ENHANCED ALCOHOL PRODUCTION DURING SYNGAS FERMENTATION USING BIOCHAR

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Abstract: Microbial fermentation of syngas (mainly CO, H_2 and CO_2) into biofuels (ethanol, butanol, etc.) and chemicals (carboxylic acids, 2,3-butanediol, etc.) is a flexible and promising route for producing liquid biofuels and chemicals. Biochar obtained from gasification or pyrolysis of the carbon-rich feedstocks contains rich mineral and metal compounds, high pH buffering capacity, and cation exchange capacity (CEC). The use of biochar in syngas fermentation could lead to enhanced production of biofuels and chemicals. In the present study, biochar from switchgrass (SGBC), forage sorghum (FSBC), red cedar (RCBC) and poultry litter (PLBC) were incorporated into fermentation media of *C. ragsdalei* and *C. carboxidivorans*. Fermentations were performed in 250 mL bottle reactors with 50 mL working volume fed with bottled syngas (40% CO, 30% H_2, 30% CO_2, by volume) at 37 °C for 15 days. Fermentations with *C. ragsdalei* showed that PLBC and RCBC improved ethanol production by 59% and 16%, respectively, compared to standard YE medium. Fermentations with *C. carboxidivorans* showed that media with PLBC and SGBC enhanced ethanol production by 90% and 73%, respectively, and butanol production by four fold compared to standard YE medium. PLBC loading of 10 and 20 g L^{-1} was shown to enhance ethanol and acetic acid productions with *C. ragsdalei* and enhance ethanol, butanol, acetic acid, butyric acid, and hexanoic acid productions with *C. carboxidivorans*. A 10 g L^{-1} of PLBC was used in fed-batch fermentation with *C. ragsdalei* in 3-L CSTR. The highest amounts of acetic acid produced in YE medium without MES, PLBC media without and with MES were 0.6, 1.2, and 2.0 g L^{-1}, respectively. The acetic acid produced in all media was completely converted to ethanol using a patented pH controller and syngas feeding method. Fermentations using PLBC media without and with MES resulted in production of 34% and 63%, respectively, more ethanol than in YE medium. Ca, Mg, Fe and Mn were mostly released from biochar during syngas fermentation with *C. ragsdalei* and *C. carboxidivorans*. The release of these elements was caused by neutralization with H^+ from undissociated acetic acid during acetogenic phase in fermentation. The reduced H^+ led to deceleration in pH drop, which extended acetogenic phase with accumulation of more acetic acid and reduced "acid stress" on the microorganisms used. Compared with other types of biochar materials, PLBC had the highest pH buffering capacity, total bound cations and acid neutralizing capacity, which contributed to the enhancement of alcohol and acid production with potential use in commercial syngas fermentation processes.

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CHAPTER I

INTRODUCTION

Transportation fuels from petroleum have greatly boosted the world economy and played a critical role in improving human's daily life during the past century. With the increase in population and living standards especially in developing countries, the demand for liquid transportation fuel will increase from 95 million barrels per day in 2015 to 121 million barrels per day in 2040 (EIA, 2016). However, over about half a century of extensive exploration, the petroleum reserves are near depletion. It was estimated that with current consumption rate, the petroleum will only last for another 45 years (EIA, 2013). The heavy dependence on fossil fuel contributes to the climate change due to considerable emission of net greenhouse gases. Besides, international trade of crude oil caused instability in politics, economies and security between oil importing and exporting countries. One example is 1970s energy crisis, during which the economy of major oil importing countries such U.S., Canada and European countries were severely affected when Organization of Petroleum Exporting Countries (OPEC) implemented embargo on crude oil exportation and huge increase in oil prices (Lifset, 2014). To be independent of petroleum reserves and trades, developing renewable and sustainable transportation fuels has been on agenda and put into practice in most countries (Demirbas, 2009).

Ethanol has been demonstrated as the most suitable liquid fuel to relieve complete dependence on petroleum-based gasoline because of its high octane number (98 compared to 80 for gasoline), low evaporative emission, and high oxygen content (i.e., complete combustion) (RFA, 2017). In fact, ethanol has been commonly blended with gasoline at E10 ("E", ethanol; "10", the percentage of ethanol content) with E15 and E85 also available in U.S. (RFA, 2017). European countries utilize E10 and Brazil uses E20, E25, and E100 for vehicles with fuel flexible engine (Goldemberg, 2008). The world's largest fuel ethanol producers are U.S. and Brazil with 57% and 28% share of total global ethanol production, respectively (RFA, 2018). Ethanol production in the U.S. is mostly from corn (Davis, 2001), while in Brazil, it is based on sugarcane (Goldemberg, 2008).

Butanol is considered as a "drop-in" biofuel that can be combusted directly or blended with gasoline at any concentration in current car engine. Moreover, its energy content is close to gasoline (29.2 MJ/L as compared to 32.5 MJ/L in gasoline and 21.2 MJ/L in ethanol). In addition, the lower hydroscopic and corrosive characteristics of butanol than ethanol allows butanol to be compatible with current infrastructures (tanks, pumps, pipelines, gas stations) without modification (Dürre, 2007). Butanol was produced heavily from ABE (acetone-butanol-ethanol) fermentation using molasses as major substrate in the early 20th century. However, a majority of butanol production plants were switched to petrochemical process during 1960s when crude oil price declined and molasses price increased. Due to considerable net CO_2 emission and limited reserve of crude oil in recent decades, ABE fermentation recaptured people's attention globally (Lee et al., 2008).

Production of ethanol and butanol from sugar-rich and starchy materials (e.g. corn, sugarcane, sugar beet, sweet sorghum etc.) is to some extent affected by the price of these materials as food supplies. Besides, the cultivation of these feedstocks is limited by climate, geography, policies and economy. One alternative to these issues is to utilize lignocellulosic biomass for ethanol and butanol production (Limayem & Ricke, 2012). Lignocellulosic biomass includes agricultural residues, woody and herbaceous biomass. Lignocellulosic biomass consists of cellulose, hemicellulose and lignin with lower quantity of extractive and ash, the compositions of which depend on genetics and growing environment (Balat, 2011). Lignocellulosic biomass can be converted into ethanol and butanol through biochemical and thermochemical platforms. Biochemical platform required three major steps including: 1) removal of lignin and release of cellulose and hemicellulose through pretreatment; 2) breakdown of cellulose and hemicellulose into fermentable sugars through hydrolysis; and 3) fermentation of sugars to ethanol and butanol.

Extensive studies have been focused on developing strategies to reduce technical complexity and capital costs, and improve productivity of the overall biochemical process. These strategies include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), separate hydrolysis and co-fermentation (SHcF) and consolidated bioprocessing (CBP) (Kennes et al., 2016). Thermochemical platform consists of two stages, gasification of biomass into syngas (mixture of mainly CO, H_2 and CO_2) and conversion of syngas into butanol and ethanol. A great advantage of thermochemical platform over biochemical platform is that it utilizes all components of biomass without the need to remove lignin. Conversion of

syngas into ethanol and butanol can be achieved either by chemical route using metal catalysts (Dyer et al., 1987) or by a biological route using microorganisms by syngas fermentation (Phillips et al., 2017a).

Gasification-syngas fermentation is a hybrid thermochemical-biochemical technology for conversion of biomass into biofuels and value-added chemicals. The syngas produced from gasification is converted by acetogenic bacteria into cell mass, alcohols, carboxylic acids (Munasinghe & Khanal, 2010a; Phillips et al., 2017a), and 2,3-butanediol (Köpke et al., 2011b). The gaseous substrates for syngas fermentation can also be obtained from industrial CO-rich off gases (Molitor et al., 2016). Syngas fermentation has several advantages over Fischer-Tropsch (FT) process such as no requirement of strict CO and H_2 ratio, moderate operating temperature and pressure, and high tolerance to gaseous contaminants. However, the disadvantages of syngas fermentation include limited liquid-gas mass transfer, low productivity, and high production cost. Addressing such drawbacks in syngas fermentation had been intensively attempted during recent decades by modification of bioreactor designs (Devarapalli et al., 2016; Shen et al., 2017b), reduction in media cost (Kundiyana et al., 2010a; Maddipati et al., 2011), process control and improvement in productivity (Atiyeh et al., 2018; Atiyeh et al., 2016b; Shen et al., 2017a; Sun et al., 2018a).

Biochar is a carbon-rich solid material formed as byproducts or waste during thermochemical conversion of biomass, coal, animal wastes … etc. with limited supply of oxygen (Lehmann et al., 2006). More biochar is produced during pyrolysis than from gasification. Most biochars have high content of minerals and trace metals, alkalinity, porosity, surface area, pH buffering capacity, cation exchange capacity (CEC) and rich

functional groups (Sun et al., 2018a; Yuan et al., 2011). These properties of biochar are largely affected by feedstock types, production methods (slow or fast pyrolysis, gasification, or hydrothermal carbonization … etc.) and temperature (Sun et al., 2014). Because of these properties, biochar is commonly used as soil amendment, greenhouse gas sequestration, and management of contaminations in wastewater (Ahmad et al., 2014; Lehmann et al., 2006; Lehmann et al., 2011). The application of biochar in syngas fermentation can replace minerals and metal nutrients, which might result in reduced fermentation medium cost. In addition, the biochar can provide buffering effect for acetogenic bacteria which might enhance cell growth and productivity of alcohols and organic acids from syngas. Since the beginning of this project in 2014, no reports were found in the literature on the utilization of biochar in syngas fermentation. The proposed research explores the application of various types of biochar in syngas fermentation for production of ethanol and butanol using *Clostridium ragsdalei* and *C. carboxidivorans*. Switchgrass, forage sorghum, red cedar, and poultry litter biochars were used in 250 mL bottle fermenters and 3-L continuously stirred tank reactor (CSTR). The biochar loading, gas flow rate, pH, medium composition and mechanisms of leaching biochar elements were investigated. In addition, the physiochemical properties of the four types of biochars made by gasification and pyrolysis were measured and their effects on enhancement of ethanol and butanol production from syngas were determined.

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CHAPTER II

LITERATURE REVIEW

2.1 What is biofuel?

Biofuels refer to fuels made from plant material including corn, sugar cane or beet and the lignocellulosic biomass. Biofuels can be classified into solid biofuels (e.g. fire wood, wood pellets, and charcoal), liquid biofuels (e.g. bioethanol, biodiesel, pyrolysis bio-oil, drop-in biofuels), and gaseous biofuels (e.g. biogas and syngas) (Guo et al., 2015). Heavy dependence on fossil fuels had resulted in global issues such as pollutions of water, air and soil, green-house gas (GHG) effect, economic instability, and political conflicts. Besides, the limited reserves of fossil fuels will not be enough for ever-increasing global energy demand. It was estimated that with current consumption rate, the reserves of coal will last for 120 years, natural gas for 60 years and petroleum for 45 years (EIA, 2013). However, global energy demand was expected to expand from 549 quadrillion British thermal unit (Btu) in 2012 to 629 quadrillion Btu in 2020 and to 815 quadrillion Btu in 2040, a 48% increase from 2012 to 2040 (EIA, 2016). Biofuels can reduce net CO_2 and NO_x and are considered as one of the alternatives to fossil fuels for current and future energy demand (Demirbas, 2009).

2.2 Biofuels from carbohydrates

Biofuels production from carbohydrates in this dissertation is specifically referred to bioethanol and bio-based butanol production from sugars, starch or lignocellulosic materials. The principle behind using each starting materials is breaking down the polysaccharides or disaccharides (e.g. starch, sucrose) into monosaccharides (e.g. glucose, fructose, xylose, mannose, arabinose) which can be used by microorganisms for synthesis of ethanol and butanol.

Bioethanol can be converted directly from monosaccharides such as hexose by microorganisms such as *Saccharomyces cerevisiae* and from pentose by *Scheffersomyces stipitis* (Chaudhary & Ghosh, 2014). These monosaccharides can be obtained from corn starch, sugar cane, sugar beet, or lignocellulosic biomass. Biofuels made from sugar and starch are called first generation biofuels, while biofuels from lignocellulosic biomass are called second generation or advanced biofuels.

The United States and Brazil are two major fuel ethanol producers (Fig. 2.1A). The U.S. is the leading country in corn production. About 96% of its total bioethanol production is currently made from corn (RFA, 2018) (Fig. 2.1B). Corn grains contain 61 % starch, 3.8 % corn oil, 8 % protein, 11.2 % fiber, and 16 % moisture (Davis, 2001). Two types of processes are employed for corn milling: wet milling and dry milling (Fig. 2.1C). The wet milling employs a series of milling and screening which results in the separations of starch and byproducts such as gluten feed, gluten meal, corn oil and corn steep liquor. The dry milling process utilizes whole grain without fractionation and starts from milling, cooking, liquefaction (enzymatic hydrolysis), and yeast fermentation to distillation, resulting in production of ethanol, CO_2 and Distillers Dried Grains with Solubles (DDGS).

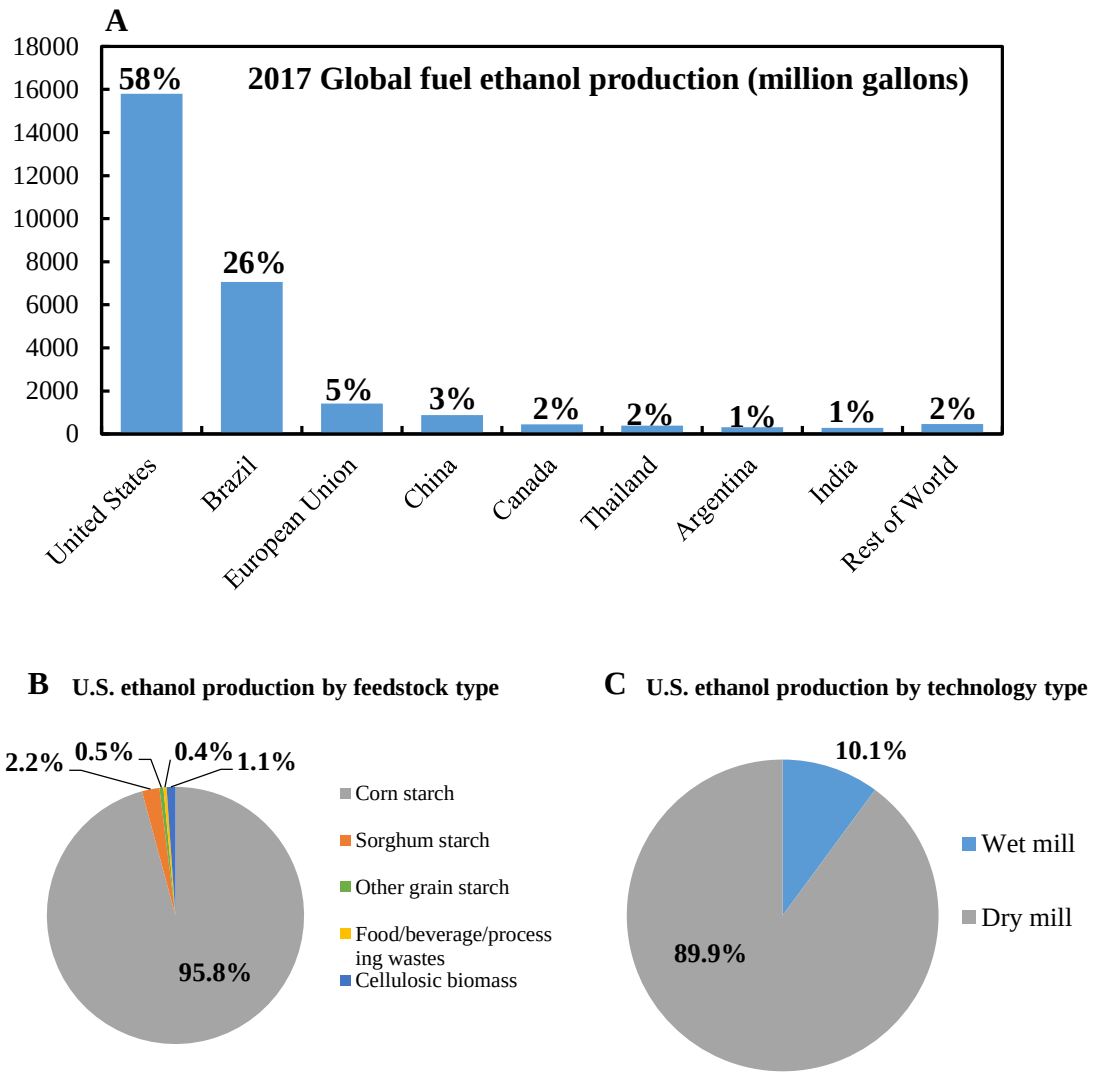

Fig. 2.1 (A) Global fuel ethanol production in the year 2017 by countries (RFA, 2018), and U.S. ethanol production by (B) feedstock type and (C) technology type .

Sugarcane is a major sugar crop and mainly grown in Brazil, although certain amounts are also grown in some parts of U.S., China, and India. Almost half of sugarcanes grown in Brazil are crushed for production of bioethanol with another half for sugar production (Goldemberg, 2008). Ethanol production from sugarcane is achieved when the sugarcanes are crushed and cane juice is extracted followed by immediate

fermentation. The sugarcane molasses can also be used for ethanol fermentation. The sugarcane bagasse is commonly burned for electricity generation.

Other starch crops that can be used in bioethanol production are wheat, cassava, sweet sorghum and barley. Bioethanol production from wheat grains is mainly employed in European countries such as France, Germany and Spain, in a similar process to that of corn ethanol. Cassava is another important starch crop grown mainly in tropical countries as a source for bioethanol and sugar supply. Cassava has high moisture content (around 70%) compared to other crops. This requires instant processing after harvesting (usually within 3 to 4 days) before deterioration. Another promising crop for bioethanol production is sweet sorghum which has advantages such as high starch content in its grain, high sugar content in its stalk, and high lignocellulosic content in its bagasse. Besides, it can also be grown in conditions with drought, flooding and salinity (Almodares & Hadi, 2009).

2.3 Biofuels from lignocellulosic materials

Lignocellulosic materials are the most abundant biomass in the world and mainly contain cellulose, hemicellulos and lignin, the compositions of which vary based on different types of feedstocks. Cellulose is the most abundant component in biomass (25 to 70 wt. %) followed by hemicellulose (25 to 50 wt. %). Lignin content of biomass varies with the type of biomass, e.g. 5 to 15 wt. % in agricultural residues, 20 to 25 wt. % in hardwood, and 30 to 60 wt. % in softwood (Limayem & Ricke, 2012). The lignin is a highly cross-linked aromatic polymer of phenylpropane units and protects the cellulosic materials from being attached and provides the whole biomass mechanical strength

(Cheng, 2009). Removal or disruption of lignin to release cellulose and hemicellulose and detoxification of possibly generated inhibitors during hydrolysis is a complicated and costly procedure before fermentation (Liu et al., 2015a; Liu et al., 2015b). However, one advantage of using lignocellulosic biomass is that they are non-food or feed materials, eliminating the possibility of "fuel or food" conflict. Another advantage is its huge availability around the globe because lignocellulosic feedstocks can be agricultural residues, woody biomass, herbaceous biomass, and part of municipal solid wastes (Rastogi & Shrivastava, 2017).

2.4 Biofuels from syngas

Syngas, consisting of mainly CO, H_2 and CO_2 and minor compounds such as methane, ethane, ethylene, nitrogen oxides, tar and ash, is produced via gasification of biomass, coal, animal wastes and municipal solid wastes (Kumar et al., 2009). It can also be directly obtained from CO-rich off gases from steel mill industry (Molitor et al., 2016). Unlike carbohydrates (sugar, starch, cellulose, hemicellulose), the spectrum of feedstock for syngas is much wider and is not limited to climate and geography. Besides, all components including lignin are gasified to syngas. The composition of each component in syngas varies depending on types of feedstock, utilization of oxidizing agents (air, oxygen, and steam), conditions of gasification (e.g. temperature, heating rate, residence time and pressure), types of gasifier (e.g. fixed bed or fluidized bed), and operation strategy (e.g. updraft, downdraft, bubbling or recirculating). Syngas can be converted into C_4 to C_{25} hydrocarbons through Fischer-Tropsch process (Dyer et al., 1987) and into alcohols, organic acids and other chemicals through syngas fermentation (Devarapalli & Atiyeh, 2015; Phillips et al., 2017a). The major differences between

Fischer-Tropsch process and syngas fermentation are in types of catalysts and products, CO to H_2 ratio, process conditions such as temperature and pressure. Fischer-Tropsch process uses metal catalysts (e.g. cobalt, iron or ruthenium), requires high H_2 to CO molar ratio (e.g. from 2:1 to 3:1), high reaction temperature (e.g. from 200 to 350 ˚C) and high pressure (e.g. 100 to 1000 psig) (Dyer et al., 1987). However, syngas fermentation uses microorganisms (e.g. acetogenic bacteria) at more flexible CO to H_2 molar ratios, ambient temperatures (e.g. from 25 to 37 ˚C) and pressures (e.g. from atmosphere to up to 30 psig) (Abubackar et al., 2011). This dissertation is mainly focused on syngas fermentation.

2.5 Syngas fermentation

Syngas fermentation converts CO and H_2 into alcohols and organic acids, via anaerobic fermentation of acetogenic microorganisms (Atiyeh et al., 2018; Devarapalli et al., 2016; Liu et al., 2012; Phillips et al., 1994; Phillips et al., 2015; Phillips et al., 1993). The advantages of syngas fermentation are: its broad spectrum of substrates, no requirement of high temperature and pressure, no strict CO and H_2 ratio, and high specific production of alcohol with few byproducts. However, low liquid-gas mass transfer rates and relatively low productivity are the major limitations of this technology. The stoichiometric reactions with Gibbs free energy of CO, CO_2, and H_2 conversions to acetic acid, ethanol, butyric acid, butanol, hexanoic acid and hexanol are listed below from (1) to (12) (Ramachandriya et al., 2013; Ukpong et al., 2012; Zhang et al., 2016):

$$4\,CO + 2\,H_2O \rightarrow CH_3COOH + 2\,CO_2 \qquad (\Delta G^{\circ} = -154.6\ \text{kJ/mol}) \qquad (1)$$

$$4\,H_2 + 2\,CO_2 \rightarrow CH_3COOH + 2\,H_2O \qquad (\Delta G^{\circ} = -74.3\ \text{kJ/mol}) \qquad (2)$$

$6\,CO + 3\,H_2O \rightarrow C_2H_5OH + 4\,CO_2$ (ΔG° = -217.4 kJ/mol) (3)

$6\,H_2 + 2\,CO_2 \rightarrow C_2H_5OH + 3\,H_2O$ (ΔG° = -97.0 kJ/mol) (4)

$10\,CO + 4\,H_2O \rightarrow C_3H_7COOH + 6\,CO_2$ (ΔG° = -421 kJ/mol) (5)

$10\,H_2 + 4\,CO_2 \rightarrow C_3H_7COOH + 6\,H_2O$ (ΔG° = -220 kJ/mol) (6)

$12\,CO + 5\,H_2O \rightarrow C_4H_9OH + 8\,CO_2$ (ΔG° = -486 kJ/mol) (7)

$12\,H_2 + 4\,CO_2 \rightarrow C_4H_9OH + 4\,H_2O$ (ΔG° = -245 kJ/mol) (8)

$16\,CO + 6\,H_2O \rightarrow C_5H_{11}COOH + 10\,CO_2$ (ΔG° = -663 kJ/mol) (9)

$16\,H_2 + 6\,CO_2 \rightarrow C_5H_{11}COOH + 10\,H_2O$ (ΔG° = -341 kJ/mol) (10)

$18\,CO + 7\,H_2O \rightarrow C_6H_{13}OH + 12\,CO_2$ (ΔG° = -759 kJ/mol) (11)

$18\,H_2 + 6\,CO2 \rightarrow C_6H_{13}OH + 11\,H_2O$ (ΔG° = -397 kJ/mol) (12)

2.5.1 Microorganisms and metabolism

The microorganisms used in syngas fermentation can be pure culture or mixed culture. Pure culture fermentation involves only one microorganism (Table 2.1). In a pure culture syngas fermentation, medium has to be sterilized before inoculation and contamination of other microbes should be avoided throughout fermentation. The most commonly used microorganisms in pure culture fermentation are *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Clostridium carboxidivorans*, *Clostridium ragsdalei*, *Alkalibaculum bacchi*, etc. (Abubackar et al., 2015; Devarapalli et al., 2016; Huhnke et al., 2010; Liu et al., 2012; Phillips et al., 2015; Phillips et al., 1993). The common products in pure culture syngas fermentation are ethanol and acetic acid and their

productivities and specificities can reach to high level. In contrast, the inocula for mixed culture syngas fermentation can come from manure, wastewater sludge or digester (He et al., 2018; Wang et al., 2018; Xu et al., 2015). The purpose of the mixed culture is usually to enrich microorganisms that can consume syngas or produce alcohols. Mixed culture can also be obtained by mixing more than one pure cultures with the objective of broadening products spectrum such as production of longer carbon-chain alcohols and carboxylic acids (Diender et al., 2016; Liu et al., 2014b; Richter et al., 2016a). However, the product yield and specificity in mixed culture are relatively low.

