value reported with *E. limosum* KIST 612. Assuming that strain CP15 has a similar CO maintenance requirement, there was not enough CO transferred to the cells and therefore, cells utilized ethanol for maintenance, which can also explain the low ethanol concentration in the fermentor (Fig. 2).

Ethanol concentration in YE medium was at least 3-fold greater with Syngas III and cell recycle, compared to either Syngas I or Syngas II (Table 4). This was because Syngas III contained more reductants (CO and H_2) compared to either Syngas I or Syngas II. In addition, the average ethanol yield from CO was the highest with Syngas III in the three media used. About 7% more ethanol was formed in the YE-free medium compared to the YE medium. The use of YE-free medium shifted the conversion of CO and H_2 from more cells to ethanol. Ethanol production in CSL medium was lower than YE and YE-free media. CO utilization in YE medium ranged from 19% to 61% of the supplied gas, while H_2 utilization ranged from 18% to 54% (Table 4). The average CO and H_2 utilization in YE-free medium were comparable to YE medium at the same operating conditions with Syngas III. However, CO and H_2 utilization with Syngas III were the lowest in CSL medium (Table 4) due to low cell mass concentration. In commercial syngas fermentation processes, the control of the H_2:CO ratio within a range that provides high carbon conversion efficiency and product yield is desired. However, this is a challenging task due to the inherent changes in the composition of biomass feedstock used. Syngas fermentation is a versatile process in which the microorganism can ferment H_2 and CO at various ratios as shown in Table 4.

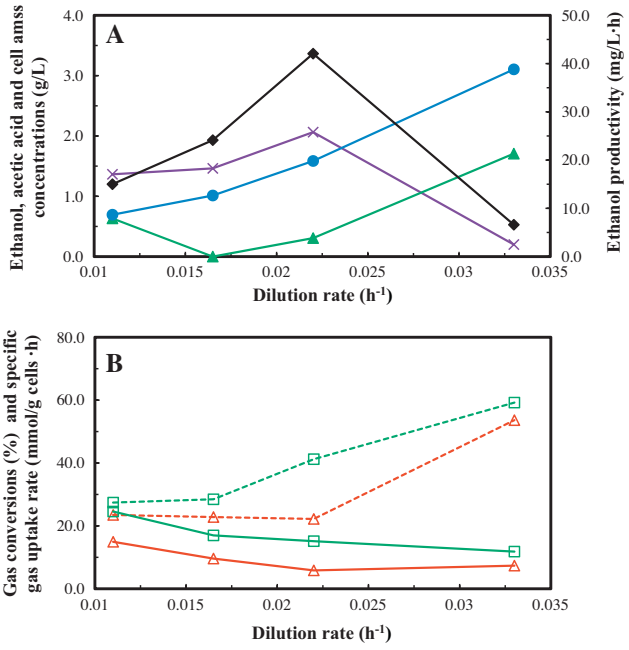

Fig. 5. Fermentation profiles in YE medium at various dilution rates at pH 7.0 using Syngas I (A) products profiles, (B) gas uptake profiles; cell mass (●), acetic acid (▲), ethanol (×), ethanol productivity (♦), specific CO uptake rate (□), specific H_2 uptake rate (Δ), CO utilization (□ and dash line), and H_2 utilization (Δ and dash line).

Compared to previous syngas fermentation studies in CSTR, the maximum ethanol productivity of 70 mg/L h obtained in the present study with Synags III in YE-free medium (Table 4) was close to that reported for *C. ljungdahlii* without cell recycle (78 mg/L h) (Mohammadi et al., 2012), but much lower than with cell recycle using *C. ljungdahlii* (1632 mg/L·h) (Phillips et al., 1993). Coskata, Inc., a company pursing commercialization of syngas fermentation technology, reported an ethanol productivity of 247 mg/L h by "*Clostridium coskatii*" in a continuous fermentation without cell recycle (Zahn and Saxena, 2011). This rate was 3.5 times higher than the rate observed with strain CP15. However, strain CP15 had 4.1 times higher ethanol productivity than "*C. ragsdalei*" (17 mg/L h) in batch fermentation with a continuous gas flow using YE medium (Maddipati et al., 2011).

Although a decrease in ethanol concentration was observed in 20 g/L CSL medium due to contamination, the average ethanol productivity in the present study was 40 mg/L h, which was still 48% higher than "*C. ragsdalei*" in 20 g/L CSL medium (Maddipati et al., 2011). To improve ethanol productivity of strain CP15 in continuous fermentation, mass transfer limitation must be addressed by improving gas transfer rate through increasing gas flow rate, agitation speed or using other reactor configurations such as trickle bed reactor (TBR) or hollow fiber membrane reactor (HFR) (Orgill et al., 2013). In addition, consumption of ethanol by cells should be avoided. This can be done by adjusting reactor conditions and balancing cell mass concentration and the mass transfer rate to provide enough CO and H_2 to meet cells' maintenance requirements and divert CO and H_2 to ethanol formation. The use of lean medium such as YE-free medium can also enhance ethanol production (Table 4). The YE-free medium has a low growth stimulator, which can be a good production medium when a cell recycle system is used.

The simultaneous production of n-propanol and n-butanol with ethanol during the late stages of continuous fermentation with strain CP15 has not been reported previously. The contamination of the fermentor with *C. propionicum* was identified as being probably responsible for n-propanol and n-butanol production at late stages of the fermentation. The culture present in the CSL medium was a mixed culture as confirmed with the 16S rRNA gene sequence. The mixed culture presents a new opportunity for the production of higher alcohols from syngas, which previously has not been reported in the literature.

C. propioncium has been reported to convert lactic acid and the amino acid alanine to propionic acid (Cardon and Barker, 1946; Tholozan et al., 1992). Butyric acid was also produced by *C. propioncium* from the four carbon–amino acid threonine (Cardon and Barker, 1946), which is an amino acid available in YE and CSL. In addition, *Clostridium neopropionicum* was able to convert ethanol into propionic acid and *C. propionicum* displayed a similar

metabolic pathway to *C. neopropionicum* (Tholozan et al., 1992). The decreasing ethanol concentration and propionic acid production in CSL medium could have been because of *C. propionicum* or the four other *Clostridium* species in the minority found in the fermentor (Fig. 4C). In addition, strains "*C. ragsdalei*" and *C. ljungdahlii* showed the ability to convert propionic acid and butyric acid to corresponding alcohols (Isom et al., 2011; Perez et al., 2012), indicating strain CP15 could have a similar capacity to convert propionic acid or butyric acid produced by *C. propionicum* in the mixed culture into n-propanol and n-butanol. The conversions of these organic acids to their corresponding alcohols during syngas fermentation using the mixed culture warrant further investigation.

4. Conclusions

A. bacchi CP15 produced 78% more ethanol by replacing yeast extract (YE) with corn steep liquor (CSL) in bottle fermentations. A sustainable ethanol concentration of 6 g/L was achieved in the YE and YE-free media during continuous fermentation with cell recycle. Ethanol production decreased due to high cell mass concentration above 5 g/L and mass transfer limitation. A mixed culture was obtained during continuous fermentation in the CSL medium, which mainly consisted of *A. bacchi* CP15 and *C. propionicum*. The mixed culture produced a maximum of 8 g/L ethanol, 6 g/L n-propanol and 1 g/L n-butanol.

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