Table 2.1 Summary of main syngas fermenting microorganisms

Microorganism	pH	Temperature (˚C)	Products	References
C. carboxidivorans	4.4 – 7.6	24 – 42	Acetate, ethanol, butyrate, butanol, caproate, hexanol	(Liou et al., 2005; Phillips et al., 2015)
C. ragsdalei	5.0 – 7.5	25 – 40	Acetate, ethanol	(Huhnke et al., 2010)
C. autoethanogenum	4.5 – 6.5	20 – 44	Acetate, ethanol	(Abrini et al., 1994)
C. ljungdahlii	4.0 – 6.0	30 – 40	Acetate, ethanol	(Tanner et al., 1993)
C. kluyveri	6.0 – 7.5	30	Butyrate, caproate, H_2	(Gildemyn et al., 2017)
Butyribacterium methylotrophicum	5.5 – 6.0	37	Acetate, ethanol, butyrate, butanol	(Grethlein et al., 1991)
Alkalibaculum bacchi	6.5 – 10.5	15 – 40	Acetate, ethanol	(Allen et al., 2010; Liu et al., 2012)
Sporomusa ovata	5.0 – 8.1	15 – 45	Acetate, ethanol	(Ammam et al., 2016; Möller et al., 1984)
Mixed culture (*A. bacchi* and *C. propionicum*)	6.0 – 8.0	37	Acetate, ethanol, propionate, propanol, butyrate, butanol, hexanol	(Liu et al., 2014b)
Mixed culture (*C. autoethanogenum* and *C. kluyveri*)	5.5 – 6.5	37	Acetate, ethanol, butyrate, butanol, caproate, hexanol	(Diender et al., 2016)
Mixed culture (*C. ljungdahlii* and *C. kluyveri*)	5.7 – 6.4	35	Acetate, ethanol, butyrate, butanol, caproate, hexanol, 2,3-butanediol, octanol	(Richter et al., 2016a)

Syngas-utilizing acetogens conserve energy and fix carbon through Wood-Ljungdahl pathway (WLP), or Acetyl-CoA pathway (Fig. 2.2). Before entering WLP, 1 mole of H_2 is catalyzed into 2 moles of reducing equivalent ([H]) via hydrogenase (H_2ase), and CO together with H_2O is converted into CO_2 and H_2 via water-gas shift reaction. The WLP starts with two branches: Methyl (Eastern) branch and Carbonyl (Western) branch (Köpke et al., 2011a). In Methyl branch, 1 mole of CO_2 is reduced to 1 mole of formate by formate dehydrogenase (FDH) and 2 moles of [H]. Then, 1 mole of formate is combined with tytrahydrofolate (THF) at the cost of 1 mole of ATP to form formyl-THF via formyl-THF synthetase. The resulted formyl-THF goes through several reductions with the cost of several moles of [H] to methylene-THF. The THF is then replaced by corrinoid-FeS-protein (Co-FeS-P) and recycled back to combine with another formate. In the Carbonyl branch, 1 mole of CO_2 is reduced to carbonyl group with the cost of 2 moles of [H] or 1 mole of CO directly converted to carbonyl group via the catalysis of carbon monoxide dehydrogenase (CODH). Then, the group binds to CODH/Acetyl-CoA synthase (ACS) complex. During the acetyl-CoA synthesis, the Co-FeS-P is detached from the methyl group and recycled back as a carrier, leaving the methyl group to combine with carbonyl-group via the catalysis of CODH/ACS complex. The acetyl-CoA is finally synthesized when HS-CoA is introduced. The acetyl-CoA can be converted into acetate and ethanol, or into butyryl-CoA for formation of butyrate and butanol, or into pyruvate for synthesis of 2,3-butanediol (Daniell et al., 2012).

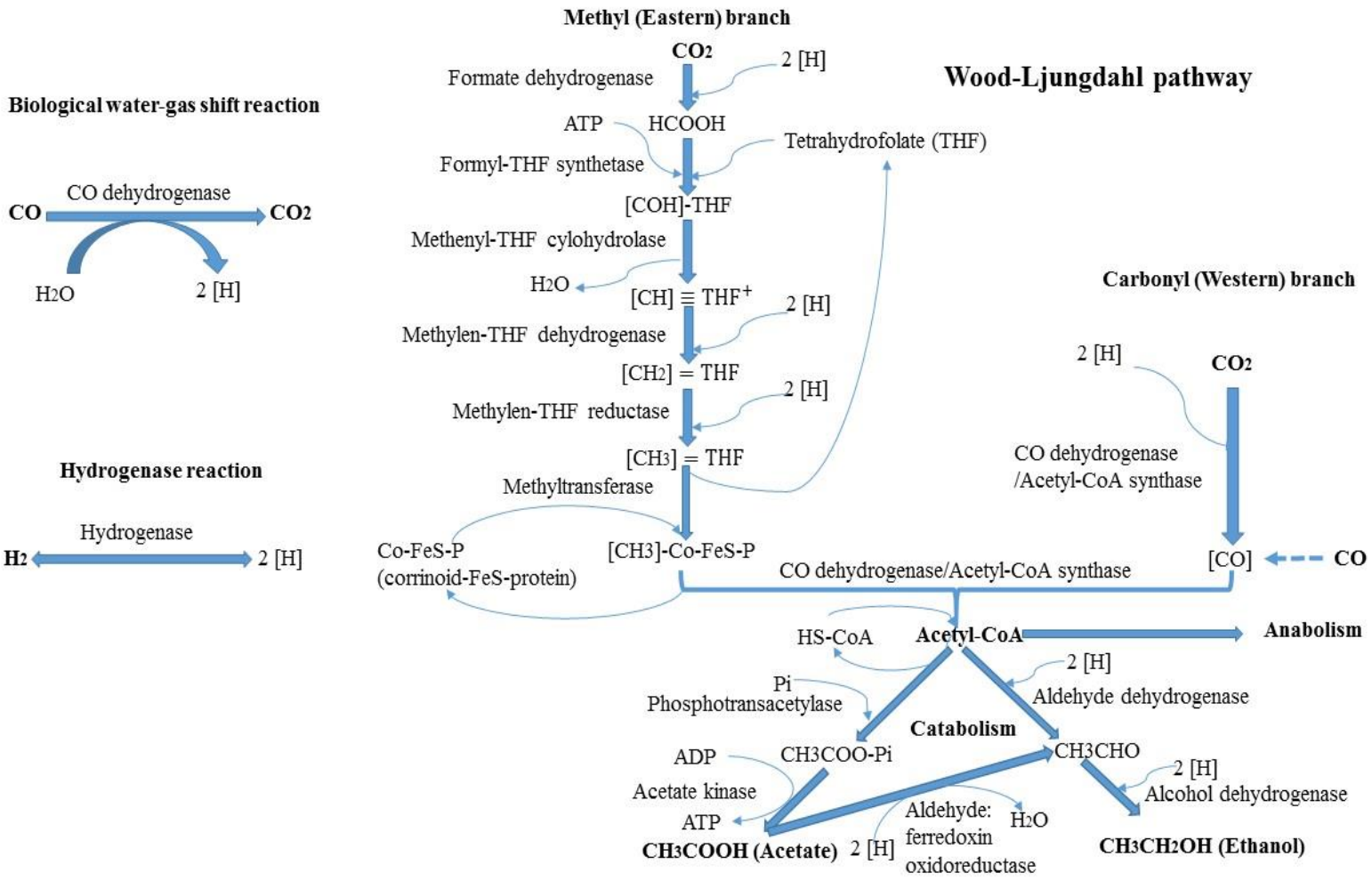

Fig. 2.2 Flow diagram of CO, H_2 and CO_2 conversion to ethanol by acetogenic bacteria via Wood-Ljungdahl pathway (Köpke et al., 2011a).

During the early stage of fermentation when cell growth dominates, the acetyl-CoA takes route of acetate synthesis since the energy is conserved in forms of ATP. The ATP is then utilized during the methyl group synthesis, resulting no net ATP generation. It has been demonstrated that the energy conservation for the cell growth is through sodium motive force (Madigan, 2014). When the cells enter stationary phase caused by the inhibitory effects of low pH and nutrient limitations, the acetyl-CoA takes route of ethanol synthesis. Ethanol can also be converted from acetate first through aldehyde ferredoxin oxidoreductase (AFOR) to aldehyde then through alcohol dehydrogenase (ADH) to ethanol. The latter route is preferable for the cells because it can reduce the undissociated acetic acid concentration in the culture hence relieve the stress of low pH on cells (Richter et al., 2016b).

2.5.2 Medium and operating conditions

2.5.2.1 Gaseous substrates

In syngas fermentation, CO and H_2 are two major substrates for acetogenic bacteria. However, acetogenic bacteria can also use CO_2. The composition of syngas affects growth and product formation. The effects of CO partial pressure on cell growth, productions of acetic acid and ethanol by *C. carboxidivorans* was investigated (Hurst & Lewis, 2010). They showed that with the increase in CO partial pressure from 35.46 to 202.65 kPa, the maximum cell mass increased by 3.4-fold and final ethanol production increased by 20-fold. Besides, ethanol production changed from non-growth associated to growth associated with increasing CO partial pressure. In syngas fermentation, reducing equivalent from H_2 catalyzed by H_2ase provides reducing power for carbon fixation to

cell mass and products. Therefore, the effect of H_2 partial pressure on syngas fermentation by *C. ragsdalei* was investigated (Skidmore et al., 2013). The authors demonstrated that increased H_2 partial pressure led to increased H_2ase activity. But the hydrogenase was saturated at high H_2 partial pressure (above 43 kPa). It was also reported that H_2ase activity of *C. ragsdalei* was reduced by about 90% in the presence of 10% CO (Skidmore, 2010). Besides the major components (CO, H_2 and CO_2), syngas from gasification usually contains impurities such as hydrocarbons (e.g. CH_4, C_2H_2, C_2H_4, C_2H_6, C_6H_6, tar, etc.), nitrogenous compounds (e.g. NH_3, NO_x, etc.), and sulfurous compounds (e.g. H_2S, SO_x, etc.). Syngas with these impurities should be cleaned before fermentation. Acetogens can be inhibited if the impurities in syngas are at high concentration. Xu et al. (2011) studied and reviewed the effects of syngas impurities on acetogens. They pointed out that tar present in gaseous substrates resulted in cell dormancy and products redistribution in *C. carboxidivorans*. However, cells can be adapted to grow in presence of tar after a prolonged period of incubation. The same authors also observed that NH_3 concentration below 150 mmol L^{-1} had no significant effect on cell density of *C. ragsdalei*. But cell growth was delayed when NH_3 concentration was between 250 and 350 mmol L^{-1}. Another study showed that NO content in syngas greater than 40 ppm in syngas inhibited H_2ase of *C. carboxidivorans* but the effect is negligible if NO concentration was below 40 ppm (Ahmed & Lewis, 2007)

2.5.2.2 Liquid medium components

Liquid medium in syngas fermentation provides necessary nutrients to support growth and metabolisms of the microorganisms used. Most media of acetogens used in

syngas fermentation during recent decades were based on medium developed by R. S. Tanner (Tanner et al., 1993). The acetogen media typically consist of amino acids (e.g. yeast extract), vitamins, mineral salts and trace metals. Some trace metals were demonstrated to have significant effects on enzymes in Acetyl-CoA pathway (Table 2.1). Saxena and Tanner (2011) evaluated the effects on cell growth rate, ethanol and acetic acid productions using *C. ragsdalei* by removing or multiplying quantities of trace metals in medium. No cell growth or accumulation of ethanol and acetic acid were observed when nickel (Ni) was removed from medium. Ethanol production was compromised when tungstate (W), iron (Fe), cobalt (Co), and molybdate (Mo) each was removed. However, ethanol production was greatly enhanced with ten-time increase in each of Ni (ethanol increased by 394%), zinc (Zn) (by 423%), selenite (Se) (by 52%) and W (by 102%). The same authors also showed the effects of trace metals on key enzymes in Acetyl-CoA pathway. FDH activity was enhanced by 32% and 41% with five-time increase of Se and ten-time increase of W, respectively. However, FDH activity was inhibited when W and Fe were removed. CODH and H_2ase activities were enhanced by 26% and 3.7 fold, respectively, with ten-time increase of Ni, but these enzymes were inhibited when Fe was removed. Removal of Se and W slightly enhanced ADH activity by 22% and 13%, respectively. However, ADH activity reduced upon removal of Fe.

Table 2.2 Effects of minerals and trace metals on cell growth and products formation and their biological functions in syngas fermentation.

Element	Ion	Effects on syngas fermentation	Biological functions	Reference
Minerals[a]				
N	NH_4^+	Cell mass and ethanol production reduced by 33% and 41%, respectively, if removed.	Provide inorganic nitrogen source for cell growth.	(Saxena & Tanner, 2012)
P	PO_4^{3-}	Cell mass and ethanol production reduced by 58% and 85%, respectively, if removed.	Constituent of nucleic acids, phospholipids and nucleotides.	
S	S^{2-}	No cell mass, ethanol or acetate was produced when sulfur-containing cysteine sulfide was eliminated.	Required by H_2ase and corrinoid enzyme for synthesis of methyl-group in acetyl-CoA	
K	K^+	Not required for cell growth, ethanol, and acetate production.	Formyl-H_4folate synthase activator	(Ljungdhal, 1986)
Na	Na^+	Not required for cell growth, ethanol, and acetate production. But cell mass and ethanol production reduced by 45% and 98%, respectively, with 171 mM Na.	Na+ or H+ is critical for ion gradient-driven phosphorylation involved in ATP synthesis.	(Saxena & Tanner, 2012)
Ca	Ca^{2+}	No effect on cell growth, ethanol, and acetate production with 3.4 mM Ca or eliminated.	Stabilize cell membrane and ATPase activity.	(Ljungdhal, 1986; Saxena & Tanner, 2012)
Mg	Mg^{2+}	Ethanol production decreased by 90% when removed. Ethanol was increased by 12% with 11.7 mM Mg.	Co-factor of key enzymes in cell wall, membranes and phosphate esters.	(Saxena & Tanner, 2012)
Trace metals[a]				
Fe	Fe^{2+}	Ethanol reduced by 82% when Fe was eliminated. No effect on cell mass or products formation with 204 μM Fe.	Component of CODH, FDH, ADH and H_2ase.	(Saxena & Tanner, 2011)
Co	Co^{2+}	Ethanol reduced by 24% when Co was eliminated. No effect on cell mass and products formation with 84 μM Co.	Required by corrinoid enzyme for synthesis of methyl-group in acetyl-CoA	(Ljungdhal, 1986; Saxena & Tanner, 2011; Wood et al., 1986)
Mn	Mn^{2+}	No effect on cell growth, ethanol and acetate production 591 μM Mn or eliminated.	Required by phosphotransacetylase	
Ni	Ni^{2+}	No cell growth or products formation if eliminated. Ethanol increased by 4.0-fold with 8.4 μM Ni.	Component of CODH, ACS and H_2ase.	(Saxena & Tanner, 2011)
Mo	Mo^{6+}	Ethanol decreased by 38% and 34% if eliminated and with 8.3 μM Mo for *C. ragsdalei*. Ethanol decreased by 51% at 20% less Mo and 20% less Zn for *C. carboxidivorans.*	Component of FDH	(Phillips et al., 2015; Saxena & Tanner, 2011)
Se	SeO^{4-}	Ethanol increased by 52% with 10.6 μM Se.	Component of FDH	(Saxena & Tanner, 2011)
W	WO^{4-}	Ethanol increased by 102% for *C. ragsdalei* with 6.8 μM W. Ethanol increased by 206% for *S. ovata* with 0.1 μM W.	Component of FDH	(Ammam et al., 2016; Saxena & Tanner, 2011)
Zn	Zn^{2+}	Ethanol increased by 4.2 fold with 66.9 μM Zn for *C. ragsdalei.* Cell mass doubled, ethanol, butanol, and hexanol increased by 3.0-fold, 7.6-fold, and 44-fold with 280 μM Zn for *C. carboxidivorans.*	Component of ADH	(Li et al., 2018; Saxena & Tanner, 2011)
Cu	Cu^{2+}	Inhibit ethanol production when Cu was present in medium.	Negatively affect ACS activity.	(Saxena & Tanner, 2011)

[a]Minerals and trace metals were based on standard acetogen medium (ATCC medium no. 1754)

Another study investigated the effect of W on performance of strain *Sporomusa ovata* by examining expression of gene encoding metalloenzymes AFOR, ADH and FDH (Ammam et al., 2016). The same study showed that cell growth, ethanol production, conversions of propionate and butyrate to their corresponding alcohols (1-propanol and 1-butanol) were enhanced with ten-time increase in W concentration. W containing AOR and FDH were involved in the improved synthesis of ethanol, acetate, 1-propanol and 1-butanol. The effects of Zn on *C. carboxidivorans* growth and product formation with analysis of gene expression were also investigated in another study (Li et al., 2018). The authors showed that increase in Zn concentration from 7 to 280 μM led to doubled cell concentration, increased production of ethanol, butanol and hexanol by 2.0 fold, 6.6 fold, and 43 fold, respectively. The enhancement of alcohol production was related to increased gene expressions of carbon fixation and ADH.

The effects of syngas fermentation end products (e.g. ethanol, butanol, hexanol, acetic acid, butyric acid, and hexanoic acid) on cell grows, gas consumption of *C. carboxidivorans*, and products formation were also investigated (Fernández-Naveira et al., 2016a; Zhang et al., 2016). Fernández-Naveira et al. (2016a) showed that cell mass concentration of *C. carboxidivorans* and CO consumption were significantly declined with increased pre-added ethanol concentration from 1 to 35 g L^{-1}, butanol concentration from 1 to 20 g L^{-1}, and mixture of ethanol and butanol (1:1, w/w) concentration from 2 to 25 g L^{-1}. In the study of Zhang et al. (2016), cell mass concentration of *C. carboxidivorans* decreased with increased concentration of pre-added acetic acid, butyric acid, hexanoic acid, ethanol and butanol. However, pre-added fatty acids were likely to

induce more production of fatty acids and alcohols, while pre-added ethanol and butanol led to increased productions of corresponding fatty acids.

2.5.2.3 Operating conditions (pH and temperature)

pH and temperature are key parameters in syngas fermenting bacteria. Abubackar et al. (2015) showed that syngas fermentation by *C. autoethanogenum* performed at constant pH of 4.5 resulted in no accumulation of acetic acid. However, equal amounts of acetic acid and ethanol were accumulated when performed at constant pH of 6.0. This indicated that lower pH was favorable for ethanol production. Researchers from different groups used *C. autoethanogenum* (Abubackar et al., 2016), *C. carboxidivorans* (Fernández-Naveira et al., 2016b), *C. ljungdahlii* (Martin et al., 2016) and *C. ragsdalei* (Maddipati et al., 2011) to evaluate effect of pH on syngas fermentation. They all showed that lower pH (4.5-5.0) triggered solventogenesis (conversion of acetic acid to ethanol) for solvent (ethanol and butanol) production while higher pH (5.0-6.0) was preferable for acetogenesis where cell mass growth and production of carboxylic acids (e.g. acetic acid) dominated. Liu et al. (2012) reported that a novel syngas fermenting bacteria *Alkalibaculum bacchi* was able to grow at pH around 8.0 and produce ethanol and acetic acid at pH range between 6.0 and 8.0.

The medium pH is also critical for product carbon-chain elongation in syngas fermentation. Ganigué et al. (2016) using mixed culture syngas fermentation showed that pH between 4.7 and 4.8 allowed conversion of acids to solvents and were compatible for growth of chain-elongation bacteria *C. kluyveri*, while low pH at 4.5 were detrimental to *C. kluyveri* and had adverse effects on accumulation of C4 and C6 compounds. Fernández-Naveira et al. (2017b) controlled initial pH constantly at 5.75 for higher

accumulation of carboxylic acids followed by reduction of the pH to constant 4.75 which allowed conversion of accumulated carboxylic acids to their corresponding alcohols. Syngas fermentation using two-stage reactors combining a growth reactor and a product reactor was also proposed and investigated (Kundiyana et al., 2011; Richter et al., 2013). In these studies, the growth reactor was operated at higher pH (e.g. 5.5) for accumulation of cell mass and carboxylic acids. The product reactor was operated at lower pH (e.g. 4.5-4.7) to trigger alcohol production from accumulated carboxylic acids in the growth reactor.

Fermentation temperature is another critical parameter for growth and metabolisms of acetogenic bacteria. Huhnke et al. (2010) showed that appropriate temperature for growth of *C. ragsdalei* was between 30 ℃ and 40 ℃ with 37 ℃ as optimum. Liou et al. (2005) showed that *C. carboxidivorans* could grow at temperature between 24 ℃ and 42 ℃ and its optimized growth was obtained at temperature between 37 ℃ to 40 ℃. Temperature optimized for growth and growth-associated acid accumulation might not be preferable for alcohol production. Ramió-Pujol et al. (2015) reported that lower temperature of 25 ℃ with *C. carboxidivorans* was preferable for alcohol productions and carbon chain elongation. Besides, incubation of *C. carboxidivorans* at 25 ℃ prevented "acid crash" during growth phase. Similar results had also been reported using *C. carboxidivorans* that a varied temperature from 37 ℃ to 25 ℃ after 24 h of fermentation enhanced productions of acetate, butyrate, caproate, ethanol, butanol, and hexanol than fermentation with constant temperature of 37 ℃ (Shen et al., 2017a).

2.5.3 Gas-liquid mass transfer

Gas-liquid mass transfer is a major challenge for syngas fermentation because water solubilities for CO and H_2 are only 83% and 71% of that of O_2 at 37 ℃ on a molar basis (Phillips et al., 2017). Abundant research work had been performed during recent decade to enhance gas-liquid mass transfer. This includes design and modification of existing bioreactor configurations, utilization of different bioreactors such as hollow fiber membrane reactor (HFMR), trickle bed reactor (TBR) and continuous stirred tank reactor (CSTR), and development of new cultivation systems. The CSTR is the most commonly used reactor both in industry and laboratory due to its controllable stirring and ease of operation. Different types of impeller could cause different stirring patterns which affect contact between liquid and gas. Ungerman and Heindel (2007) evaluated different dual impeller configurations and showed that combination of a Rushton impeller with a different type of impeller (concave turbine, pitched blade turbine, fluidfoil, etc.) other than two Rushton impellers significantly reduced energy cost and enhanced gas-liquid volumetric mass transfer.

Gas diffusion type is another important factor that affects liquid-gas mass transfer. Munasinghe and Khanal (2010b) evaluated different gas diffusing configurations. It was shown that bulb diffuser in the reactor had the highest volumetric mass transfer coefficient (k_La) (91.08 h^{-1}) with increased CO flow rate. The reactor with a submerged composite hollow fiber membrane module had the lowest k_La (0.4 h^{-1}). Orgill et al. (2013) compared the k_La values of HFMR with different modules, TBR with different size of packing beads, and CSTR. The same authors found that the highest k_La (1062 h^{-1})

was obtained using HFMR with non-porous polydimethylsiloxane followed by TBR with large size (6 mm) beads (421 h^{-1}) and CSTR (114 h^{-1}).

Due to the high k_La, HFMR had been investigated intensively in syngas fermentation. The pressure inside the hollow fiber lumen and membrane surface area was studied using HFMR to evaluate the k_La (Yasin et al., 2014). The same study showed that high k_La was obtained at either high pressure (93.76 kPa) with low surface area per unit working volume (27.5 m^{-1}) or low pressure (37.23 kPa) with high surface area per unit working volume (62.5 m^{-1}). Shen et al. (2014b) investigated the effects of syngas flow rate, liquid recirculation rate and dilution rate on CO and H_2 consumptions, ethanol and acetic acid productions by *C. carboxidivorans* using HFMR. The same researchers showed that high syngas flow rate, high recirculation rate and low dilution rate were favorable for ethanol productivity. Other bioreactors such as monolithic biofilm reactor (Shen et al., 2014a), bubble column reactor (BCR) (Rajagopalan et al., 2002b), TBR with co-current and counter-current modes (Devarapalli et al., 2016; Devarapalli et al., 2017), gas-lift reactor (Munasinghe & Khanal, 2014), and horizontal rotating packed bed biofilm reactor (Shen et al., 2017b) had also been studied (Table 2.3).

Table 2.3 Summary of major bioreactors used in syngas fermentation

Bioreactor	Microorganisms	Fermentation mode[d]	Gas substrate $CO:H_2:CO_2:N_2$ (% by volume)	Products (g/L)	Reference
Continuous stirred tank reactor (100 L)	*C. ragsdalei*	Semi-continuous	12-18:7-12:10-17:55-60	Ethanol: 15.0, acetate: 2.8	(Kundiyana et al., 2010b)
Continuous stirred tank reactor (7.5 L)[a]	*C. ragsdalei*	Semi-continuous	20:5:15:60	Ethanol: 9.6, acetate: 3.4	(Maddipati et al., 2011)
Continuous stirred tank reactor (3 L)[a]	Mixed culture of *A. bacchi* and *C. propionicum*	Semi-continuous	40:30:30:0	Ethanol: 1.1, acetate: 5.5, propanol: 1.0, propionate: 1.5, butanol: 0.1, butyrate: 0.4	(Liu et al., 2014b)
Continuous stirred tank reactor with activated carbon	*C. ragsdalei*	Semi-continuous	40:30:30:0 or 20:5:15:60	Ethanol: 19.0, acetate: <1.0	(Atiyeh et al., 2016b)
Continuous stirred tank reactor (7.5 L)	*C. carboxidivorans*	Semi-continuous	20:5:15:60	Ethanol: 1.8, acetate: 0.4, butanol: 0.4	(Ukpong et al., 2012)
Hollow fiber membrane reactor	*C. carboxidivorans*	Continuous	20:5:15:60	Ethanol: 23.9, acetate: 7.0	(Shen et al., 2014b)
Monolithic biofilm reactor[c]	*C. carboxidivorans*	Continuous	20:5:15:60	Ethanol: 5.0, acetate: 3.0	(Shen et al., 2014a)
Bubble column reactor	*C. carboxidivorans*	Continuous	15:4:17:57[e]	Ethanol: 5.0, acetate: 1.0, butanol: 0.6, butyrate: 0.4	(Datar et al., 2004)
Trickle bed reactor[b]	*C. ragsdalei*	Semi-continuous	38:29:28:5	Ethanol: 5.7, acetate: 12.3	(Devarapalli et al., 2016)
Trickle bed reactor[b]	*C. ragsdalei*	Continuous	38:29:28:5	Ethanol: 13.2, acetate: 4.3	(Devarapalli et al., 2017)
Gas-life reactor	*C. carboxidivorans*	Semi-continuous	13-41:20:12:27-45	Ethanol: 0.1, acetate: 0.1	(Munasinghe & Khanal, 2014)
Horizontal rotating packed bed biofilm reactor[c]	*C. carboxidivorans*	Continuous	20:5:15:60	Ethanol: 7.0, acetate: 6.0	(Shen et al., 2017b)

[a] Fermentation used medium with corn steep liquor (CSL).
[b] Fermentation used co-current mode.
[c] Product concentration was estimated when reactor achieved highest ethanol productivity.
[d] Semi-continuous: continuous gas feeding with fixed liquid volume; continuous: continuous gas feeding and medium flow.
[e] Gas substrate contained about 4% CH_4.

Addition of nanoparticles to enhance syngas fermentation based on improving gas-liquid mass transfer was also investigated. Kim et al. (2014) evaluated the effects by adding six types of nanoparticles to medium on CO, H_2 and CO_2 solubility and cell mass, ethanol and acetic acid productions in syngas fermentation of *C. ljungdahlii*. Researchers showed that silica nanoparticles improved CO, H_2 and CO_2 solubility compared with other nanoparticles. Besides, methyl-functionalized silica performed better than silica in CO, H_2, and CO_2 solubility. Cell mass, ethanol and acetic acid were significantly enhanced by 34%, 166%, and 29%, respectively, with addition of nanoparticles compared to no nanoparticles in fermentation. Kim and Lee (2016) then examined magnetic silica nanoparticles on CO, H_2, and CO_2 solubility and cell mass, alcohol, and acid productions in syngas fermentation. Their results showed that silica nanoparticles with Co and Fe oxides enhanced CO, H_2, and CO_2 solubility by 315%, 294%, and 97%, respectively, and improved productions of ethanol, acetic acid and cell mass by 214%, 60%, and 228%, respectively, compared with control in syngas fermentation. Besides, the used magnetic nanoparticles showed almost the same performance as the new ones.

2.5.4 Fermentation strategies

Multi-stage fermentation was considered to be beneficial because it maximizes utilization of nutrients, improves efficiency of cells usually by cell recycling, enhances syngas utilization, and extends products portfolio. Kundiyana et al. (2011) developed two-stage syngas fermentation of *C. ragsdalei* using two CSTRs with each CSTR equipped with one HFM module for cell recycling. In their study, two CSTRs were operated continuously with the first CSTR used for cell growth and the second CSTR for ethanol production. It was shown that two-stage fermentation strategy improved ethanol

and acetic acid yield than single stage fermentation. Multi-stage fermentation can also use combination of two different reactors. Richter et al. (2013) developed a two-stage fermentation of *C. ljungdahlii* by combining CSTR with bubble column reactor equipped with HFM for cell recycle. In their study, the first stage was CSTR used to grow cells and the second stage was bubble column reactor used for converting acetic acid to ethanol at high cell density. A different culture other than acetogens can be used in multi-stage fermentation given than the products of one stage can be used as substrate for another stage. Based on this strategy, Oswald et al. (2016) studied production of malic acid from syngas. In their study, *C. ljungdahlii* was used in the first stage under anaerobic condition for accumulation of acetate while the second stage used *Aspergilus oryzae* under aerobic condition to convert acetate to malic acid.

Semi-continuous and continuous fermentations in trickle bed reactor (TBR) were performed using *C. ragsdalei* with co-current and counter-current gas and liquid flow (Devarapalli et al., 2016; Devarapalli et al., 2017). Results showed that co-current flow was more feasible to avoid flooding of TBR in both studies. Also, ethanol and acetic acid concentrations with co-current mode were 5.7 and 12.3 g L^{-1} in the semi-continuous and were 13.2 and 4.3 g L^{-1} in the continuous fermentation.

2.5.5 Fermentation media design

Developing cost-effective media has been another important part of research in syngas fermentation. Low-cost medium can be achieved either by reducing or removing unnecessary components from the medium or by replacing existing nutrients with low-cost ones. Gao et al. (2013) developed a cost-effective medium for *C. ragsdalei* by

removing the most expensive morpholinoethane sulfonic acid (MES) buffer (around 90% of total media cost), reducing the concentration of mineral and trace metals, and replacing yeast extract (YE) with defined chemicals. The same study showed that the total medium cost after modification reduced by 95% with a compromise of 30% reduction in final ethanol production compared with unmodified medium. Phillips et al. (2015) designed a low-cost medium for *C. carboxidivorans* by reducing certain minerals, trace metals, removing YE and MES, and using low-cost ammonium hydroxide instead of KOH for pH adjustment. The produced ethanol, butanol, and hexanol were at the similar level as in the original standard medium.

Kundiyana et al. (2010a) found that incorporation of cotton seed extract (CSE) in the standard media while removing YE, MES, minerals, trace metals, and vitamins also provide the nutrients required for *C. ragsdalei* without compromising ethanol production. Maddipati et al. (2011) used corn steep liquor (CSL), another nutrient replacement, for syngas fermentation of *C. ragsdalei*. They found that 20 g L^{-1} of CSL medium enhanced final ethanol production and cumulative CO consumption in 7.5-L fermenter by around 60% and 35%, respectively. Shen et al. (2017a) optimized the medium formulation for *C. carboxidivorans* by using Box-Behnken design. They increased quantities of Ni^{2+}, Co^{2+}, SeO_4^{2+}, WO_4^{2+}, Cu^{2+}, reduced amounts of MoO_4^{2+}, Zn^{2+} and Fe^{2+}, and added Fe^{3+} in optimized medium, resulting in doubling ethanol, butanol and hexanol production.

A most recent patent application in our research group showed that addition of activated carbon to fermentation medium of *C. ragsdalei* in CSTR remarkably enhanced ethanol production to around 19 g L^{-1} from around 1.0 g L^{-1} in medium without active carbon (Atiyeh et al., 2016b). The same patent application also showed significantly

enhanced CO and H_2 conversion efficiencies in medium with activated carbon compared to that without activated carbon. Based on this study, the idea of using biochar (raw material for activated carbon) obtained from gasification of biomass in syngas fermentation was proposed and hence the topic of this dissertation (Fig. 2.3)

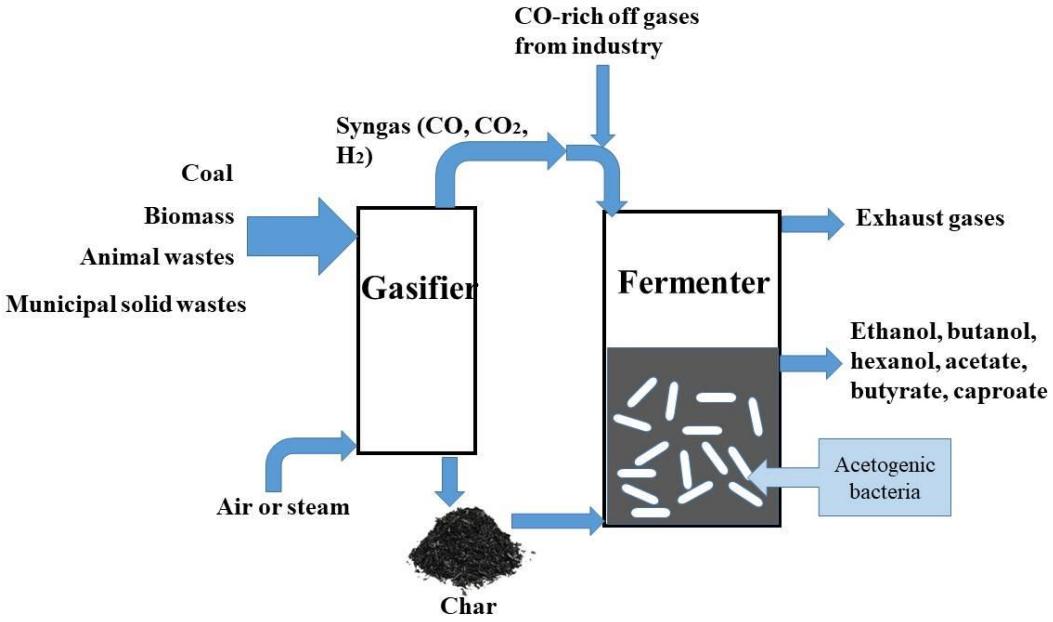

Fig. 2.3 Scheme diagram of usage of biochar from gasification in syngas fermentation.

2.6 Biochar characteristics and application

Biochar is a carbon-rich material obtained from thermochemical conversion (e.g. pyrolysis and gasification) of biomass with little or no O_2 supply (Lehmann et al., 2006). Biochar is distinguished from charcoal, char, and activated carbon because of its typical application in soil amendment and atmospheric carbon sequestration (Lehmann, 2009). The physical and chemical properties of biochar include pH, surface area (SA), porosity, fixed carbon, ash contents, leaching ability of alkali and alkaline compounds (Na, K, Ca and Mg), contents of minerals and metals (P, S, Fe, Mn, Zn etc.), pH buffer capacity,

cation exchange capacity (CEC) and electrical conductivity (EC) (Azargohar et al., 2014; Yuan et al., 2011). A brief summary of major characteristics of biochar from different sources is listed in Table 2.4. The physical and chemical properties of biochar are primarily affected by feedstock type, production methods, heating rate and final reaction temperature (Sun et al., 2014). Because of these properties, biochar can be used to enhance soil properties such as pH, cation exchange capacity (CEC), bulk density, water holding capacity, and activity of microorganisms (Jien & Wang, 2013). In addition, biochar surface has functional groups that were reported to adsorb heavy metals and organic compounds in contaminated soil and water, and to sequestrate CO_2 to alleviate greenhouse effects (Creamer et al., 2014; Taha et al., 2014).

Table 2.4 Characteristics of biochar from different sources and production methods

Feedstock	Production method	Temperature (℃)	Ash (%)	pH (in H_2O)	CEC (cmol kg^{-1})	EC (dS m^{-1})	SA (m^2 g^{-1})	Reference
Poultry litter	Pyrolysis	600	NA[a]	9.9	47.8	780-960	NA	(Jassal et al., 2015)
Spruce-pine-fir			NA	7.5	1.3	36-49	NA	
Pine sawdust	Pyrolysis	680	1.01	9.7	22.8	1.6	795	(Srinivasan et al., 2015)
Broiler litter			11.2	10.1	23.9	14.8	7.0	
Paunch grass			28.7	8.8	31.0	8.6	2.0	
Sewage sludge	Pyrolysis	700	86.8	8.4	NA	1.6	26.7	(Yuan et al., 2015)
De-inking paper sludge	Pyrolysis	500	88.3	9.4	16.5	755	22.8	(Méndez et al., 2014)
Sugarcane bagasse	Pyrolysis	<500	12.0	9.6	69.6	0.1	92.3	(Abdelhafez et al., 2014)
Orange peel			11.0	8.8	68.3	0.1	0.2	
Rice husk and cotton seed shell	Pyrolysis	400	12.6	10.6	12.5	1.0	NA	(Akhtar et al., 2014)
Arundo donax L. straw	Pyrolysis	800	6.2	11.2	NA	0.2	506.3	(Zhao et al., 2017)
Canola straw	Pyrolysis	700	28.6	10.8	179	NA	NA	(Yuan et al., 2011)
Corn straw			73.3	11.3	210	NA	NA	
Soybean straw			23.7	11.1	222	NA	NA	
Peanut straw			38.5	11.2	254	NA	NA	
Switchgrass	Gasification	NA	12.7	9.6	155.7	NA	NA	(Sun et al., 2018a; Sun et al., 2018b)
Forage sorghum	Gasification	NA	22.8	9.9	253.9	NA	NA	
Red cedar	Gasification	NA	4.0	8.3	175.2	NA	NA	
Poultry litter	Pyrolysis	NA	75.1	10.0	559.8	NA	NA	

[a] Not available

Recently, utilization of biochar in biological production of hydrogen, biogas, bioethanol, biodiesel, and long-chain carboxylic acids has emerged. These applications expanded the spectrum of biochar utilization from traditional soil amendment, wastewater treatment and manufacturing of activated carbon (Table 2.5).

Biochar enhanced hydrogen production from mixed culture of *Enterobacter aerogenes* and *E. coli* in municipal solid wastes was studied by Sharma and Melkania (2017). The authors showed that the biochar used enhanced both hydrogen yield and volatile fatty acid production, reduced lag phase and facilitated removal of chemical oxygen demand (COD). Shen et al. (2017c) amended corn stover biochar (CSBC) and pine biochar (PBC) to anaerobic digestion of sewage sludge. Both CSBC and PBC enhanced substrate consumption, biogas production, and process stability. Besides, CSBC and PBC released micro- and macro- nutrients which added more value to the sewage sludge as fertilizer. Another study used biochars from pyrolysis (pyrochar) and hydrothermal carbonization (hydrochar) in anaerobic digestion for biogas production (Mumme et al., 2014). The same study showed that hydrochar significantly enhanced biogas yield, which might be due to conversion of parts of the hydrochar into methane. However, the inhibitory effect on biogas yield could only be avoided by pyrochar at mild ammonia concentration (e.g. 500 mg kg^{-1}), while inhibition of stronger ammonia concentration could not be reduced either by pyrochar or hydrochar. One possible theory of reduced inhibition from ammonia could be due to alkalinity of biochar, which could contribute to shifting of ammonia-ammonium dissociation equilibrium and reduce concentration of undissociated ammonia which is toxic to microbes. Another possible mechanism of the relieved inhibition from ammonia and improved cell growths and

products formation could be the support of biochar surface for the formation of biofilm, which makes the microbes robust enough to resist the ammonia inhibition and facilitate the metabolisms compared to suspended microbes.

Activated biochar was also used to adsorb inhibitors generated from pretreatment of lignocellulosic biomass for ethanol production (Klasson et al., 2013). In the same study, the acid used in biomass pretreatment generated furfural and hydroxymethylfurfural (HMF), which were toxic to the fermenting yeast. Steam activated biochar could absorb both furfural and HMF. The resulting decontamination of the hydrolysate resulted in significant reduction of lag phase in fermentation. In another study, the growth of *S. cerevisiae* (yeast) on the poultry litter biochar (PLB) medium (2 g L^{-1} PLB) was higher than that of the glucose-added yeast extract (GYE) medium under both aerobic and anaerobic conditions (Diallo, 2014). The same author showed that hydrolysate from steam-exploded poplar mixed with PLB was converted to ethanol faster than that without PLB. The ethanol productivity using hydrolysate with PLB was two times higher than that without PLB, but the final ethanol concentration was lower. The reason was reported to be that the PLB could absorb toxic compounds (furfural and HMF) in hydrolysate of steam-exploded poplar.

Table 2.5 Recent applications of biochar from different sources

Application	Biochar	Benefits in application area	References
Improve soil property	White lead tree biochar	Increased soil pH, CEC, base cations, microbial biomass carbons; decreased soil bulk density; reduced soil loss	(Jien & Wang, 2013)
Improve soil water holding capacity	Gasified biochar from peanut shell and palm kernel shell	Water holding capacity and water adsorption were correlated with micropore volume of biochar	(Bikbulatova et al., 2018)
Remove pesticides from water	Rice straw biochar	Absorbed most pesticides. Pesticides with higher hydrophobicity were more likely being absorbed	(Taha et al., 2014)
Remove aqueous heavy metals	Biochars from biomass and industrial by-products	Removed heavy metals Cd, Cu, Zn, Ni, Pb, Hg, Cr by precipitation, complexation, ion exchange, electrostatic interaction, etc.	(Inyang et al., 2016)
Remove H_2S from biogas	Biochars from pyrolyzed wood chips and anaerobic digester residue	Removed about 98% of H_2S from biogas by oxidation of H_2S into S^0 and sulfate	(Kanjanarong et al., 2017)
Capture CO_2	Sugarcane bagasse biochar	Absorbed CO_2 due to sufficient surface area and porous structure	(Creamer et al., 2014)
Anaerobic digestion for biogas production	Pine wood biochar and white oak biochar	Improved methane contend in biogas, enhanced process stability, alleviate ammonia inhibition	(Shen et al., 2016)
Lignocellulosic ethanol production	Flax shive biochar	Absorbed furfural and HMF, reduced inhibitory effect on the following yeast fermentation	(Klasson et al., 2013)
Biodiesel production	Pyrolyzed hardwood char pretreated with sulfuric acid	Enhanced esterification and transesterification	(Dehkhoda et al., 2010)
Hydrogen production	Pyrolyzed woody biomass biochar	Improved hydrogen production rate, mitigated ammonia inhibition, and shortened lag phase	(Sharma & Melkania, 2017)
Caproate production	Pine wood biochar	Improved productions of caproate, caprylate, H_2 and CO_2 by enrichment of functional microorganisms and enhanced conductivity	(Liu et al., 2017)
Syngas fermentation	Biochars from switchgrass, forage sorghum, red cedar, and poultry litter	Improved utilization of CO and H_2 and enhanced productions of ethanol, acetate, butanol, butyrate and caproate by *C. ragsdalei* and *C. carboxidivorans*	(Sun et al., 2018a; Sun et al., 2018b)

Biochar was also used as catalysts. A catalyst made by sulfonating biochar with fuming sulfuric acid was efficient in conversion of free fatty acid esterification and vegetable oil transesterification (Dehkhoda et al., 2010). The study also showed that the higher surface area in biochar and the longer sulfonation time could lead to the higher catalytic activity in transesterification reaction of vegetable oil, which means more production of biodiesel. In another study, biochar based catalyst which was made by sulfonating biochar with fuming sulfuric acid showed promising catalytic activity for combination of transesterification and esterification reactions in a mixture of canola oil and oleic acid (Dehkhoda & Ellis, 2013). The results also showed that increasing the alcohol to oleic acid molar ratio led to a continuously increasing yield of the reaction and thus improved the biodiesel production.

Liu et al. (2017) showed that enhanced caproate and caprylate productions were found by addition of pine biochar to medium with mixed culture from anaerobic sludge of paper wastewater digester. In the same study, caproate and caprylate productions in culture treated with pine biochar was improved by about 50% and 100%, respectively, after around 100 h of incubation, compared with culture without biochar. Besides, H_2 and CO_2 production during the anaerobic digestion were significantly enhanced in culture with pine biochar. It was shown that biochar could enrich functional microorganisms that committed to carbon chain elongation. Biochar has the potential use in syngas fermentation, which is the focus of this dissertation.

2.7 References

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CHAPTER III

OBJECTIVES

Biochar contains minerals, trace metals and other chemical groups and has buffering capacity that can make a potential resource use in medium formulation for syngas fermentation. The use of biochar as medium component in syngas fermentation has the potential to eliminate the need to add certain nutrients and buffer as well as would possibly provide other useful properties such as buffering capacity and adsorptive sites similar to carbon. Recently, the Oklahoma State University syngas fermentation team reported in a US Patent Application US 2016/0215303 A1 (Atiyeh et al., 2016b) that the addition of activated carbon to syngas fermentation medium largely enhanced ethanol production and prolonged *Clostridium ragsdalei* activity. However, the properties of biochar can be different depending on feedstock and gasification or pyrolysis operating conditions used. Thus, biochar from different feedstocks and manufacturing conditions are expected to have different effects on syngas fermentation. In addition, the same type of biochar could have different effects on the activity of the acetogenic bacteria used in syngas fermentation. Finally, the enhanced syngas fermentation process with biochar should be examined with various sizes of reactors and acetogenic bacteria to demonstrate the feasibility of their and scale-up for commercialization. The utilization of biochar in syngas fermentation has not been studied since the beginning of this project. In addition,

possible mechanisms for biochar enhancement of syngas fermentation were not explored. Thus, the objectives of this study include:

1. Design fermentation media for ethanol production by *C. ragsdalei* using biochar from switchgrass (SGBC), forage sorghum (FSBC), red cedar (RCBC) and poultry litter (PLBC). Test the effects of the each biochar on syngas fermentation with respect to consumption of CO, H_2, and production of ethanol, acetic acid and cell mass. Also, characterize the various biochar used and monitor the changes of elements concentrations in medium during syngas fermentation using Inductive Coupled Plasma (ICP) analysis. Explore possible mechanisms for enhanced syngas fermentation by *C. ragsdalei* with biochar.

2. Design fermentation media for ethanol and butanol production by *C. carboxidivorans* using SGBC, FSBC, RCBC and PLBC. Characterize the physiochemical properties of each biochar in terms of acid neutralizing capacity (ANC), pH buffering capacity, cation exchange capacity (CEC), total soluble and bound cations, and functional groups, etc. Test the effects of each biochar on syngas fermentation of *C. carboxidivorans*. Monitor the changes in elements concentrations in medium with biochar using ICP. Propose a possible mechanism for enhanced syngas fermentation by *C. carboxidivorans* using biochar.

3. Determine the effect of inoculum age of *C. ragsdalei* on syngas fermentation using medium with biochar. Evaluate the effect of biochar loading on syngas fermentation using *C. ragsdalei* and *C. carboxidivorans*. Develop a method to release biochar elements and examine syngas fermentation with biochar leachate.

4. Scale up fermentation from bottle reactors to a 3-L continuous stirred tank reactor (CSTR) with medium containing biochar. Perform fed-batch syngas fermentations using *C. ragsdalei* with standard yeast extract (YE) medium as control and biochar media. Evaluate the effects of medium buffering capacity by comparing syngas fermentation using standard YE medium without MES buffer and biochar media with and without MES buffer.

CHAPTER IV

ENHANCED ETHANOL PRODUCTION BY *CLOSTRIDIUM RAGSDALEI* FROM SYNGAS BY INCORPORATING BIOCHAR IN THE FERMENTATION MEDIUM

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ABSTRACT

Biochar contains minerals and metals that can serve as nutrients for acetogens to produce ethanol via syngas fermentation. In this study, four fermentation media containing biochar from switchgrass (SGBC), forage sorghum (FSBC), red cedar (RCBC) and poultry litter (PLBC) were compared with standard yeast extract (YE) medium for syngas fermentation using *Clostridium ragsdalei*. Fermentations were performed in 250 mL bottle reactors at 150 rpm and 37 ℃ with syngas containing $CO:H_2:CO_2$ (40:30:30) by volume. Results showed that media containing RCBC and PLBC improved ethanol production by 16.3% and 58.9%, respectively, compared to YE medium. *C. ragsdalei* consumed 69% more H_2 and 40% more CO in PLBC medium compared to YE medium. However, no enhancement of ethanol production was observed in SGBC and FSBC media. The highest release of Na, K, Ca, Mg, S and P was from PLBC, which was considered to contribute in enhancement of ethanol production.

4.1. Introduction

The global demand for liquid transportation fuel is expected to increase from 95 million barrels per day (MBPD) in 2015 to 121 MBPD in 2040 (EIA, 2016). Ethanol is blended with gasoline as E10 (10% ethanol, 90% gasoline) in U.S., European Union and most of China (GRFA, 2017; RFA, 2017). Ethanol can be produced from synthesis gas (syngas containing CO, H_2, and CO_2) via gasification-syngas fermentation hybrid conversion process. Gasification of biomass and waste materials generates syngas that can be converted into alcohols by acetogenic bacteria such as *Clostridium ljungdahlii*, *Clostridium ragsdalei*, *Clostridium carboxidivorans*, *Clostridium autoethanogenum* and

Alkalibaculum bacchi via Wood-Ljungdahl pathway (Abubackar et al., 2015; Devarapalli et al., 2016; Gao et al., 2013; Liu et al., 2012; Phillips et al., 2017b; Richter et al., 2016b). Gasification-syngas fermentation process utilizes all biomass components including lignin resulting in a higher ethanol yield than in conventional biochemical conversion process of lingocellulosic materials. However, bottlenecks of syngas fermentation include low gas to liquid mass transfer, high cost of fermentation medium, and low productivity.

Efforts have been made to address the gas to liquid mass transfer limitation by designing and characterization of various types of reactors, diffusers, and impeller (Devarapalli et al., 2016; Munasinghe & Khanal, 2010b; Orgill et al., 2013; Shen et al., 2017b). Research was also focused on evaluating effect of media components such as minerals and trace metals on cell growth, products formation and key enzymes in acetyl-CoA pathway (Saxena & Tanner, 2011; Saxena & Tanner, 2012). Optimization of fermentation medium was also performed by removing or reducing unnecessary components for production of ethanol, butanol and hexanol (Gao et al., 2013; Phillips et al., 2015). Replacements of expensive yeast extract (YE) by low-cost corn steep liquor (CSL) (Maddipati et al., 2011) and cotton seed extract (CSE) (Kundiyana et al., 2010a) improved ethanol production.

Biochar is a carbon-rich material obtained from thermochemical conversion (e.g. pyrolysis and gasification) of biomass with little or no O_2 supply (Lehmann et al., 2006). Biochar is distinguished from charcoal, char, and activated carbon because of its typical application in soil amendment and atmospheric carbon sequestration (Lehmann, 2009). The physical and chemical properties of biochar (pH, surface area, porosity, fixed carbon and ash contents) are primarily affected by feedstock type, production methods, heating

rate and final reaction temperature (Sun et al., 2014). Biochar enhances soil properties such as pH, cation exchange capacity (CEC), bulk density, water holding capacity, and activity of microorganisms (Jien & Wang, 2013). In addition, biochar surface has functional groups that were reported to adsorb heavy metals and organic compounds in contaminated soil and water, and to sequestrate CO_2 to alleviate greenhouse effects (Creamer et al., 2014; Taha et al., 2014). Biochar has also been reported to remove furfural and hydroxymethylfurfural from hydrolyzates in lignocellulosic ethanol production (Klasson et al., 2013), to catalyze esterification and transesterification in biodiesel production (Dehkhoda & Ellis, 2013), and to facilitate biogas production during anaerobic digestion (Mumme et al., 2014).

Biochar can release alkali (e.g. Na and K) and alkaline (e.g. Ca and Mg) elements (Li et al., 2015), and trace metals such as Fe, Mn, Zn, and Ni in liquid media (Lehmann, 2009). The leached alkali and alkaline compounds likely contributed to relieve acid stress that otherwise inhibited growth and metabolic functions of microbes in anaerobic digestion (Luo et al., 2015). Metals such as Ni, Zn and W were critical to stimulate growth and production of acetic acid and ethanol by *C. ragsdalei* (Saxena & Tanner, 2011). The same study also reported that a ten-fold increase in Se concertation from base value only stimulated ethanol production, while a similar increase in Mo concentration had no effect on either growth or production of ethanol and acetic acid. The released minerals and metals from biochar in the fermentation medium can serve as nutrients for cell growth and ethanol production and reduce the use of costly pure chemicals for medium formulation. In addition, syngas fermenting acetogens are susceptible to low pH that leads to complete growth inhibition (Fernández-Naveira et al., 2017c). A shift from

acetogenesis to solventogenesis in acetogens typically occurs when culture pH drops to below 5.0. Ethanol production by *C. ragsdalei* started during decelerated growth and stationary phases when the pH was around 4.7 (Maddipati et al., 2011). *C. ljungdahlii* required a drop in pH from 5.5 to 4.5 to induce solventogenesis (Martin et al., 2016). Therefore, the use of biochar in syngas fermentation medium could allow biochar alkaline components to neutralize undissociated acetic acid and reduce acid stress, which leads to high cell growth and ethanol production.

To our knowledge, the utilization of biochar in syngas fermentation was not previously reported. In the present study, biochars made from gasification of switchgrass (SGBC), forage sorghum (FSBC), red cedar (RCBC), and pyrolysis of poultry litter (PLBC) were incorporated in syngas fermentation media for ethanol production. The best performing biochar was further evaluated. The effects of fermentation conditions on leaching of biochar elements were also elucidated. Inductive coupled plasma (ICP) analysis was performed to determine changes in element concentrations during fermentation.

4.2. Materials and methods:

4.2.1. Microorganism

Clostridium ragsdalei (ATCC-PTA-7826) obtained from Dr. Ralph Tanner's Laboratory at the University of Oklahoma was used in this study. Culture maintenance was performed weekly to obtain highly active culture before a second transfer to a fresh medium or used as inoculum. The maintenance technique was a modification of *C. carboxidivorans* culture maintenance developed in our laboratories (Phillips et al., 2015).

4.2.2. Inoculum preparation

C. ragsdalei inoculum and maintenance medium, also called standard yeast extract (YE) medium, was a modification of the standard acetogen medium (ATCC medium No. 1754) based on previous studies (Saxena & Tanner, 2011; Saxena & Tanner, 2012). The standard YE medium contained (per liter): 25 ml of mineral stock solution, 10 ml of trace metal stock solution, 10 ml of vitamin stock solution, 0.5 g of yeast extract, 10 g of MES, 3 ml of 4% cysteine sulfide solution as reducing agent, and 1 ml of 0.1% resazurin solution as indicator. The detailed compositions of the mineral, vitamin, and stock solutions are shown in Table 4.1.

C. ragsdalei inoculum was obtained at the end of weekly maintenance to reduce lag phase following inoculation. In the modified culture maintenance technique, 80 ml of standard YE medium was sterilized and reduced with 4% cysteine and sodium sulfide in 250 ml serum bottle. A total working volume of 100 ml was achieved in each bottle after 20% (v/v) inoculation with *C. ragsdalei*. The headspace in the bottles was initially purged with syngas mix I containing $CO:H_2:CO_2:N_2$ (20:5:15:60) by volume (Stillwater Steel Supply Company, Stillwater, OK, USA) to 142 kPa and incubated on side statically at 37 ℃ for 66 h. Then, the headspace was exchanged with syngas mix II containing $CO:H_2:CO_2$ (40:30:30) by volume (Stillwater Steel Supply Company) to 101 kPa and the culture was shaken at 125 rpm until 90 h. The headspace was then replaced with syngas mix II to 142 kPa until 114 h and to 170 kPa until 138 h. At 138 h, the pH of medium containing the culture dropped to 4.8 as measured when 0.5 ml liquid sample was withdrawn from the bottle using 3 ml sterile syringe. The pH was adjusted from about 4.8 to between 5.0 and 5.1 with 0.5 ml of 10% NH_4OH solution added to the bottle using

another 3 ml sterile syringe to avoid inhibitory effects of undissociated acetic acid on cells with lowering the culture pH below 4.5 during the next 24 h of incubation. The amount of 10% NH_4OH solution added to adjust the pH was determined from preliminary experiments. After pH adjustment, the agitation speed was increased from 125 to 150 rpm with the headspace pressure kept at 170 kPa. At 162 h, the pH was adjusted again from about 4.6 to between 5.0 and 5.1 using 0.8 ml of 10% NH_4OH solution and the headspace pressure was increased to 225 kPa using syngas mix II with reduced shaking speed back to 125 rpm. *C. ragsdalei* inoculum with optical density (OD) between 0.5 and 0.6 was obtained after 162 h of incubation.

Table 4.1. Composition of syngas fermentation stock solutions.

Medium components	Formula	Amount (g L^{-1})
Minerals stock solution		
Ammonium chloride	NH_4Cl	100
Potassium chloride	KCl	10
Potassium phosphate monobasic	KH_2PO_4	10
Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	20
Calcium chloride	$CaCl_2 \cdot 2H_2O$	4
Vitamin stock solution		
Pyridoxine	$C_8H_{11}NO_3 \cdot HCl$	0.01
Thiamine	$C_{12}H_{17}N_4OSCl \cdot HCl$	0.005
Riboflavin	$C_{17}H_{20}N_4O_6$	0.005
Calcium pantothenate	$Ca(C_9H_{16}NO_5)_2$	0.005
Thioctic acid	$C_8H_{14}O_2S_2$	0.005
p-(4)-Aminobenzoic Acid	$C_7H_7NO_2$	0.005
Nicotinic acid	$C_6H_5NO_2$	0.005
Cyanocobalamin	$C_{63}H_{88}CoN_{14}O_{14}P$	0.005
Biotin	$C_{10}H_{16}N_2O_3S$	0.002
Folic acid	$C_{19}H_{19}N_7O_6$	0.002
2-Mercaptoethanesulfonicacid sodium salt (MESNA)	$C_2H_5NaO_3S_2$	0.01
Trace Metal stock solution		
Nitrilotriacetic acid	$C_6H_9NO_6$	2
Manganese sulfate	$MnSO_4 \cdot H_2O$	1
Ferrous ammonium sulfate	$FeH_{20}N_2O_{14}S_2$	0.8
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	0.2
Zinc sulfate	$ZnSO_4 \cdot 7H_2O$	1
Nickel chloride	$NiCl_2 \cdot 6H_2O$	0.2
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.02
Sodium selenate	Na_2SeO_4	0.1
Sodium tungstate	$Na_2WO_4 \cdot 2H_2O$	0.2
Deficient stock solution		
Ammonium chloride	NH_4Cl	4.5
Nitrilotriacetic acid	$C_6H_9NO_6$	2
Manganese sulfate	$MnSO_4 \cdot H_2O$	1
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	0.2
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.02
Sodium selenate	Na_2SeO_4	0.1
Sodium tungstate	$Na_2WO_4 \cdot 2H_2O$	0.2

4.2.3. Fermentations using media incorporating SGBC, FSBC, RCBC and PLBC

Biochars from gasification of switchgrass (SGBC), forage sorghum (FSBC), and red cedar (RCBC) were obtained from downdraft gasifier at Oklahoma State University. The poultry litter biochar (PLBC) was produced from pyrolysis of poultry litter and obtained from Oklahoma State University Soil, Water and Forage Analytical Laboratory. SGBC, FSBC and RCBC have well developed and aligned honeycomb-like groups of pores with diameter between 10 to 50 μm (Fig. 4.1). These pores were mostly derived from capillary structures of their original raw materials. However, PLBC lacked the porous structure due to its non-biomass origin. Other possible reason is that pyrolysis employed for PLBC production required relatively lower temperature (about 500 ℃) than gasification (between 750 ℃ to 1000 ℃) and therefore produces less developed porous structures (Lehmann, 2009).

The moisture and ash contents, volatile matter, fixed carbon and concentration of elements in SGBC, FSBC, RCBC, and PLBC are shown in Table 4.2. The ash content in PLBC (75%) was the highest compared to SGBC, FSBC and RCBC. Except for total C, all element concentrations in PLBC were higher than in SGBC, FSBC, and RCBC.

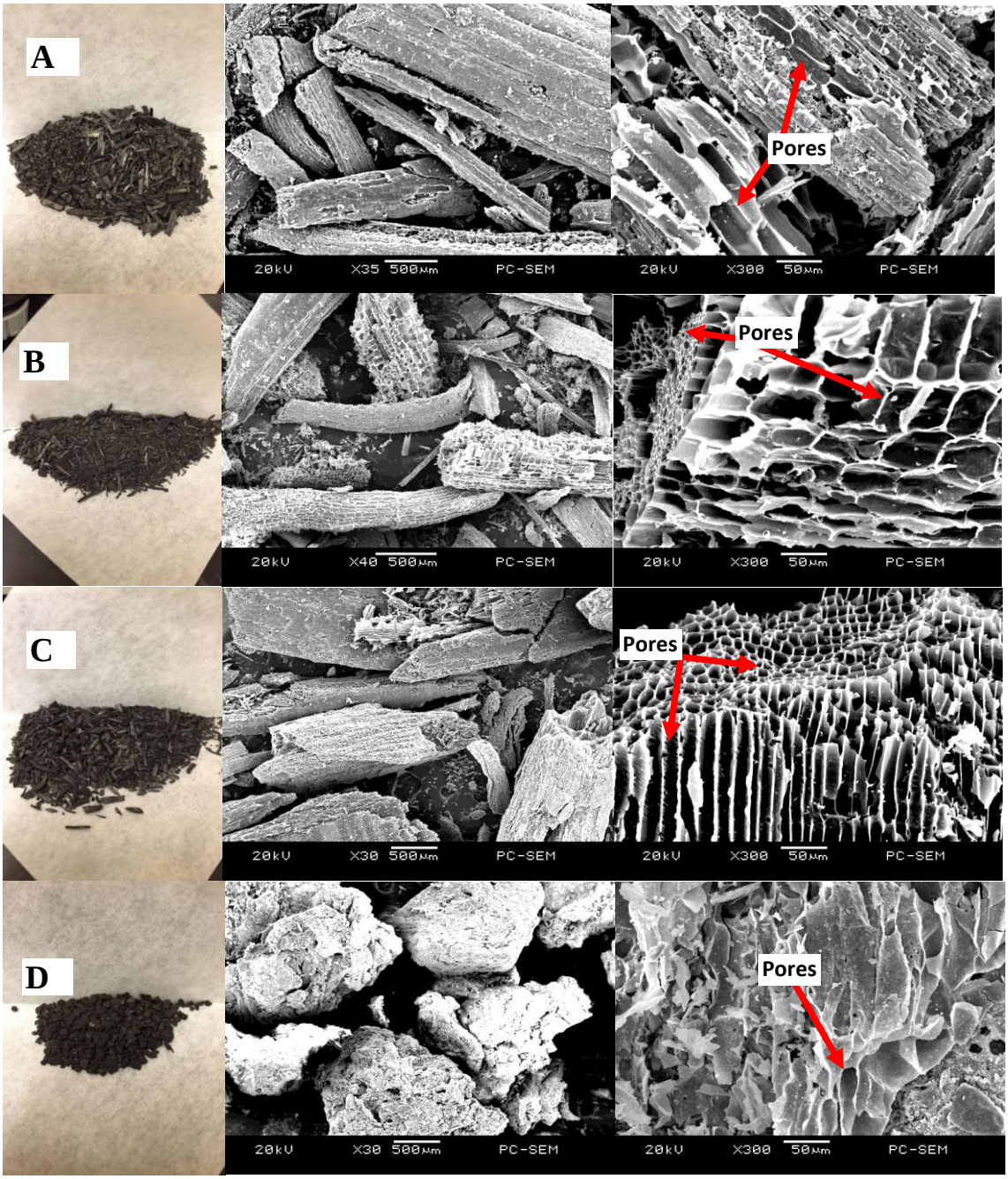

Fig. 4.1. The appearance, scanning electron microscopic photos at 35 magnification, and at 300 magnification of (A) SGBC, (B) FSBC, (C) RCBC, and (D) PLBC.

Table 4.2. Proximate and elemental analyses of various biochars used in this study.

	SGBC	FSBC	RCBC	PLBC
Proximate analysis[a]				
Moisture (%)	4.9	4.2	2.5	0.7
Ash (%)	12.7	22.8	4	75.1
Volatile matter (%)	13.8	18.8	22.3	14.9
Fixed carbon (%)	68.6	54.2	71.2	9.3
Elemental analysis[b]				
Total C (%)	51.23 ± 0.30	53.67 ± 1.51	60.85 ± 2.32	19.56 ± 2.24
Total N (%)	0.59 ± 0.03	0.73 ± 0.03	0.62 ± 0.03	1.50 ± 0.16
Na (%)	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	2.67 ± 0.23
K (%)	0.88 ± 0.04	3.26 ± 0.10	0.27 ± 0.00	5.83 ± 0.52
Ca (%)	1.41 ± 0.03	1.97 ± 0.56	2.55 ± 0.08	10.56 ± 2.06
Mg (%)	0.58 ± 0.01	0.79 ± 0.01	0.13 ± 0.01	2.29 ± 0.37
P (%)	0.41 ± 0.02	0.39 ± 0.01	0.06 ± 0.00	3.49 ± 0.30
S (%)	0.05 ± 0.00	0.10 ± 0.00	0.04 ± 0.00	1.42 ± 0.11
Fe (%)	0.30 ± 0.05	0.17 ± 0.08	0.40 ± 0.07	3.28 ± 0.52
Zn (ppm)	110.38 ± 3.14	107.29 ± 6.97	33.50 ± 10.25	1817.07 ± 126.04
Mn (ppm)	386.36 ± 14.77	167.24 ± 23.87	219.46 ± 1.69	7874.24 ± 3946.27
Cu (ppm)	10.78 ± 0.67	9.90 ± 0.48	9.51 ± 0.07	295.64 ± 16.69
Ni (ppm)	11.59 ± 2.51	< 1.0	< 1.0	38.43 ± 6.61
Mo (ppm)	< 1.0	< 1.0	< 1.0	29.67 ± 1.02
Co (ppm)	< 1.0	< 1.0	< 1.0	13.41 ± 8.66
Se (ppm)	< 1.0	< 1.0	< 1.0	32.77 ± 4.44
W (ppm)	< 1.0	< 1.0	< 1.0	< 1.0

[a] Data was on wet basis.
[b] Data was on dry basis.

Syngas fermentations in standard YE, SGBC, FSBC, RCBC and PLBC media were performed in 250 ml serum bottles with 50 ml working volume. The control treatment (Ctrl) was the standard YE medium with initial pH adjusted to between 6.0 and 6.1 using 5N KOH. The composition of Ctrl (standard YE) and biochar media are shown in Table 4.3. The biochar media contained a deficient stock solution instead of the rich mineral and trace metal stock solutions used in the standard YE medium. The composition of the stock solutions used is shown in Table 1. The deficient stock solution contained nutrients required for *C. ragsdalei* growth and ethanol production not supplied

by the biochar. The deficient stock solution contained ammonium chloride, nitrilotriacetic acid, cobalt chloride, sodium molybdate, sodium selenate, sodium tungstate, and manganese sulfate. The initial pH of the media before addition of biochar was adjusted to about 5.5 using 5N KOH. Addition of biochar to the medium increased the pH to a range of 5.7 and 6.4 based on the type of biochar used. The biochar loading in each bottle was 20 g L^{-1}.

Table 4.3. Summary of components for Control (Ctrl), SGBC, FSBC, RCBC and PLBC treatments.

Medium Name	**Ctrl**	**SGBC**	**FSBC**	**RCBC**	**PLBC**
Nutrients			(g L^{-1})		
Yeast extract	0.5	0.5	0.5	0.5	0.5
MES	10.0	10.0	10.0	10.0	10.0
Biochar	0.0	20.0	20.0	20.0	20.0
Stock solutions			(mL L^{-1})		
Mineral stock solution	25.0	0.0	0.0	0.0	0.0
Trace metal stock solution	10.0	0.0	0.0	0.0	0.0
Vitamin stock solution	10.0	10	10	10	10
0.1% Resazurin	1.0	1.0	1.0	1.0	1.0
4.0% Cysteine-sulfide	10.0	10.0	10.0	10.0	10.0
5.0N KOH solution	5.0	2.1	2.1	2.1	2.1
10% NH_4OH solution	36.0	0.0	0.0	16.0	16.0
Deficient solution	0.0	10.0	10.0	10.0	10.0

The liquid medium was boiled in a microwave followed by bubbling pure N_2 into the medium until cooled to room temperature. The liquid medium was transferred to empty 250 mL serum bottles that had previously purged with N_2 for 2 min. For the media with biochar, the biochar was added in each 250 mL serum bottle purged with N_2. The liquid medium was then transferred to the serum bottle with biochar and purged with N_2 for another 2 min before the bottles were sealed with rubber stoppers and aluminum caps. Sterilization was performed using autoclave (PRIMUS Sterilizer Co., Inc., Omaha, NE, USA) at 121℃ for 30 min. After sterilization, 4% cysteine sulfide solution was added to

the cooled medium to remove traces of O_2. Inoculation rate in each bottle was 20% (v/v). The inoculum age used was 162 h with an OD at 660 nm of about 0.6. After inoculation, syngas mix II was fed into the headspace to 240 kPa and was replaced with the same gas mix every 24 h after a liquid and gas samples were withdrawn from each bottle. The fermentation was performed in triplicate at 150 rpm and 37 ℃ for 15 days.

4.2.4. Effect of inoculum age on syngas fermentation

In general, the shorter the inoculum age, the higher the growth and production rates obtained during syngas fermentation. Therefore, different inoculum ages were examined in four treatments using medium with 20 g L^{-1} of PLBC. Four inocula with ages of 138 h (Treatment TR1), 162 h (Treatment TR2), 186 h (Treatment TR3) and 210 h (Treatment TR4) were used. Three bottles of inoculum were prepared following the same procedure described in section 2.2 during the first 138 h. At 138 h, culture from one of the three maintenance bottles was selected randomly and inoculated in three fermentation bottles with medium containing PLBC. The headspace of each fermentation bottle was filled with syngas mix II to 240 kPa, and the fermentation bottles were placed on the shaker at 150 rpm and 37 ℃. The pH of the remaining inoculum culture was increased to between 5.0 and 5.1 using 10% NH_4OH solution and the headspace was replaced with syngas mix II to 170 kPa. The inoculum bottles were then returned to the shaker. This inoculum culture was consecutively used at 162 h, 186 h, and 210 h to inoculate fresh PLBC medium following the same procedure at age of 138 h. The pH of the inoculum culture at 162 h and 186 h after inoculation was increased to between 5.0 and 5.1 to prevent inhibitory effect on cell growth from decreased pH of the culture. All treatments were performed in triplicate, incubated at 150 rpm and 37 ℃ for 15 days and

fed with syngas mix II to 240 kPa every 24 h after a liquid and gas samples were withdrawn.

4.2.5. Effects of fermentation conditions on leaching minerals and metals from biochar

An experiment was performed to study the effects of fermentation conditions on leaching minerals and metals from PLBC. Three treatments (TRA, TRB, and TRC) were prepared. Treatment TRA was the same as treatment TR1 with 20 g L^{-1} PLBC inoculated with *C. ragsdalei* as discussed in the previous section. Treatment TRB used 20 g L^{-1} PLBC medium without *C. ragsdalei*. Treatment TRC used 20 g L^{-1} PLBC medium without *C. ragsdalei* but with addition of acetic acid solution (100 g L^{-1}) at 0 h and 48 h to achieve acetic acid concentration in the medium similar to what was produced in treatment TRA. A solution containing 10% NH_4OH was added to treatment TRC at 192 h and 264 h to neutralize previously added acetic acid for simulation of acetic acid conversion to ethanol occurred in TRA. Treatments TRB and TRC were incubated with treatment TRA at the same conditions but without daily sampling and headspace gas exchange.

4.2.6. Analytical procedures

4.2.6.1. Cell mass

The cell mass in the liquid samples for each treatment without biochar was measured at 660 nm using UV-1800 spectrophotometer (Shimadzu, Houston, TX, USA). Samples with absorbance above 0.5 were diluted with DI water to be within the linear range of the calibration curve; cell mass (g L^{-1}) = 0.396 $\times OD_{660}$ as previously reported

(Panneerselvam et al., 2009). The cell mass in biochar treatments was determined by Bradford protein assay (Bradford, 1976). Bradford protein assay was also performed for *C. ragsdalei* culture (without biochar) with known OD_{660} values to establish the relationship between OD_{660}, cell mass and measured protein content used for biochar media.

4.2.6.2. Gas and solvent analysis

A volume of 100 µl gas sample from the headspace of each bottle was withdrawn using 100 µl gas tight syringe (Hamilton Company, Reno, NV, USA) every 24 h before replacing syngas in headspace. The gas composition was analyzed using an Agilent 6890N GC (Agilent Technologies, Wilmington, DE, USA) equipped with thermal conductivity detector (TCD) and Supelco PLOT 1010 column as previously reported (Liu et al., 2014b). A volume of 0.5 ml liquid sample after centrifugation was acidified with equal volume of 0.1M HCl solution prior to GC analysis. Ethanol and acetic acid concentrations in the acidified sample were measured in an Agilent 6890N GC (Agilent Technologies) equipped with flame ionization detector (FID) and a DB-FFAP capillary column (Liu et al., 2014b).

4.2.6.3. Elemental analysis

Elemental analysis of solid biochar was performed using an Inductive Coupled Plasma (ICP) spectrometer at the Soil, Water and Forage Analytical Lab of Oklahoma State University according to EPA 3050B method (Hu et al., 2011). Na, K, Ca, Mg, S, P, Fe, Zn, Cu, Mn, Ni, Co, Mo, Se, and W concentrations in the fermentation media were also determined by ICP spectrometer. These elements were components of the mineral, trace metal and deficient stock solutions added in the fermentation medium (Table 1). A

volume of 5 ml of liquid sample was withdrawn from each fermentation bottle before inoculation (0 d) and after fermentation (15 d) for treatments Ctrl, SGBC, FSBC, RCBC, and PLBC. For Treatment TRA, 5 ml of liquid samples were taken at 0, 10 and 15 d. For treatments TRB and TRC, 5 ml liquid samples were taken at 0, 7, 10, and 15 d, and at 0, 3, 7, 10, and 15 d, respectively. More frequent sampling was performed in TRB and TRC without *C. ragsdalei* to keep the working volume similar as in treatment TRA, which was sampled every 24 h. All liquid samples for ICP analysis were filtered through 0.45 µm cellulose acetate filters (VWR International, Radnor, PA, USA). About 50 to 100 µL of 34% (v/v) nitric acid was used to acidify the filtered sample to stabilize metals in the solution.

4.2.6.4. Statistical analysis and calculation

The statistical analysis was performed using Tuckey's multiple comparison of means at 95% confidence level based on R programming language to determine pairwise statistical differences of cell mass, mineral and metal concentrations, H_2 and CO utilization, and ethanol and acetic acid production between each treatment. Ethanol yield per cell mass was estimated as previously reported (Gao et al., 2013). Cell mass and ethanol yields from CO, and CO and H_2 utilization were estimated (Table 4.4) as previously reported (Liu et al., 2012).

Table 4.4. Equations used to estimate cell mass and ethanol yields and gas utilization.

Equation	Reference
$\text{Ethanol yield per cell mass (g/g)} = \frac{\text{Ethanol produced}}{\text{Cell mass}}$	(Gao et al., 2013)
$\text{Cell mass yield} = \frac{\text{Maximum cell mass} - \text{Initial cell mass}}{\text{Moles of CO consumed}}$	(Liu et al., 2012)
$\text{Ethanol yield} = \frac{\frac{\text{Total moles of ethanol produced}}{\text{Total moles of CO consumed}}}{\frac{\text{1 mol ethanol produced}}{\text{6 mol of CO consumed}}} \times 100\%$	
$\text{CO utilization}, \% = \frac{\text{Total moles of CO consumed}}{\text{Total moles of CO supplied}} \times 100\%$	
$H_2 \text{ utilization}, \% = \frac{\text{Total moles of } H_2 \text{ consumed}}{\text{Total moles of } H_2 \text{ supplied}} \times 100\%$	

4.3. Results and discussion

4.3.1. Effects of SGBC, FSBC, RCBC and PLBC on syngas fermentation

C. ragsdalei growth profiles in treatments with standard YE (Ctrl), SGBC, FSBC, RCBC, and PLBC media are shown in Fig. 4.2A. No lag phase was observed in all treatments. However, the cell growth was very low in SGBC medium compared to other media. Cell growth in Ctrl, RCBC, and PLBC media entered stationary phase at 72 h with cell mass concentration reaching about 0.3 g L^{-1}. The differences in cell mass concentrations in Ctrl, RCBC, and PLBC media were not significant ($p > 0.05$) from 72 h to 360 h, although abrupt drop of cell mass in PLBC medium from 240 h to 312 h was observed. This indicated that similar to Ctrl medium, RCBC and PLBC media had no inhibitory effects on cell growth. Cell mass concentration in FSBC medium from 24 h to 216 h was significantly lower ($p < 0.05$) than in Ctrl, RCBC, and PLBC media.

Fig. 4.2B showed that pH dropped in all treatments after inoculation due to growth-associated acetic acid production. The initial pH in biochar media after

inoculation was between 5.6 to 6.1 due to the different quantities and categories of minerals and metals in each biochar (Table 4.2). The pH values between 5.6 to 6.1 have no significant inhibition on *C. ragsdalei* growth (Huhnke et al., 2010). The pH in the Ctrl and RCBC media dropped to about 4.5 at 48 h compared to 4.8 in PLBC medium and 5.0 in FSBC medium at 72 h. A volume of 0.8 mL of 10% NH_4OH was added to each fermentation bottle with RCBC and PLBC media, and 1.8 mL of 10% NH_4OH was added to each fermentation bottle with Ctrl medium to increase the pH to between 5.0 and 5.1 and prevent irreversible inhibition of cells' growth. More NH_4OH solution was required for pH adjustment in Ctrl medium than in biochar media because alkali (Na and K) and alkaline (Ca and Mg) compounds leached from biochars neutralized the accumulated acetic acid (Li et al., 2015; Luo et al., 2015). NH_4OH used to adjust the pH was not consumed by *C. ragsdalei* as previously reported (Xu et al., 2011). In addition, the amount of NH_4OH added in Ctrl medium in the present study was the highest (61.4 mM). This was below the level (150 mM) that strongly inhibited growth of *C. ragsdalei* (Xu et al., 2011). In the present study, the pH in all media increased after reaching lowest levels due to conversion of acetic acid to ethanol and neutralization of the acid by alkali and alkaline compounds leached from biochar.

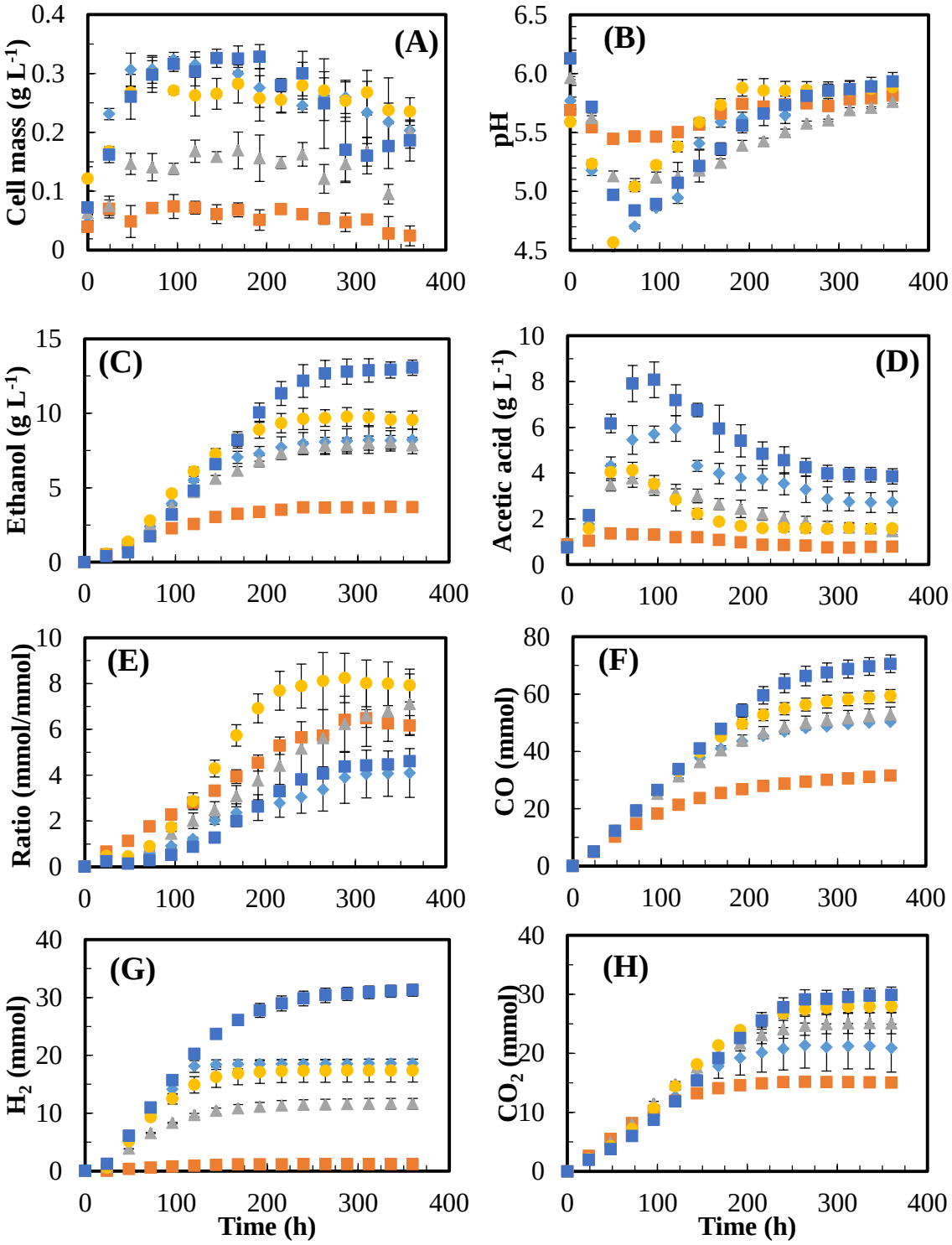

Fig. 4.2. (A) Cell mass, (B) pH, (C) ethanol and (D) acetic acid concentrations, (E) ethanol to acetic acid ratio, cumulative uptake of (F) CO and (G) H_2, and (H) cumulative CO_2 produced during fermentation with *C. ragsdalei* in treatments: (♦) Ctrl, (■) SGBC, (▲) FSBC, (●) RCBC and (■) PLBC, n = 3.

Ethanol production in PLBC medium from 240 h to 360 h was significantly higher ($P < 0.05$) than that in Ctrl, FSBC, and RCBC media (Fig. 4.2C). Insignificant differences in ethanol production were observed in the Ctrl, FSBC, and RCBC media during the same period ($p > 0.05$). *C. ragsdalei* produced the highest amount of ethanol (13.1 g L^{-1}) in the PLBC medium, which was 58.9 % higher than in the Ctrl medium. The medium with RCBC produced more ethanol than Ctrl medium. However, FSBC medium resulted in similar amount of ethanol compared to the Ctrl medium. Ethanol production in SGBC medium was significantly lower ($P < 0.05$) than other media from 96 h to 360 h due to growth inhibition. Ethanol is typically a non-growth-associated product of acetogens, which accumulates at early stationary phase when cell growth slows. Ethanol production during slow growth phase could be dominated by a route in Wood-Ljungdahl pathway that converts acetyl-CoA to acetaldehyde by acetaldehyde dehydrogenase (ADHE) then to ethanol by alcohol dehydrogenase (ADH) (Köpke et al., 2011a). More ethanol was produced during stationary phase as previously reported (Gao et al., 2013; Kundiyana et al., 2010a; Liu et al., 2012). Ethanol production during stationary growth phase in solventogenesis could take another route in Wood-Ljungdahl pathway that reduces accumulated acetate to acetaldehyde via aldehyde ferredoxin oxidoreductase (AOR) followed by conversion to ethanol using ADH (Köpke et al., 2011a).

Acetic acid started to accumulate after inoculation in all media during acetogenesis as shown in Fig. 4.2D. The highest acetic acid concentration of 8.1 g L^{-1} was measured in PLBC medium at 96 h, followed by Ctrl medium (5.9 g L^{-1}) at 120 h. The highest acetic acid concentrations in FSBC and RCBC media at 72 h were significantly lower ($p < 0.05$) than in PLBC and Ctrl media. Acetic acid concentration in

SGBC medium was below 1.6 g L^{-1} due to low cell growth (Fig. 4.2A). Acetic acid concentrations decreased after reaching maximum due to shift to solventogenesis (conversion of acid to solvent). These trends were similar to previous syngas fermentations using *C. ragsdalei*, *C. carboxidivorans* and *A. bacchi* in media containing YE, CSL and CSE (Kundiyana et al., 2010a; Liu et al., 2012; Maddipati et al., 2011). Acetic acid is lipophilic when not dissociated and can be diffused through cytoplasmic membrane into the cell cytosol. As pH in the cytosol increases, acetic acid dissociates, releasing protons that could crash the proton gradient in and out of the cytoplasmic membrane and finally cause cell death. To prevent acid crash, the cells entered solventogenesis, converting acetic acid to alcohol (Fernández-Naveira et al., 2017c). The ethanol to acetic acid molar ratio in the PLBC medium was 4.5 at 360 h, which was slightly higher than in the Ctrl medium (Fig. 4.2E). The ethanol to acetic acid molar ratio in the RCBC medium reached to 7.9, which demonstrated a better specificity for ethanol production in the RCBC medium.

CO consumption after 48 h in the Ctrl, PLBC, FSBC, and RCBC media was significantly higher ($p < 0.05$) than that in the SGBC medium, and achieved total CO uptake between 50 and 71 mmol in 360 h (Fig. 4.2F). CO consumption in from 168 h to 360 h PLBC medium was significantly higher ($p < 0.05$) than in Ctrl and FSBC media. No significant differences ($p < 0.05$) in CO uptake were found between Ctrl, FSBC, and RCBC media during the fermentation. CO is an important source of carbon and reducing power for acetogens and can support ethanol production as a sole gaseous substrate (Phillips et al., 2017b; Rajagopalan et al., 2002a). The highest cumulative H_2 consumption by *C. ragsdalei* was in the PLBC medium at 360 h, which was 68.5%,

79.9%, and 168.5% higher ($p < 0.05$) than those in the Ctrl, RCBC, and FSBC media, respectively (Fig. 1G). *C. ragsdalei* in the SGBC medium consumed the least H_2 due to low cells' activities (Fig. 1A). Differences in H_2 consumption between the RCBC and Ctrl media were insignificant ($p > 0.05$) during fermentation. In addition, H_2 was simultaneously consumed with CO in the Ctrl, FSBC, RCBC, and PLBC media but at lower rate than CO. No H_2 was consumed in the SGBC medium, while CO was consumed (Figs. 1F and 1G). The low consumption of H_2 compared to CO can be attributed to inhibitory effects of CO on hydrogenase (H_2ase) and thermodynamic disfavor of utilization of H_2 in the presence of CO (Hu et al., 2011). No significant differences in CO_2 production ($p > 0.05$) were observed in all media from 0 h to 144 h and in Ctrl, FSBC, RCBC, and PLBC media from 168 h to 360 h (Fig. 4.2H). CO_2 is another carbon source for acetogens and can be reduced to carbonyl group which is combined with methyl group to form acetyl-CoA via combined catalysis of CODH and acetyl-CoA synthase (ACS) (Köpke et al., 2011a). However, ethanol and acetic acid production is more favorable from both CO and H_2 than from CO_2 and H_2 (Phillips et al., 2017b).

The cell mass and ethanol yields from CO, CO and H_2 utilization are shown in Table 4.5. Cell mass yield in the Ctrl medium was significantly higher ($p < 0.05$) than those in SGBC, FSBC, RCBC, and PLBC media. This indicates that more cell mass was produced in Ctrl medium compared to other media with the same amount of CO consumed. However, ethanol yield from CO was the highest in PLBC medium (66.4 %) followed by Ctrl, RCBC and FSBC media. This indicates that more ethanol was produced in PLBC medium from the same amount of CO consumed. In addition, H_2 and CO

utilizations in PLBC medium were significantly higher ($p < 0.05$) than those in other media (Fig. 4.2). *C. ragsdalei* produced more ethanol in PLBC medium in the present study than in other reported media (Table 4.6)

Table 4.5. Fermentation parameters for *C. ragsdalei* in media with various biochars.

Fermentation parameters	Ctrl	SGBC	FSBC	RCBC	PLBC
Cell mass yield (g mol^{-1})[a]	1.01 ± 0.16[A]	0.14 ± 0.11[B]	0.13 ± 0.01[B]	0.40 ± 0.03[B]	0.39 ± 0.01[B]
Ethanol yield (%)[b]	58.74 ± 1.23[A]	41.82 ± 0.29[B]	53.18 ± 1.03[C]	57.63 ± 1.91[A]	66.39 ± 0.31[D]
CO utilization (%)	36.13 ± 4.51[A]	22.73 ± 1.22[B]	37.86 ± 2.00[A]	42.55 ± 1.90[A]	50.41 ± 2.64[C]
H_2 utilization (%)	19.86 ± 1.55[A]	1.27 ± 0.36[B]	12.43 ± 1.29[C]	18.56 ± 2.22[A]	33.24 ± 2.84[D]

[a] Based on CO consumed at maximum cell mass concentration for Ctrl at 48 h, SGBC at 24 h, FSBC at 120 h, RCBC at 72 h, PLBC at 96 h).
[b] Based on CO consumed calculated at 360 h.
Same capital letter in each row represents no significant difference between treatments ($p > 0.05$).

Table 4.6. Comparison of ethanol and acetic acid formed by *C. ragsdalei* in various media and fermentation strategies.

Reactor type/Working volume	Gaseous substrate $CO:H_2:CO_2:N_2$	Medium[a]	Products (g L^{-1})	Reference
Bottles (50 ml)	40:30:30:0	PLBC	Ethanol: 13.1, acetic acid: 3.6	Present study
		RCBC	Ethanol: 9.6, acetic acid: 1.6	
		FSBC	Ethanol: 7.8, acetic acid: 1.5	
		SGBC	Ethanol: 3.7, acetic acid: 0.8	
		YE	Ethanol: 8.2, acetic acid: 2.7	
Bottles (100 ml)	20:5:15:60	CSE	Ethanol: 2.7, acetic acid: 0.8	Kundiyana et al., 2010a
100-L fermenter (70 L)	20:5:15:60 and producer gas[b]	CSL	Ethanol: 15.3, 2-propanol: 9.3, acetic acid: 4.8, butanol: 0.4	Kundiyana et al., 2010b
Bottles (100 ml)	20:5:15:60	CSL	Ethanol: 1.89, acetic acid: 1.45	Kundiyana et al., 2011b
7.5-L fermenter (3 L)	20:5:15:60	CSL	Ethanol: 9.6, acetic acid: 3.4	Maddipati et al., 2011
Bottles (100 ml)	20:5:15:60	YE[c]	Ethanol: 1.3, acetic acid: 3.2	Gao et al., 2013
Trickle bed reactor- co-current mode (500 ml)	38:28.5:28.5:5	YE	Ethanol: 5.7, acetic acid: 12.3	Devarapalli et al., 2016

[a] PLBC: poultry litter biochar; RCBC: red cedar biochar; FSBC: forage sorghum biochar; SGBC: switchgrassbiochar ; YE: yeast extract; CSE: cotton seed extract ; CSL: corn steep liquor.
[b] Producer gas from gasification of switch grass in downdraft gasifier with composition of 7%-12% H_2, 12%-18% CO, 10%-17% CO_2.
[c] Modified standard yeast extract medium (using revised mineral solution and reduced yeast extract).

Element concentrations obtained using ICP analysis in the media before inoculation (0 d) and at 5 d of fermentation are shown in Fig. 4.3. The red solid horizontal lines in the subfigures represent the ICP analysis of the prepared deficient fermentation medium before addition of biochar. Elements with concentrations above the red solid lines indicate that these elements were released in the liquid medium from the added biochar. Elements with concentration below the solid red lines indicate these elements were either utilized by the cells or adsorbed on the added biochar. Ca, Mg, and Fe concentrations in deficient medium without biochar were 2.6, 1.5, and 0.1 mg L^{-1}, respectively, which were low and not shown in Fig. 2. Results showed that higher concentrations of elements (Na, K, Ca, Mg, S, P, and Mn) leached from PLBC medium compared to those other media due to high ash content of PLBC (Table 4.2). However, Zn, Co, and W were possibly utilized by *C. ragsdalei* or adsorbed on PLBC after 15 d of fermentation as their concentrations dropped. Leached Na, K, Ca, and Mg can buffer acidified culture and reduce the acid stress on cells (Li et al., 2015; Luo et al., 2015). These properties of PLBC contributed to improved ethanol production (Fig. 4.2C).

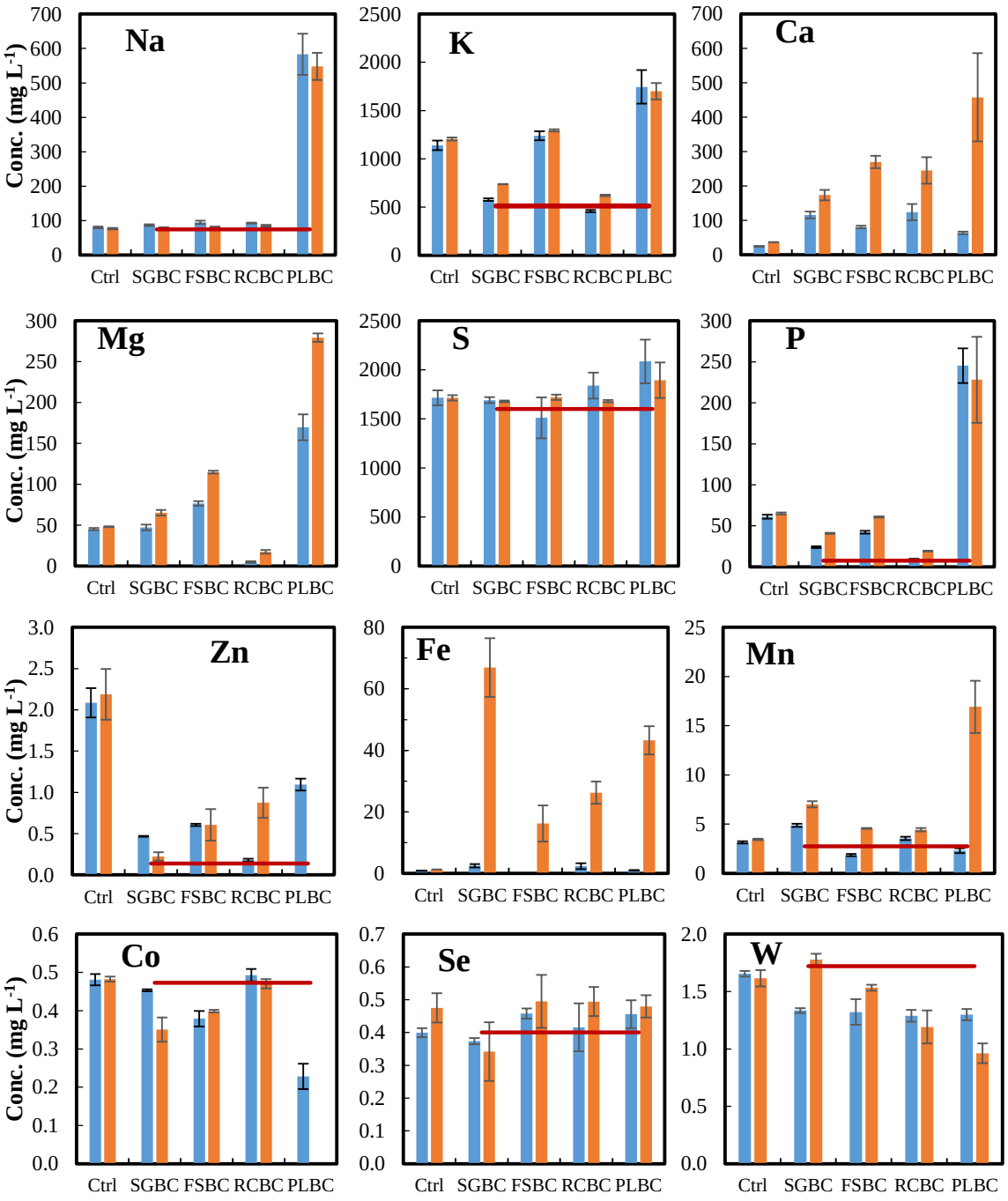

Fig. 4.3. Na, K, Ca, Mg, S, P, Zn, Fe, Mn, Co, Se, and W concentrations before inoculation (0 d, blue bar) and after fermentation (15 d, red bar) with *C. ragsdalei* in Ctrl, SGBC, FSBC, RCBC and PLBC treatments. Red solid horizontal lines represent concentrations of elements in deficient medium before biochar addition; Ca, Mg, and Fe concentrations in deficient medium were 2.6, 1.5, and 0.1 mg L^{-1}, respectively, not shown in figure, n = 3.

Na was added in all fermentation media with cysteine-sodium sulfide solution, resazurin sodium solution, 2-Mercaptoethanesulfonicacid sodium salt (MESNA), sodium molybdate, sodium selenate, and sodium tungstate (Tables 4.1 and 4.3). Na concertation and was quantified before the addition of biochar using ICP at 75.0 mg L^{-1} (shown as a horizontal red line in Fig. 4.3). K was added in the Ctrl medium with the mineral stock solution and as KOH to adjust the pH during media preparation. However, K in the biochar media was released from biochar and from added KOH to adjust the pH. The measured K concentration in the deficient medium before addition of biochar was 402.2 mg L^{-1} (horizontal red line in Fig. 4.3). Na and K are alkali species and can exist in biochar ash as alkali chlorides (i.e., NaCl and KCl), which are water soluble (Koutcheiko et al., 2007; Li et al., 2015). Na concentration in PLBC medium at 15 d was about sevenfold (548 mg L^{-1}) higher than those in Ctrl, SGBC, FSBC, and RCBC media. There were no significant differences ($p > 0.05$) in Na concentration in the Ctrl medium and deficient medium before addition of biochar. This indicated that none or little Na leached from biochar in the SGBC, FSBC, and RCBC media. The highest Na concertation measured in PLBC medium was below the level (3933 mg L^{-1}) that inhibited *C. ragsdalei* growth and ethanol production (Saxena & Tanner, 2012). In addition, no significant differences ($p > 0.05$) were found in Na concentrations between 0 d and 15 d in all media, indicating that Na leached instantaneously in the media and was not affected by the fermentation. It was reported that the removal of Na from YE medium did not affect *C. ragsdalei* growth or ethanol production indicating that energy conservation in *C. ragsdalei* is possibly be H^+-dependent not Na^+-dependent (Saxena & Tanner, 2012).

Ca and Mg are alkaline earth species, which can exist in biochar ash as water-

insoluble compounds (i.e., sulfate, carbonate, oxides of Ca and Mg) (Koutcheiko et al., 2007; Li et al., 2015). Ca and Mg concentrations in deficient medium before addition of biochar were 2.6 and 1.5 mg L^{-1}, respectively. Neither Ca nor Mg were in the deficient stock solution (Table 4.1) used for preparation of biochar media. Therefore, Ca and Mg in biochar media in Fig. 4.3 released from the biochar added. The Ca and Mg concentrations in PLBC medium at 15 d were significantly higher ($p < 0.05$) than those in Ctrl, SGBC, FSBC, and RCBC media. However, the Ca and Mg concentrations in all biochar media at 15 d were significantly higher ($p < 0.05$) than that at 0 d, indicating enhanced leaching of these elements during fermentation. The leaching of Ca and Mg was due to decreased culture pH with acetic acid production by *C. ragsdalei*. Li et al. (2015) reported that lower pH favored leaching of Ca and Mg from biochar. Ca was reported to stabilize bacterial cell wall structure by cross-linking the lipopolysaccharide within the outer membrane (Hughes, 1989), therefore, reducing the chance of cells lysed due to unfavorable environments. Saxena and Tanner (2012) reported 12% increase in ethanol production by *C. ragsdalei* when Mg concentration increased by fivefold from 48.7 mg L^{-1}. Mg functions as a cofactor for several key enzymes in cell wall and membranes. The high concentration of Ca and Mg leached from PLBC likely contributed to enhanced ethanol and acetic acid production Fig. 4.2.

The sulfur, S, was provided in all media with cysteine-sulfide, MES, trace metals stock solution and vitamins (Tables 4.1 and 4.3). S concentrations in SGBC, FSBC, and RCBC media were not significantly different ($p > 0.05$) from that in the Ctrl medium, indicating no release of S from these biochar media (Fig. 2). However, the PLBC medium had 10 to 12 % higher S concentration than in the other media at 0 d and 15 d,

indicating S leached from biochar in PLBC medium. Biochar is highly oxidized and therefore the leached S could be in the form of sulfate that is not be available for acetogens (John et al., 2014). No phosphorous, P, was added during deficient medium preparation. The concentration of P in the PLBC medium was significantly higher ($p < 0.05$) than that it in other media both at 0 d and 15 d and was leached from the PLBC biochar. It was reported that a complete removal of PO_4^{3-} from medium reduced *C. ragsdalei* growth and ethanol production because it is a major ingredient for synthesis of ATP, nucleotides, and phospholipids (Saxena & Tanner, 2012). The high P concentration in the PLBC medium in the present study contributed to enhance ethanol production (Fig. 4.2C).

Zn, Fe and Mn concentrations in the deficient medium before addition of biochar were 0.14, 0.14 and 2.8 mg L^{-1} (shown as a red line except for Fe in Fig. 4.3), respectively, of which Mn was provided by the deficient stock solution (Table 4.1). Zn concentration in PLBC medium at 15 d was below detection limit of 0.01 mg L^{-1} (not shown in Fig. 4.3). Similarly, Zn in SGBC treatment at 15 d was lower than that at 0 d. The decrease in Zn concentration during fermentation could be due to adsorption of Zn by the added biochar (Lima et al., 2009). All biochar media had significantly higher ($p < 0.05$) Fe concentration than the Ctrl medium and most Fe leached during fermentation. Fe was considered essential for FDH, CODH, H_2ase, and ADH in acetyl-CoA pathway. Complete removal of Fe from the fermentation medium resulted in decreased FDH, CODH, H_2ase, and ADH activities and lowered ethanol production (Saxena & Tanner, 2011). In the present study, the highest Fe concentration (66.9 mg L^{-1}) was measured in the SGBC medium, which was sixfold higher than the highest Fe concentration (11.4 mg

L^{-1}) tested by Saxena and Tanner (2011). The high concentration of Fe in the SGBC treatment might have inhibited *C. ragsdalei* growth and products formation Fig. 1.

Mn concentration in the PLBC medium at 15 d (16.9 mg L^{-1}) was significantly higher than those in other media ($p < 0.05$). Zn and Mn did not have any effect on metalloenzymes, *C. ragsdalei* growth, or ethanol and acetate production (Saxena & Tanner, 2011). In the present study, no Cu was added during the media preparation. In addition, Cu concentration in all media was below the detection limit of 0.01 mg L^{-1} at 0 d and 15 d, indicating that biochar did not release Cu during fermentation. It was reported that Cu was inhibitory to *C. ragsdalei* (Saxena & Tanner, 2011).

Co, Se, Mo, and W concentrations in the deficient medium were 0.5, 0.4, 0.1 and 1.7 mg L^{-1}, respectively. Biochars lacked Co, Se, Mo, and W (Table 4.2). These elements were added as part of the deficient stock solution (Table 4.1). The Co concentration in SGBC, FSBC, and PLBC media, and W concentration in FSBC, RCBC and PLBC media at 0 d and 15 d were below the measured concentration in the deficient medium before addition of biochar (Fig. 4.3). The lower concentration of Co in SGBC, FSBC, and PLBC media compared to the Ctrl medium could be due to Co adsorption on biochar. Similarly, W was likely adsorbed on biochar (Fig. 4.3). Mo and Ni concentrations were below the detection limit (0.01 mg L^{-1}) in all media at 0 d and 15 d (data not shown). The difference in Se concertation in biochar and Ctrl media was not significant ($p > 0.05$).

Biochar surface has functional groups such as COO^- (-COOH) and $-O^-$ (-OH) that can release or adsorb minerals and metals depending on their charges (Yuan et al., 2011). These functional groups are positively and negatively charged under acidic and alkaline conditions, respectively (Lehmann, 2009). *C. ragsdalei* culture was mostly acidic with

pH ranging between 4.5 and 6 (Fig. 1B), so the functional groups in SGBC, FSBC, RCBC and PLBC media were positively charged. Mo, Se, and W were added in the medium in the forms of MoO_4^{2-}, SeO_4^{2-}, and WO_4^{2-}, respectively. Therefore, these elements might have been adsorbed by the positively charged groups on biochar surface. W concentrations in SGBC and FSBC media, and Zn concentration in RCBC medium at 15 d were higher than those at 0 d possibly due to their desorption resulted from medium pH increase (Fig. 4.2B). However, adsorbed Zn, Co, and W in PLBC medium was less affected by the pH increase since no desorption was observed at 15 d. Adsorption of Zn, Co, and W on PLBC surface can provide more access for cells to utilize these metals. PLBC has high metal and mineral contents (Table 4.2 and Fig. 4.3), which enhanced ethanol production compared to other media (Fig. 4.2). Therefore, PLBC was selected for further investigation.

4.3.2. Effects of inoculum age on syngas fermentation

It is important to have a healthy and active inoculum to start the fermentation process to reduce lag time, increase productivity and efficiently convert substrate to products. Cell mass concentration in the inoculum increased with incubation time. However, increasing incubation time for syngas fermenting microorganisms can result in harvesting cells that are not active and stressed by accumulation of acetic acid. *C. ragsdalei* was typically inoculated into fresh medium when its OD_{660} reached between 0.4 and 0.8 (Devarapalli et al., 2016). However, the higher the OD_{660} means *C. ragsdalei* growth is closer to stationary phase where cell growth rate declines. The effects of *C. ragsdalei* inoculum on growth and ethanol production in PLBC medium were investigated. The inoculum OD_{660} for treatments TR1, TR2, TR3, and TR4 were 0.53,

0.67, 0.74, and 0.78, respectively (Table 4.7).

Table 4.7. Fermentation parameters in PLBC medium in treatments using inoculum ages of 138 h (TR1), 162 h (TR2), 186 h (TR3) and 210 h (TR4)

Fermentation parameters	Inoculum age			
	TR1-138 h	TR2-162 h	TR3-186 h	TR4-210 h
Inoculum OD_{660}[a]	0.53 ±0.03 [A]	0.67 ±0.03 [B]	0.74 ±0.01 [C]	0.78 ±0.02 [D]
Initial cell mass (g L^{-1})[b]	0.04 ±0.01 [A]	0.06 ±0.01 [B]	0.06 ±0.01 [B]	0.06 ±0.00 [B]
Maximum cell mass (g L^{-1})[c]	0.27 ±0.01 [A]	0.26 ±0.01 [A]	0.24 ±0.01 [A]	0.26 ±0.00 [A]
Initial medium pH	6.14 ±0.04 [A]	6.15 ±0.04 [A]	6.22 ±0.02 [A]	6.03 ±0.06 [B]
Final EtOH (g L^{-1})[d]	16.25 ±0.48 [A]	14.81 ±0.11 [B]	14.63 ±0.56 [B]	14.43 ±0.53 [B]
Maximum HAc (g L^{-1})[e]	8.42 ±0.18 [A]	9.11 ±0.15 [A]	8.66 ±0.30 [A]	8.09 ±0.52 [A]
EtOH/HAc molar ratio[d]	5.84 ±0.17 [A]	4.46 ±0.06 [B]	4.41 ±0.45 [B]	5.10 ±0.59 [A]
H_2 consumed (mmol)[d]	31.71 ±0.83 [A]	31.18 ±0.88 [A]	29.63 ±1.96 [A]	29.90 ±0.1 [A]
CO consumed (mmol)[d]	76.48 ±0.49 [A]	72.39 ±1.86 [A]	72.15 ±2.97 [A]	71.27 ±0.93 [A]
CO_2 produced (mmol)[d]	34.80 ±0.37 [A]	31.02 ±1.08 [A]	31.56 ±1.45 [A]	30.71 ±0.63 [A]
Cell mass yield (g mol^{-1})[f]	0.48 ±0.02 [A]	0.32 ±0.00 [B]	0.29 ±0.01 [B]	0.44 ±0.01 [A]
EtOH yield (%)[g]	62.36 ±1.60 [A]	60.06 ±1.23 [A]	60.20 ±0.03 [A]	59.39 ±1.42 [A]
CO utilization (%)	54.21 ±1.08 [A]	51.46 ±1.79 [A]	51.07 ±2.40 [A]	50.65 ±1.03 [A]
H_2 utilization (%)	33.54 ±1.32 [A]	33.07 ±1.77 [A]	31.44 ±1.89 [A]	31.67 ±0.73 [A]

[a] Inoculum OD_{660} before inoculation.
[b] After inoculation at 0 h.
[c] Measured for TR1 at 144 h, TR2 at 96 h, TR3 at 96 h, TR4 at 72 h.
[d] Measured at 360 h.
[e] Measured for TR1at 120 h, TR2 at 96 h, TR3 at 96 h, TR4 at 96 h.
[f] Estimated at maximum cell concentration for TR1 at 144 h, TR2 at 96 h, TR3 at 96 h, TR4 at 72 h.
[g] Estimated at 360 h.
The same capital letter in each row represents no significant difference ($p > 0.05$).

The maximum cell mass concentrations obtained in the four treatments were not significantly different ($p > 0.05$), indicating that inoculum OD_{660} from 0.53 to 0.78 had no effect on the maximum cell mass concentration obtained. The final ethanol concentration in treatment TR1 was significantly higher ($p < 0.05$) than those in treatments TR2, TR3 and TR4. However, the differences in ethanol concentration among treatments TR2, TR3 and TR4 were insignificant ($p > 0.05$). This suggested that using younger inoculum age in the PLBC medium improved ethanol production. The differences in the maximum acetic acid concentration in all treatments were insignificant No significant differences ($p > 0.05$) were found in CO, H_2 and CO_2 consumption or

ethanol yield between all treatments. The cell mass yield in TR1 and TR4 were significantly higher ($p < 0.05$) than those in TR2 and TR3, while no significant difference ($p > 0.05$) in cell mass yield was observed between TR1 and TR4, or between TR2 and TR3. The results suggested that using inoculum with age between 162 h and 210 h (OD_{660} from 0.5 to 0.8) does not significantly undermine ethanol and acetic acid production (Table 4.7). However, inoculum with age of 138 h improved ethanol production by about 10% compared to inoculum age between 162 h and 210 h.

4.3.3. Effects of fermentation conditions on leaching minerals and metals from biochar

As previosly shown in Fig. 4.3, Ca, Mg, Fe, and Mn concentations at 15 d were significantly higher ($p < 0.05$) than those 0 d in SGBC, FSBC, RCBC, and PLBC media. The leaching of Ca, Mg, Fe, and Mn from biochar during fermentation could be due to drop in pH with acetic acid production. The cause of enhanced leaching of Ca, Mg, Fe, and Mn was investigated by comparing leaching profiles of Ca, Mg, Fe, and Mg in treatment TRA (PLBC medium inoculated with *C. ragsdalei*) with treatment TRB (PLBC medium without inoculation) and treatment TRC (PLBC medium without *C. ragsdalei* but with addition of acetic acid). The concentrations of Ca, Mg, Fe, and Mn in treatment TRA increased by 2.7, 1.5, 9.3, and 5.7 fold, respectively, after 15 d when *C. ragsdalei* was inoculated (Fig. 4.4A). However, no significant differences ($p > 0.05$) in Ca, Mg, Fe, and Mn concentrations were measured in treatment TRB from 0 d to 15 d. This indicated that the activity of *C. ragsdalei* during fermentation enhanced the leaching of Ca, Mg, Fe, and Mn in PLBC medium. A filter-sterilized concentrated acetic acid was added in treatment TRC at 0 d and 2 d to achieve pH of 5.5 and 4.5, respectively, which roughly

matched the pH profile in treatment TRA at 0 d and 2 d. The pH in TRC after addition of acetic acid at 2 d increased to 4.9 at 3 d (Fig. 4.4B) accompanied by significant increase of Ca, Mg, Fe, and Mn concentrations released from PLBC medium (Fig. 4.4A). The pH in TRC increased to 5.2 at 7 d due to more leaching of Ca, Mg, Fe, and Mn. This indicated that leaching of Ca, Mg, Fe, and Mn was triggered by pH drop due to accumulation of acetic acid during fermentation. The released Ca, Mg, Fe, and Mn elements in PLBC reacted with the protons from acetic acid and increased pH. The release of alkali and alkaline elements explains the slow drop in pH during fermentation in biochar media (Fig. 4.4B), which reduced acid stress on *C. ragsdalei* and improved ethanol production.

The results showed that utilization of biochar, a gasification and pyrolysis byproduct, enhances growth and ethanol production during syngas fermentation. However, properties, mineral composition and metal compositions of biochar must be characterized to design and optimize fermentation medium so that the nutrients available in biochar can supplement the medium, especially for specific microorganism that need biochar-deficient elements. Further research is required to study effects of biochar loading on syngas fermentation and examine why certain biochars enhanced the fermentation, while others inhibited the fermentation. The use of biochar as a nutrient supplement is expected to reduce medium cost and enhance ethanol production in commercial syngas fermentation processes.

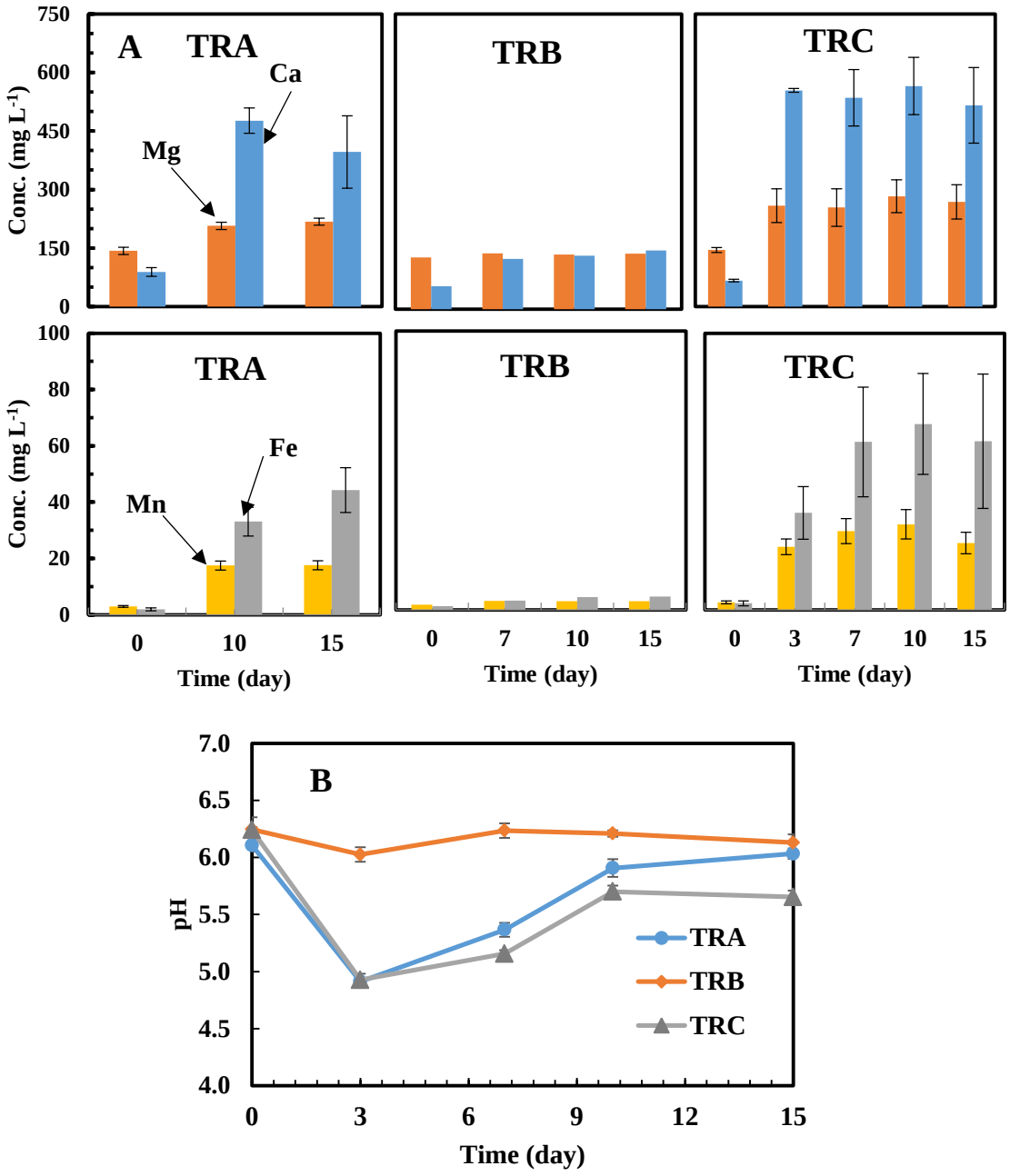

Fig. 4.4. (A) concentrations of Mg (orange), Ca (blue), Fe (grey) and Mn (yellow), and (B) pH profiles in 20 g L^{-1} PLBC medium in treatment TRA with *C. ragsdalei*, at 0 d, 10 d, and 15 d; treatment TRB without *C. ragsdalei*, at 0 d, 7 d, 10 d, and 15 d; treatment TRC without *C. ragsdalei* but with addition of acetic acid, at 0 d, 3 d, 7 d, 10 d, and 15 d.

4.4. Conclusions

Red cedar biochar (RCBC) and poultry litter biochar (PLBC) enhanced ethanol produced by *C. ragsdalei* by 16 and 59%, respectively, compared to standard yeast extract medium. The higher ash content including Na, K, Ca, Mg, P, S, Zn, Fe, Mn, Co, Mo, Se and W in PLBC compared to biomass based biochar enhanced ethanol production. The production of acetic acid and pH drop during fermentation enhanced the release of Ca, Mg, Fe and Mn from PLBC medium, which provided more nutrients, reduced acid stress on *C. ragsdalei* and produced more ethanol. Ethanol/acetic acid molar ratio was 4.5 in PLBC medium.

4.5. References

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CHAPTER V

BIOCHAR ENHANCED ETHANOL AND BUTANOL PRODUCTION BY *CLOSTRIDIUM CARBOXIDIVORANS* FROM SYNGAS

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ABSTRACT

Biochar has functional groups, pH buffering capacity and cation exchange capacity (CEC) that can be beneficial in syngas fermentation. This study examined the properties of biochar made from switchgrass (SGBC), forage sorghum (FSBC), red cedar (RCBC) and poultry litter (PLBC), and their effects on ethanol and butanol production from syngas using *Clostridium carboxidivorans*. Experiments were performed in 250 mL bottle reactors with a 50 mL working volume at 37 ℃ fed syngas containing $CO:H_2:CO_2$ (40:30:30 by volume). Results showed that PLBC and SGBC enhanced ethanol production by 90% and 73%, respectively, and butanol production by fourfold compared to standard yeast extract medium without biochar (control). CO and H_2 utilization in PLBC and SGBC media increased compared to control. PLBC had the highest pH buffering capacity, CEC and total amount of cations compared with SGBC, FSBC and RCBC, which could have contributed to its highest enhancement of ethanol and butanol production.

5.1. Introduction:

Ethanol is used to make E10 to E85 gasoline (RFA, 2017). Unlike ethanol, butanol has higher energy content, is less hydroscopic, and can be directly used in gasoline engines without modification (Lee et al., 2008). Butanol is produced by ABE (acetone-butanol-ethanol) fermentation from sugars, starchy materials and lignocellulosic biomass (Liu et al., 2015a; Liu et al., 2015b). However, the use of sugars and starchy materials causes issues with food-fuel competition while the use of lignocellulosic biomass requires complex and costly pretreatment to remove lignin and inhibitory

compounds from biomass hydrolysates (Liu et al., 2015b; Ujor et al., 2015). Syngas fermentation is an alternative biological route to produce alcohols, organic acids and other chemicals from gaseous substrates (Phillips et al., 2017a; Phillips et al., 2015). The gaseous substrates can be syngas (mainly CO, H_2 and CO_2) obtained from gasification of biomass, animal wastes, coal or municipal solid wastes (Klasson et al., 1993; Kumar et al., 2009), or CO-rich off gases from industries (Molitor et al., 2016). The high flexibility and availability of non-food and waste materials makes syngas fermentation advantageous compared to fermentation of carbohydrates.

Biocatalysts used in syngas fermentation include *C. ljungdahlii*, *C. ragsdalei*, *C. carboxidivorans*, *C. autoethanogenum* and *Alkalibaculum bacchi* (Abubackar et al., 2016; Devarapalli et al., 2016; Liu et al., 2012; Martin et al., 2016; Phillips et al., 2015). *C. carboxidivorans* is the only microorganism that was reported to convert syngas into ethanol, butanol and hexanol in a pure culture (Phillips et al., 2015). In a mixed culture *C. ljungdahlii* and *C. kluyveri* produced ethanol, butanol, hexanol and octanol (Richter et al., 2016a).

Biochar is a carbon-rich material obtained from gasification or pyrolysis of biomass, animal waste, coal or municipal solid waste under oxygen-limiting conditions (Qian et al., 2015). Biochar contains rich porous structures, functional groups, minerals and metals. The contents of functional groups and elements vary significantly based on types of feedstock, biomass feeding rate, biomass to air ratio, processing conditions such as temperature and residence time (Qian et al., 2013). Different feedstocks and processing conditions result in various biochar alkalinity, pH buffer capacity, cation exchange capacity (CEC) and electrical conductivity (EC) (Azargohar et al., 2014; Yuan

et al., 2011). Because of these properties, biochar is commonly applied to improve soil chemical, physical and biological properties (Jien & Wang, 2013; Lehmann et al., 2011), and as adsorbent of organic chemicals and waste gases in water treatment (Kanjanarong et al., 2017; Taha et al., 2014). Biochar is also beneficial in the detoxification of hydrolysate for ethanol production (Klasson et al., 2013), and enhancement of methane and caproate production during anaerobic digestion (Liu et al., 2017; Mumme et al., 2014).

Sun et al. (2018a) recently reported that ethanol production during syngas fermentation by *C. ragsdalei* was highly enhanced in medium with poultry litter biochar (PLBC). The enhancement in ethanol production from syngas was due to the rich minerals and metal content of PLBC. However, the biochar used with *C. ragsdalei* was not thoroughly characterized or examined with other syngas fermenting microorganisms. Therefore, it is important to characterize biochar from various feedstocks and examine their ability to enhance product formation using other syngas fermenting microorganisms. The use of biochar to replace expensive nutrients and to provide buffering capacity with other syngas fermenting bacteria should be investigated. Biofuel production by *C. carboxidivorans* with biochar has not been reported. The objective of this study was to examine the effects of four types of biochar, namely, switchgrass (SGBC), forage sorghum (FSBC), red cedar (RCBC) and poultry litter (PLBC) on ethanol and butanol production from syngas by *C. carboxidivorans*. Biochar physiochemical properties such as acid neutralizing capacity, pH buffering capacity, CEC, total soluble and bound cations, and functional groups were measured to explain possible reasons for enhanced syngas fermentation process. The effect of biochar loading on fermentation was also

investigated.

5.2. Materials and Methods

5.2.1. Microorganism and inoculum preparation

C. carboxidivorans P7 (ATCC BAA-624) (Liou et al., 2005) was used. The culture maintenance medium was the base defined medium (BDM) previously reported (Phillips et al., 2015) with 1.0 g L^{-1} of yeast extract (BDM-1.0YE). The culture maintenance procedure was modified from previous study (Phillips et al., 2015). A volume of 45 mL of BDM-1.0YE in each 250 mL serum bottle was sterilized (121 ℃, 30 min) and then reduced by addition of 0.25 mL of 4% cysteine-sodium sulfide solution. *C. carboxidivorans* was then inoculated into the medium in each bottle with 10% (v/v) rate to achieve the total working volume of 50 mL. The bottles were purged with syngas mix I ($CO:H_2:CO_2:N_2$ 20:5:15:60) for 2 min after inoculation and then pressurized to 142.6 kPa (abs) (CO partial pressure was 28.5 kPa). The bottles were placed horizontally at 37 ℃ without shaking for 68 h. At 68 h and 92 h, the headspace in the bottles was replaced with syngas mix II ($CO:H_2:CO_2$ 40:30:30) by purging headspace for 2 min and then pressurized to 142.6 kPa (CO partial pressure was 57.1 kPa). After 68 h, the bottles were shaken at 125 rpm and 37℃. At 116 h, the OD_{600} of *C. carboxidivorans* culture in each bottle was above 0.6 and ready for use as inoculum for the medium with and without biochar. The pH of the medium during inoculum preparation was adjusted between 5.0 and 5.1 using 10% NH_4OH solution when pH dropped to below 5.0.

5.2.2. Syngas fermentation using media incorporated with biochar

Six treatments were performed (Ctrl A, Ctrl B, SGBC, FSBC, RCBC and PLBC). Ctrl A contained BDM with 0.5 g L^{-1} YE (BMD-0.5YE) and serves as a rich nutrient control. Ctrl B used deficient medium without biochar and serves as deficient medium control. Treatments with SGBC, FSBC, RCBC and PLBC were prepared with deficient medium recipe and each biochar. The composition of each medium is shown in Table 5.1. The initial pH for each medium was adjusted using 5 N KOH solution between 5.0 and 7.0, which is the optimum pH range for *C. carboxidivorans* (Liou et al., 2005).

Table 5.1. The composition of Ctrl A, Ctrl B, SGBC, FSBC, RCBC and PLBC media[a].

Components	Ctrl A	Ctrl B	SGBC	FSBC	RCBC	PLBC
Stock solutions			(mL L^{-1})			
Mineral stock solution	20.0	0.0	0.0	0.0	0.0	0.0
Trace metal stock solution	10.0	0.0	0.0	0.0	0.0	0.0
Vitamin stock solution	10.0	10.0	10.0	10.0	10.0	10.0
0.1% Resazurin	1.0	1.0	1.0	1.0	1.0	1.0
4.0% Cysteine-sulfide	5.0	5.0	5.0	5.0	5.0	5.0
5.0 N KOH solution	6.7	6.7	4.0	2.7	2.3	4.7
Deficient solution	0.0	10.0	10.0	10.0	10.0	10.0
Other nutrients			(g L^{-1})			
Yeast extract	0.5	0.5	0.5	0.5	0.5	0.5
MES	10.0	10.0	10.0	10.0	10.0	10.0
Biochar	0.0	0.0	20.0	20.0	20.0	20.0
10% NH_4OH solution[b]	28.0	14.0	12.0	10.0	16.0	0.0[c]

[a] Ctrl A with modified BDM medium; Ctrl B with deficient medium, SGBC, FSBC, RCBC and PLBC with deficient medium and corresponding biochar.
[b] Used for pH adjustment to between 5.0 and 5.1 during syngas fermentation
[c] No NH_4OH solution was added because pH didn't drop below 5 during fermentation

Stock minerals, trace metals and vitamin solutions for *C. carboxidivorans* were based on BDM medium (Phillips et al., 2015). The deficient stock solution used in Ctrl B, SGBC, FSBC, RCBC and PLBC media was formulated to provide *C. carboxidivorans* with necessary minerals and trace metals, which were absent or at low levels in biochar

(Sun et al., 2018a). The compositions of the stock solutions used are shown in Table 5.2. A volume of 45 mL of fermentation medium was prepared in 250 mL serum bottle as in Table 5.1, sterilized (121 ℃, 30 min) and reduced (0.25 mL of 4% cysteine-sodium sulfide solution). Before inoculation, 5 mL of each medium was withdrawn from each bottle for elemental analysis. Inoculation rate of 20% (v/v) was employed to each medium in each bottle to obtain a total working volume of 50 mL. The fermentation was performed in triplicate (n =3) at 37 ℃ shaken at 125 rpm for 360 h. The headspace in each bottle was initially filled with syngas mix II ($CO:H_2:CO_2$ 40:30:30) at 170.2 kPa and replaced every 24 h to 170.2 kPa in the first 168 h. The initial headspace pressure in all treatments was replaced every 24 h to 142.7 kPa from 168 h to 360 h to reduce CO inhibition. The headspace pressure was measured every 24 h before sampling in each bottle. Then, a 100 µL gas sample was withdrawn to measure remaining CO, H_2 and CO_2 in the headspace, and a 1.5 mL liquid sample was withdrawn to measure pH, cell mass and product concentrations.

Table 5.2. Compositions of stock solutions.

Medium component	Formula	Amount (g L^{-1})
Minerals stock solution		
Ammonium chloride	NH_4Cl	100
Potassium chloride	KCl	10
Potassium phosphate monobasic	KH_2PO_4	10
Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	20
Calcium chloride	$CaCl_2 \cdot 2H_2O$	4
Vitamin stock solution		
Calcium pantothenate	$Ca(C_9H_{16}NO_5)_2$	0.005
p-(4)-aminobenzoic acid	$C_7H_7NO_2$	0.005
Biotin	$C_{10}H_{16}N_2O_3S$	0.002
Trace metal stock solution		
Nitrilotriacetic acid	$C_6H_9NO_6$	2
Manganese sulfate	$MnSO_4 \cdot H_2O$	1
Ferrous ammonium sulfate	$FeH_{20}N_2O_{14}S_2$	0.8
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	0.2
Zinc sulfate	$ZnSO_4 \cdot 7H_2O$	0.2
Nickel chloride	$NiCl_2 \cdot 6H_2O$	0.02
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.2
Sodium selenate	Na_2SeO_4	0.02
Sodium tungstate	$Na_2WO_4 \cdot 2H_2O$	0.02
Deficient stock solution		
Ammonium chloride	NH_4Cl	4.5
Potassium phosphate monobasic	KH_2PO_4	10
Nitrilotriacetic acid	$C_6H_9NO_6$	2
Zinc sulfate[a]	$ZnSO_4 \cdot 7H_2O$	0.2
Manganese sulfate	$MnSO_4 \cdot H_2O$	1.0
Cobalt chloride[b]	$CoCl_2 \cdot 6H_2O$	0.2
Nickel chloride[c]	$NiCl_2 \cdot 6H_2O$	0.02
Sodium molybdate[d]	$Na_2MoO_4 \cdot 2H_2O$	0.2
Sodium selenate[e]	Na_2SeO_4	0.02
Sodium tungstate[f]	$Na_2WO_4 \cdot 2H_2O$	0.02

[a] Provide at low levels in SGBC, FSBC and RCBC. Critical for production of alcohols by *C. carboxidivorans* (Li et al., 2018).
[b] Not provided by SGBC, FSBC and RCBC based on elemental analysis of biochar (Sun et al., 2018a).
[c] Not provided by FSBC and RCBC.
[d] Not provided by SGBC, FSBC and RCBC. Critical for production of alcohols by *C. carboxidivorans*.
[e] Not provided by SGBC, FSBC and RCBC.
[f] Not provided by SGBC, FSBC, RCBC and PLBC. Critical for ethanol production by *C. carboxidivorans*.

5.2.3 Effect of biochar loading on syngas fermentation

The two best performing biochars (SGBC and PLBC) were selected to examine the effect of their loading on syngas fermentation. Six treatments, each in triplicate, were performed to examine the effect of SGBC and PLBC loading on syngas fermentation by *C. carboxidivorans*. Medium formulations for SGBC and PLBC were the same as those in Table 5.1 except for biochar loading. Biochar loading of 5, 10 and 20 g L^{-1} were employed for SGBC (treatment SGBC5, SGBC10 and SGBC20) and PLBC (treatment PLBC5, PLBC10 and PLBC20). Fermentation conditions and sampling were similar to various biochar media.

5.2.4 Analytical techniques for syngas fermentation

Every 24 h before replacing syngas in headspace, a 100 µl gas sample from the headspace of each bottle was withdrawn using a gas tight syringe (Hamilton Company, Reno, NV, USA). CO, H_2 and CO_2 compositions were analyzed using an Agilent 6890N GC (Agilent Technologies, Wilmington, DE, USA) equipped with thermal conductivity detector (TCD) and Supelco PLOT 1010 column as reported previously (Liu et al., 2014b). A volume of 1.5 mL liquid sample from each bottle was taken to determine pH followed by optical density (OD) measurement. Then, centrifugation at 13,000 rpm for 10 min was applied to all liquid samples to precipitate cells and biochar. A volume of 0.5 mL supernatant from each sample was acidified with equal volume of 0.1M HCl solution prior to GC analysis. Agilent 6890N GC (Agilent Technologies) equipped with flame ionization detector (FID) and a DB-FFAP capillary column (Liu et al., 2014b) was used to determine the concentrations of ethanol, butanol, hexanol, acetic acid, butyric acid and

hexanoic acid in each acidified sample.

C. carboxidivorans cell mass concentrations in Ctrl A and Ctrl B were determined by measuring the OD at 600 nm (OD_{600}) using a UV-1800 spectrophotometer (Shimadzu, Houston, TX, USA) and calculated based on formula: cell mass = 0.34 x OD_{600} (Ukpong et al., 2012). Samples with OD_{600} above 0.5 were diluted with DI water to obtain value within the linear range of the calibration curve. The cell mass concentration in medium with biochar was determined by protein assay (Bradford, 1976). Protein assay of *C. carboxidivorans* with known OD_{600} was also performed to establish relationship that enabled comparison of cell mass concentration in Ctrl A and Ctrl B media with that in medium containing biochar.

A 5 mL liquid sample was taken from each bottle before inoculation (0 d), at day 10 (10 d) and day 15 (15 d) of fermentation to measure the level of Na, K, Ca, Mg, S, P, Zn, Ni, Fe, Mn, Cu, Co, Se, Mo and W using an ICP spectrometry housed in the Soil, Water and Forage Analytical Lab of Oklahoma State University according to EPA3050B method (EPA, 1996). The liquid sample was filtered using 0.45 μL cellulose acetate membrane filter and acidified with 0.1 to 0.4 mL of 34% (v/v) HNO_3 to stabilize the metals before ICP analysis.

5.2.5 Physical and chemical properties of biochar

Biochars from gasification of switchgrass (SGBC), forage sorghum (FSBC) and red cedar (RCBC) were obtained from a downdraft gasifier at Oklahoma State University. The poultry litter biochar (PLBC) was produced by slow pyrolysis of poultry litter collected from a commercial poultry house in eastern Oklahoma. PLBC was obtained from Oklahoma State University Soil, Water and Forage Analytical Lab.

Biochar particle size distribution was performed using US standard sieve with 0.25 mm (No. 60), 1.0 mm (No. 18) and 2.0 mm (No. 10) mesh size. Each ground biochar that passed through 1 mm sieve was used for further analysis. The biochar pH was determined in DI water at a 1:5 (w/w) ratio (Yuan et al., 2011). Each biochar sample was mixed thoroughly with DI water and equilibrated for 1 h. The pH was then measured using an Orion Star A221 pH meter (Thermo Scientific). Total soluble, bound cations and cation exchange capacity (CEC) of each biochar were performed based on American Standard Test Method (ASTM-D7503-10, 2010). For CEC analysis of each biochar, 3 g of air-dry ground biochar was weighed into a 50 mL screwed cap centrifuge tube. Twelve mL of 1.0 M ammonium acetate solution was added to the tube before shaking for 5 min on an orbital shaker at 220 rpm. Then, the tube was held static for 24 h before shaken for another 15 min. The liquid mixture in the tube was vacuum filtered through Whatman No.2 filter paper. During filtration, 20 mL of 1.0 M ammonium acetate was used each time to wash the biochar for four times. The ammonium acetate washed biochar was then washed three times each with 20 mL of isopropanol to remove ammonium acetate. The isopropanol washed biochar was then washed for four times each using 20 mL of 1.0 M KCl solution. The biochar was not allowed to dry between each wash in each washing step. The KCl extracts were transferred into 100 mL volumetric flask, which was then filled to a volume of 100 mL with DI water. The KCl extracts were analyzed for nitrogen (NO_3-N and NH_4-N) concentration (mg L^{-1}) using a Lachat Flow Injection Auto-analyzer (QuikChem 8500 Series 2, Hach Company, Loveland, CO). CEC was calculated using formula: CEC= N $\times$1(cmol)/140 (mg) $\times$0.10 (L)/3.0 (g) $\times$1000 (g kg^{-1}), where N is the concentration of NO_3-N and NH_4-N (mg L^{-1}).

The pH buffering capacity of each biochar was measured as reported by Xu et al. (2012). A 0.5 g of air-dry ground biochar was added into each of eight 50 mL centrifuge tubes and an appropriate amount of DI water was added such that a final volume after addition of various amounts of 0.1 M HCl or 0.1 M NaOH was 10 mL. Addition of 0, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mL of 0.1 M HCl and 0.5 mL of 0.1 M NaOH were used based on initial pH of biochar used in this study. Chloroform (0.25 mL) was added to each tube after addition of acid or base to inhibit microorganisms. Addition of 1.0 mL of 0.05 M $CaCl_2$ was made to each tube to minimize variations in ionic strength. The mixture was equilibrated and shaken at 220 rpm on orbiter shaker at 25℃ for 24 h. The mixture was then left to equilibrate statically for another 6 days at 25℃ and was shaken for 2 min each day to re-suspend the biochar. After equilibration, the pH of the mixture in each tube was measured. The pH buffering capacity was calculated as the amount of HCl or NaOH (mmol) added per unit of pH per kilogram of biochar. Acid-base titration of biochar was based on the method reported by Yuan et al. (2011). The biochar was suspended in DI water at a ratio of 1:40, thus 0.5 g of each biochar was suspended with 20 mL of DI water in 125 mL serum bottle. The content in each bottle was thoroughly mixed. The mixture was then titrated with 0.1 M HCl to pH 2.0 by automatic titration (TIM840 Titration Manager, Hach Company, Loveland, CO, USA) with continuous stirring. The titration rate was kept at 0.5 mL min^{-1} with data collected every 6 seconds.

The functional groups of each biochar were determined by Fourier Transform Inferred Spectroscopy (NICOLET 6700 FTIR spectrometer, Thermo Electron Corporation, Madison, WI, USA) with an attenuated total reflectance (ATR) accessory. Ambient air was scanned as background signal before analyzing samples. The 64 scans of

spectra of samples were obtained at 4 cm^{-1} resolution from 4000 to 650 cm^{-1}. The spectrum of each sample was defined by comparing peak positions with known peaks. Each spent biochar after fermentation was collected and washed five times each with 50 mL DI water to remove microbes mixed with or attached to the biochar. During washing, the spent biochar was thoroughly mix with DI water and placed statically for 5 min for biochar to precipitate before decanting the supernatant. The washed biochar was then dried at 105 ℃ for 12 h. Then, the dried biochar was passed through 1 mm sieve) for functional group analysis using FTIR.

5.2.6 Statistical analysis and calculations

The statistical analysis was performed using Tukey's multiple comparison of means at 95% confidence level based on R programming language to determine pairwise statistical differences of cell mass, mineral and metal concentrations, H_2 and CO utilization, alcohols and carboxylic acids production between each treatment. Calculations of cell mass yield, CO and H_2 utilization were performed as reported previously (Sun et al., 2018a). Ethanol and butanol yields were estimated using equations 1 and 2.

$$\text{EtOH yield}, \% = \frac{\frac{\text{Total moles of ethanol produced}}{\text{Total moles of CO consumed} - \text{total moles of CO consumed for butanol}}}{\frac{\text{1 mol ethanol produced}}{\text{6 mol of CO consumed}}} \times 100\% \quad (1)$$

$$\text{BuOH yield}, \% = \frac{\frac{\text{Total moles of butanol produced}}{\text{Total moles of CO consumed} - \text{total moles of CO consumed for ethanol}}}{\frac{\text{1 mol butanol produced}}{\text{12 mol of CO consumed}}} \times 100\% \quad (2)$$

5.3 Results and discussion

5.3.1 Physical and chemical properties of biochar

Fig. 5.1 shows acid titration curves and FTIR spectra for the functional groups for SGBC, FSBC, RCBC and PLBC. The acid titration curves represent Acid Neutralizing Capacity (ANC) or alkalinity of each biochar (Fig. 5.1A). The initial pH values of SGBC, FSBC, RCBC and PLBC in water matrix before titration were 9.8, 10.0, 8.2 and 10.2, respectively. To reach a pH of 2.0, PLBC required the highest volume of 0.1 M HCl (31.3 mL) followed by FSBC (13.4 mL), RCBC (12.7 mL), and SGBC (5.7 mL). The pH of PLBC in water matrix decreased from 10.0 to 2.0 slower than other biochar did throughout the titration, indicating PLBC's strong alkalinity. During acid titration, the pH increased from 3.6 to above 4.1 and from 3.1 to 3.3 in FSBC and RCBC, respectively, indicating a strong ANC within these ranges. SGBC had the lowest ANC because its pH decreased faster than other biochar. The ANC of biochar provides its potential for neutralizing undissociated acetic acid into acetate during syngas fermentation. The use of biochar can therefore reduce possible acid stress on *C. carboxidivorans* and promote product formation.

Other physiochemical properties of SGBC, FSBC, RCBC and PLBC are summarized in Table 5.3. The biochars tested have high pH values in water, which was due to release of soluble cations. Bound cations were adsorbed to the charged surface of biochar and can be exchanged with other cations (ASTM-D7503-10, 2010). CEC is an inherent characteristic of biochar and represents an ability to hold on nutrients and provides buffering against acidification. CEC values of SGBC, FSBC and RCBC used in the present study were similar to biochar derived from canola straw, corn straw, soybean straw and peanut straw (Yuan et al., 2011), but were 1.5 to 2.3 times lower than PLBC (Table 5.3). PLBC exhibited the highest pH, CEC, total soluble cations and adsorbed

cations among the other biochars, indicating its highest potential for neutralizing undissociated acids and releasing minerals and metals during syngas fermentation.

The FTIR spectra for the functional groups in raw and exhausted biochars after syngas fermentation are shown in Fig. 5.1B. Aromatic C=C bending (1500-1600 cm^{-1}, peak A), aromatic C-H bending (680-860 cm^{-1}, peak E) (Qian et al., 2013) are typical structures of biochar aromatic carbon. Carboxylate (-COOH) groups (780 cm^{-1} and 1400 cm^{-1}, peak B) and hydroxyl (-OH) stretching (3300-3400 cm^{-1}, peak F) (Azargohar et al., 2014) were considered to contribute to alkalinity of biochar. Phosphine oxides (950-1100 cm^{-1}) (Qian et al., 2013) and Si-O stretching (1030 cm^{-1}) (Yuan et al., 2011) are overlaid and shown as peak C in Fig. 1B. Carbonate groups are shown at 860 cm^{-1} (peak D) and 1100 cm^{-1} (peak G) (Yuan et al., 2011). These functional groups were detected in all biochars used in the present study. Peaks B and D were less pronounced in the spectra of spent SGBC and FSBC than in raw SGBC and FSBC, indicating carboxylate and carbonate groups were released from the biochar during fermentation. Peak B in spectrum of spent RCBC was smaller, suggesting fewer carboxylate groups remained in RCBC after fermentation. Peak G in spent PLBC spectrum almost disappeared, which indicates near elimination of carbonate groups in spent PLBC after fermentation. The phosphate, or P, leached from SGBC, FSBC, and PLBC (Fig. 5.1B) could be in the form of phosphine oxides (peak C). However, no leaching of P from RCBC indicates that the peak C in RCBC could represent only Si-O stretching. The carbonate groups could be in the forms of $CaCO_3$, $MgCO_3$, $FeCO_3$ and $MnCO_3$ bound to biochar, and had low solubility in water-based medium. Protons (H^+) from acetic acid produced during fermentation dissolves more Ca, Mg, Fe and Mn compounds. This was shown by the

increase in Ca, Mg, Fe and Mn concentrations during fermentation with *C. carboxidivorans* (Fig. 5.3) and *C. ragsdalei* (Sun et al., 2018a).

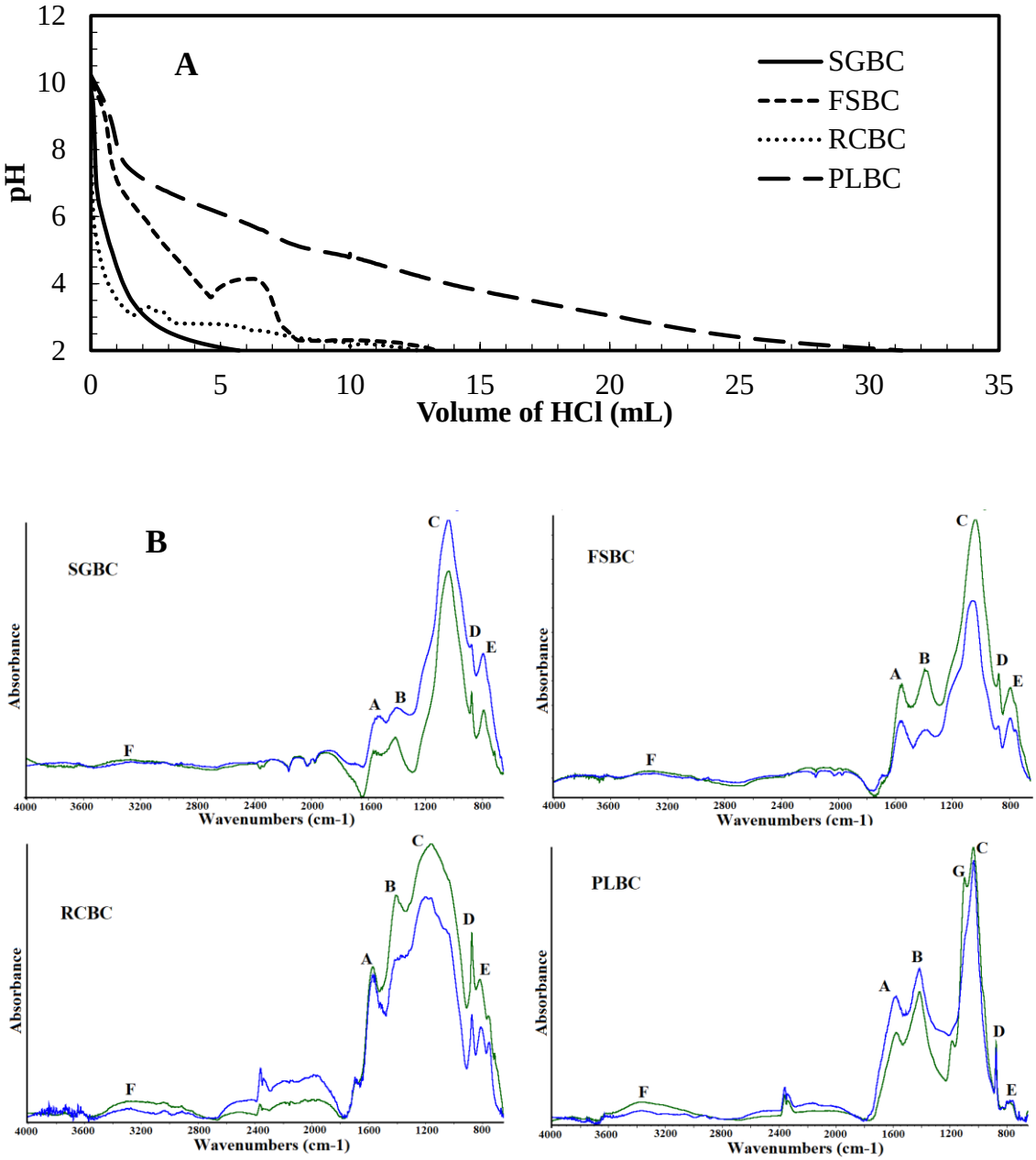

Fig. 5.1. (A) Acid titration curves of SGBC, FSBC, RCBC and PLBC, and (B) FTIR spectra of raw SGBC, FSBC, RCBC and PLBC (green line) and spent biochar after fermentation (blue line): recognized peaks represent functional groups in biochar (A: aromatic C=C bending; B: carboxylate; C: phosphine oxides and Si-O stretching; D: carbonates; E: aromatic C-H bending; F: hydroxyl stretching; G: carbonates).

Table 5.3. Summary of physiochemical properties of biochar.

	SGBC	FSBC	RCBC	PLBC
pH in biochar-H_2O matrix	9.64 ± 0.25	9.94 ± 0.08	8.26 ± 0.66	10.01 ± 0.02
CEC (cmol kg^{-1})	155.74	253.91	175.16	559.84
pH buffer capacity (mmol kg^{-1} pH^{-1})	167.20	241.00	250.77	394.35
Total soluble cations (cmol kg^{-1})[a]	1180.18	2757.14	706.64	16969.07
Total bound cations (cmol kg^{-1})[b]	1969.70	6024.36	722.79	12400.07
Particle size distribution (% by weight)				
>2 mm	11.00	17.01	5.71	25.57
1 to <2 mm	31.60	20.28	32.13	58.87
0.25 to <1 mm	37.49	36.55	36.94	10.63
<0.25 mm	19.91	26.16	25.22	4.93

[a] Total soluble cations are the sum of total Na^+, K^+, Ca^{2+} and Mg^{2+} soluble in water.
[b] Total bound cations are the sum of total Na^+, K^+, Ca^{2+} and Mg^{2+} insoluble in water

5.3.2 Effects of biochar on syngas fermentation

The profiles of pH, cell growth, alcohol and carboxylic acid concentrations, CO and H_2 utilization, and CO_2 production are shown in Fig. 2. The intial pH values in all treatments with and without biochar after inoculation ranged from 5.7 to 6.3 (Fig. 2A), which were within the optimum pH range for growth of *C. carboxidivorans* (Liou et al., 2005). The high initial pH for PLBC treatment was due to its highest total soluble cation content (Table 5.3). The pH in all treatments dropped during first 48 h due to accumulation of acetic acid associated with cell growth. About 0.5 to 1.4 mL 10% NH_4OH solution was added after sampling to each treateatment except PLBC between 24 and 96 h to increase pH between 5.0 and 5.1 and prevent inhibition of cell growth. More NH_4OH was added in the Ctrl A and Ctrl B treatments than in biochar medium due to high soluble bound cations in the biochar (Tables 5.1 and 5.3). The pH started to increase after 72 h in CtrlA, CtrlB, SGBC and RCBC treatments, and after 96 h in FSBC and PLBC treatments. The pH increase in all treatments was due to conversion of acetic acid

and butyric acid to ethanol and butanol, respectively (Figs. 5.3 C, D, E and F). In addition, alkali and alkaline compounds in the biochar contributed to the increase in the pH by neutralizing produced carboxylic acids (Sun et al., 2018a). The pH in PLBC treatment dropped after 168 h due to resumption of growth and accumulation of acetic and butyric acids after the reduction in initial headspace pressure from 170.2 kPa to 142.7 kPa. No changes in growth or product formation occurred in other treatments when the initial headspace prerssure decreased. *C. carboxidivorans* grew in all treatments with similar profiles except in SGBC, FSBC and PLBC treatments (Fig. 5.2B). Cell mass in SGBC and FSBC treatments decreased at 144 h and 264 h, respectively. However, cell mass concertation in PLBC treatment after 168 h was higher than in other media, possibly due to more nurients released by PLBC. In addition, *C. carboxidivorans* growth in RCBC and PLBC treatments was comparable to rich medium in CtrlA. However, *C. carboxidivorans* growth in SGBC and FSBC treatments was lower than in CtrlA.

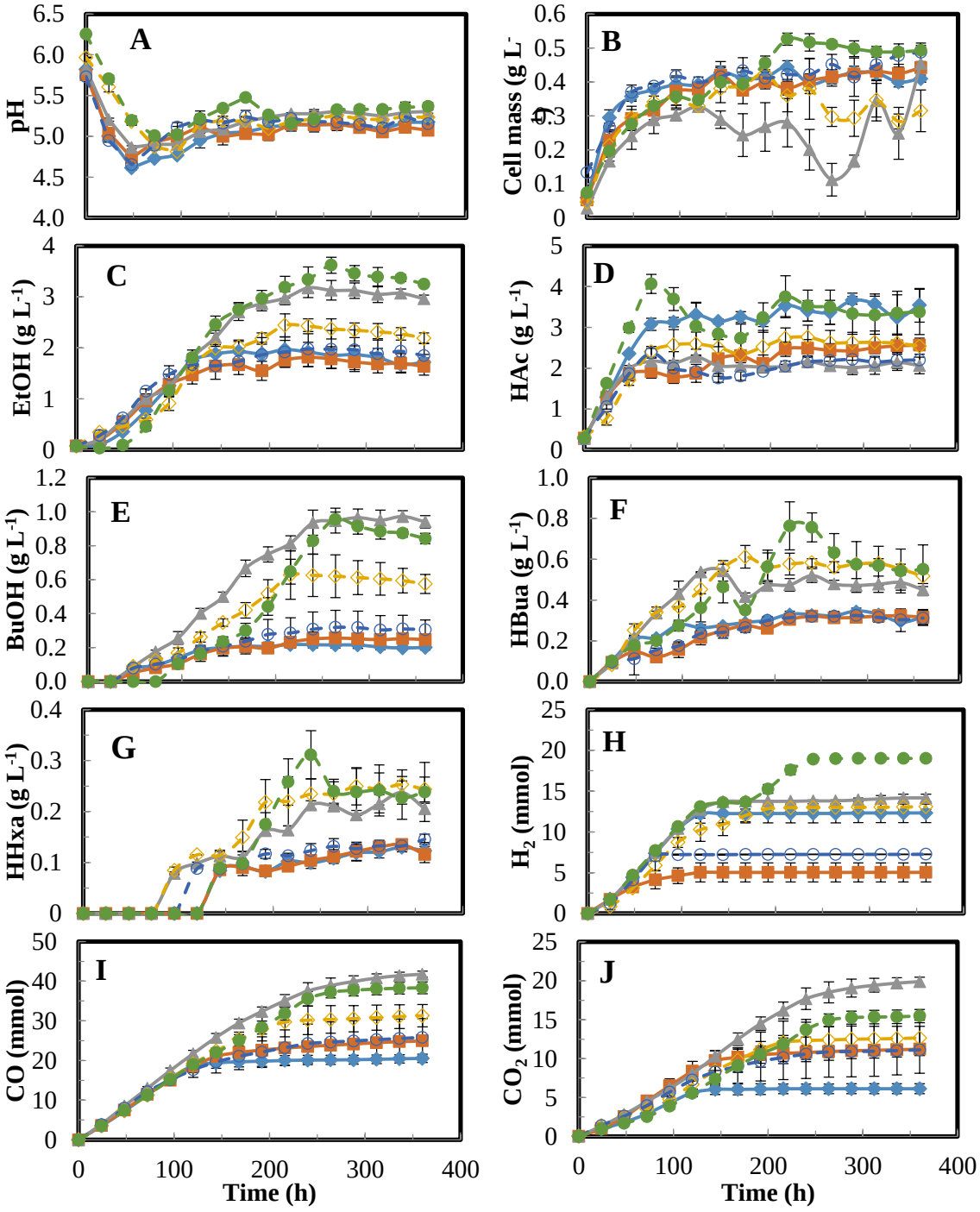

Fig. 5.2. Profiles of (A) pH, (B) cell mass, (C) ethanol (EtOH), (D) acetic acid (HAc), (E) butanol (BuOH), (F) butyric acid (HBua) and (G) hexonoic acid (HHxa) concentrations, cumulative uptake of (H) H_2 and (I) CO and (J) cumulative production of CO_2 during syngas fermentation by *C. carboxidivorans* in treatments: Ctrl A (♦), Ctrl B(■), SGBC (▲), FSBC (◊), RCBC (○) and PLBC (●).

Ethanol, butanol, acetic acid, butyric acid and hexanoic acid were produced in all treatments by *C. carboxidivorans* (Fig. 5.2). However, hexanol was not detected in any treatment. Ethanol accumulation started after inoculation (0 h) in CtrlA, CtrlB, SGBC, FSBC and RCBC treatments, while it was delayed in PLBC during the first 48 h (Fig. 2C). Butanol production started after 24 h in CtrlA, CtrlB, SGBC, FSBC and RCBC treatments (Fig. 5.2E) but was delayed until 72 h in PLBC treatment. This is due to slower pH decrease during acedogenesis caused by high ANC and pH buffering capacity of PLBC as shown in Fig. 5.1 and Table 5.3, which delayed initiation of solventogenesis. The maximum ethanol concentation in PLBC treatment was 3.6 g L^{-1}, followed by SGBC treatment (3.2 g L^{-1}). The highest butanol concentration in PLBC and SGBC treatment was 1.0 g L^{-1}. Ethanol production in PLBC and SGBC treatments was significantly higher ($p < 0.05$) than in CtrlA, CtrlB, FSBC and RCBC tretments fter 168 h. Butanol production in PLBC and SGBC treatments were significantly higher ($p < 0.05$) than in all other treatments after 264 h.

Acetic acid accumulated during growth faster in PLBC treatment and reached 4.1 g L^{-1} at 72 h, which was significantly higher ($p < 0.05$) than in other treatments (Fig. 5.2D). This could be due to high pH buffering capacity in PLBC that reduced acid stress on *C. carboxidivorans* metabolism and enabled more acetic acid production (Figs. 5.2 A & D). *C. ragsdalei* also produced more acetic acid and ethanol in PLBC treatment than in other biochar treatments (Sun et al., 2018a). Butyric acid was slowly produced in all treatments (Fig. 5.2F). However, hexanoic acid production started after 72 h in SGBC and FSBC treatments, at 96 h in RCBC treatment, and 120 h in CtrlA, CtrlB and PLBC treatments (Fig. 5.2G). The butyric and hexanoic acids in PLBC reached highest

concentrations of 0.76 g L^{-1} and 0.31 g L^{-1}, respectively, which were significantly higher ($p < 0.05$) than in other treatments due to resumption of activity with the decrease in initial syngas pressure after 168 h.

H_2 was consumed in all treatments from the beginning of fermentation. However, H_2 consumption stopped after 72 h in CtrlB and RCBC treatments, after 120 h in CtrlA and SGBC treatments h, and after 168 h in FSBC treatment. H_2 consumption by *C. carboxidivorans* in PLBC treatment was significantly increased ($p < 0.05$) at 168 h due to the recovered cell growth after reduction in initial headspace pressure (Fig. 5.2H). The hydrogenase (H_2ase) activity in PLBC medium was possibly less inhibited by CO than in other treatments. H_2 is the main source of electrons and CO provides reducing power to consecutively synthesize acetyl-CoA, butyrl-CoA and hexanoyl-CoA in *C. carboxidivorans* (Fernández-Naveira et al., 2017a; Wilkins & Atiyeh, 2011). Acetyl-CoA is a building block for biomass, acetate and ethanol and butyrl-CoA. Butyrl-CoA is a precurser of butyrate, butanol and hexanoyl-CoA. Hexanoyl-CoA is the building block for hexanoate and hexanol production. CO consumption in SGBC and PLBC treatments was significantly higher ($p < 0.05$) than in other treatments from 240 h to 360 h with profile trends similar to ethanol and butanol production in SGBC and PLBC (Figs. 5.2 C, E and I). CO_2 production profiles matched CO consumption profiles in all treatments (Figs. 5.2 I and J).

Fermentation parameters for *C. carboxidiviorans* in various treatments are shown in Table 5.4. Cell mass yields in RCBC, CtrlA and CtrlB treatments were significantly higher ($p < 0.05$) than in SGBC, FSBC and PLBC treatments. Ethanol yield was the highest in PLBC and CtrlA treatments, while butanol yield was the highest in PLBC and

SGBC treatments. The alcohol to acid ratios (ethanol/acetic acid and butanol/butyric acid) and CO and H_2 utilization in SGBC and PLBC treatments were higher than in other treatments.

Table 5.4. Parameters of fermentation by *C. carboxidivorans* in different treatments.

Parameters	CtrlA	CtrlB	SGBC	FSBC	RCBC	PLBC
Cell mass yield (g mol^{-1})[a]	0.75 ± 0.05^{A}	0.69 ± 0.08^{AB}	0.57 ± 0.03^{BC}	0.49 ± 0.03^{C}	0.76 ± 0.03^{A}	0.49 ± 0.05^{C}
Ethanol yield (%)[b]	28.40 ± 1.21^{AB}	22.34 ± 2.23^{C}	25.58 ± 0.18^{BC}	24.83 ± 1.32^{BC}	24.81 ± 1.51^{BC}	30.53 ± 1.23^{A}
Butanol yield (%)[b]	5.50 ± 0.30^{A}	5.15 ± 0.80^{A}	11.94 ± 0.34^{B}	9.70 ± 0.28^{C}	6.29 ± 0.08^{A}	12.41 ± 0.71^{B}
EtOH/HAc ratio (mmol/mmol)[b]	0.64 ± 0.12^{D}	0.83 ± 0.09^{CD}	1.89 ± 0.16^{A}	1.13 ± 0.09^{BC}	1.09 ± 0.05^{BCD}	1.29 ± 0.21^{B}
BuOH/HBua ratio (mmol/mmol)[b]	0.77 ± 0.13^{D}	0.93 ± 0.04^{CD}	2.48 ± 0.07^{A}	1.32 ± 0.04^{C}	1.16 ± 0.15^{CD}	1.88 ± 0.30^{B}
Total alcohol (g L^{-1})[c]	1.92 ± 0.17^{A}	1.88 ± 0.20^{A}	3.90 ± 0.10^{B}	2.76 ± 0.16^{C}	2.15 ± 0.30^{A}	4.09 ± 0.02^{B}
CO utilization (%)	21.08 ± 2.18^{D}	25.81 ± 3.99^{CD}	44.26 ± 4.76^{A}	32.75 ± 6.54^{BC}	27.03 ± 6.12^{CD}	41.01 ± 3.69^{AB}
H_2 utilization (%)	17.85 ± 2.37^{A}	7.33 ± 3.18^{B}	20.54 ± 1.44^{A}	19.12 ± 5.96^{A}	10.55 ± 0.79^{B}	28.69 ± 2.25^{C}

Same capital letter in each row represents no significant differences between treatments ($p > 0.05$).

[a] Based on CO consumed at maximum cell mass concentration for CtrlA at 144 h, CtrlB at 144 h, SGBC at 120 h, FSBC at 192 h, RCBC at 96 h and PLBC at 216 h.

[b] EtOH/HAc (ethanol/acetic acid); BuOH/HBua (butanol/butyric acid); based on CO consumed meaasured at 360 h.

[c] Contained ethanol and butanol meaasured at 360 h.

The concentrations of Na, K, Ca, Mg, P, S, Fe, Mn, Zn and Co at 0 d, 10 d and 15 d in all treatments are shown in Fig. 5.3. Na, K, Ca, Mg, P and Mn concentrations were the highest at 10 d and 15 d in PLBC treatment. Na concentrations in SGBC, FSBC, and RCBC treatments were similar to CtrlB treatment without biochar, indicating no Na released from these three types of biochar. Na concentration in PLBC treatment was about tenfold higher than in other treatments. About 1300 mg L^{-1} K was achieved by addition of KOH solution to CtrlA and CtrlB treatments to adjust the initial pH of the medium to 6.2. However, K concentrations resulting from addition of KOH to adjust initial pH to between 5.5 and 6.0 before addition of biochar in SGBC, FSBC, RCBC and PLBC treatments were 780, 526, 448 and 916 mg L^{-1}, respectively. The addition of biochar increased the initial pH to about 6.0. Based on the ICP analysis, about 200, 740, 200 and 1290 mg L^{-1} of K were released from SGBC, FSBC, RCBC and PLBC by 15 d of fermentation. Na and K concentrations in PLBC were significantly higher ($p < 0.05$) than in other treatments, which was supported by PLBC's highest soluble cations (Table 5.3). No significant differences ($p < 0.05$) in Na and K concentrations were measured between 0 d, 10 d and 15 d within each biochar treatment. This suggests that Na and K compounds in the biochar were readily soluble and released at the beginning of fermentation.

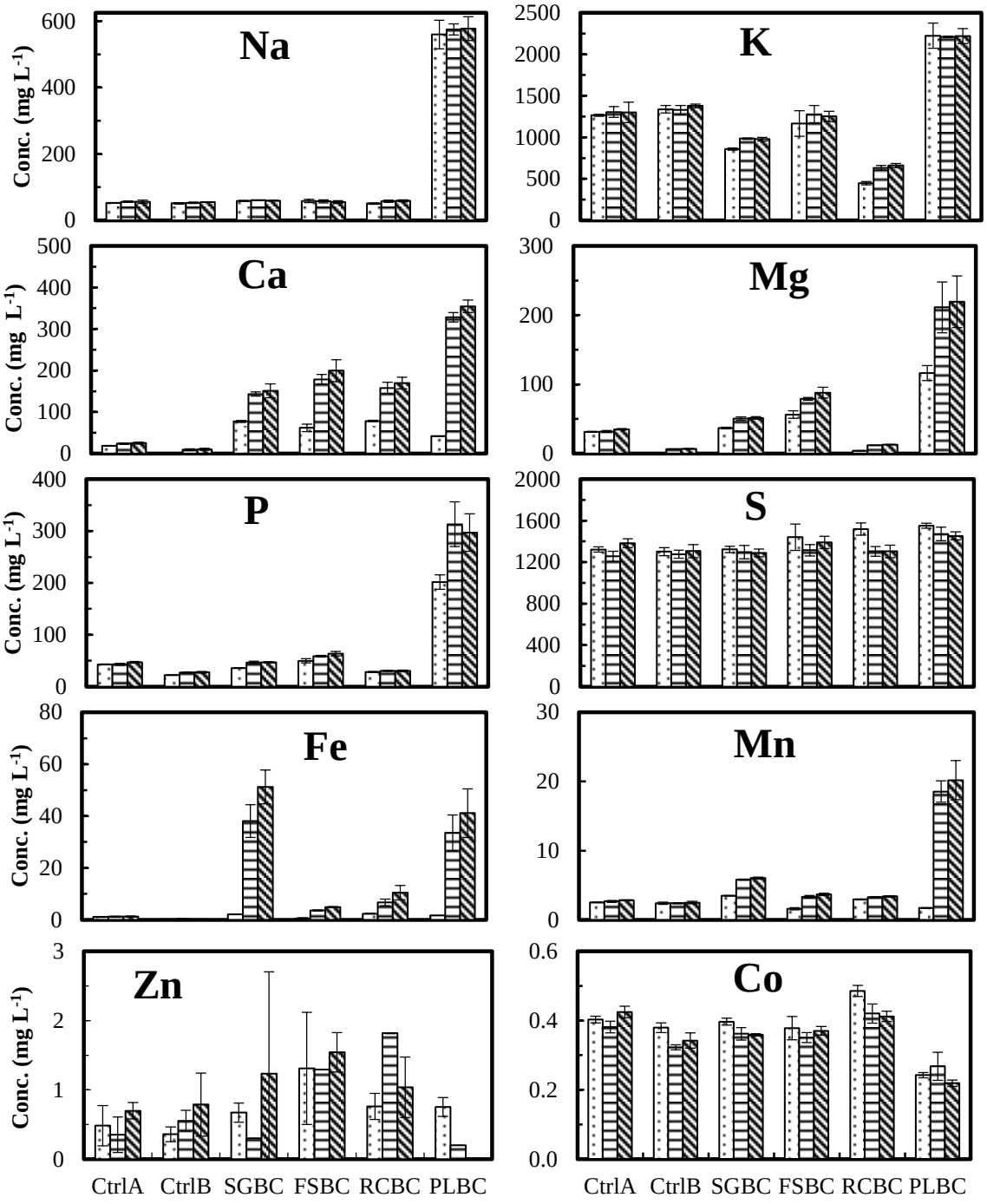

Fig. 5.3. Concentrations of Na, K, Ca, Mg, P, S, Fe, Mn, Zn and Co at 0 d (dots), 10 d (horizontal), and 15 d (diagonal) of fermentation in treatments Ctrl A, Ctrl B, SGBC, FSBC, RCBC and PLBC. The elements Cu, Ni, Mo, Se and W were not detected by ICP (detection limit was 0.01 mg L^{-1} for Cu, Ni, Mo, Se and 0.1 mg L^{-1} for W) and were not shown in the figure.

Ca and Mg Concentrations were the highest in PLBC treamtent at 10 d and 15 d (Fig. 5.3) compared to to other treatments. All treatments with biochar exhibited significantly higher ($p < 0.05$) Ca and Mg concentrations at 10 d and 15 d than at 0 d. All biochar treatments released Ca and Mg into medium during fermentation. The significant ($p < 0.05$) increases in Ca and Mg concentrations from 0 d to 10 d is due to the increase in their solubility as pH decreased with acid production during fermentation (Figs. 5.3A, D, F and G).

Phosphorous concentration was the highest in PLBC treatment compared to other treatments (Fig. 5.3). Compared to CtrlB treatment that contained deficient stock solution (Tables 5.1 and 5.2), phosphate compounds were released only in SGBC, FSBC and PLBC treatments (Fig. 5.3). It was reported that complete removal of PO_4^{3-} from medium resulted in decrease of *C. ragsdalei* growth and ethanol production due to its function in synthesis of ATP, nucliotides and phospholipids (Saxena & Tanner, 2012). The highest P concentration in PLBC treatment possibly enahnced *C. carboxidivorans* growth and alcohol production. No significant differences ($p > 0.05$) in S concentrations were found among all treatments suggesting S containing compounds were not released from biochar during fermentation.

Fe and Mn concentrations in CtrlA and CtrlB treatments without biochar were very low (below 3 mg L^{-1}). However, Fe and Mn were released from biochar during fermentation. The SGBC treatment had the highest Fe concentration at 10 d and 15 d, followed by PLBC treatment. Mn concentration was the highest in PLBC treatment at 10 d and 15 d. Similar to Ca and Mg, the concentrations of Fe and Mn increased significantly ($p < 0.05$) from 0 d to 10 d in all biochar treatments due to increased

solubility with decrease in pH and acid formation. Fe was considered essential element for activity of formate dehydrogenase (FDH), carbon monoxide dehydrogenase (CODH), H_2ase, and alcohol dehydrogenase (ADH) in the Wood-Ljungdahl pathway (Saxena & Tanner, 2011). The activities of FDH, CODH, H_2ase and ADH in *C. ragsdalei* were reduced and ethanol production was inhibited when Fe was removed from medium (Saxena & Tanner, 2011). However, no effect of Mn was observed on cell growth and product formation of *C. ragsdalei* (Saxena & Tanner, 2011).

Zn and Co concentrations in CtrlA and CtrlB treatments were between 0.35 to 0.79 mg L^{-1} and between 0.32 to 0.42 mg L^{-1}, respectively. The higher Zn concentration at 0 d in SGBC, FSBC, RCBC and PLBC treatments than it in CtrlB was due to Zn leaching from these types of biochar. However, lower Zn concentrations were detected in PLBC treatment at 10 d and 15 d than it in CtrlB treatment suggesting a possible adsorption of Zn onto PLBC. Increased Zn concentration from 70 to 280 μM (4.6 to 18.3 mg L^{-1}) was reported to enhance alcohols production by *C. carboxidivorans* (Li et al., 2018). There were no significant differences ($p > 0.05$) in Co concentrations at 0 d, 10 d and 15 d in SGBC, FSBC and RCBC treatments, and CtrlB. However, lower Co concentration was measured in PLBC treatment compared to CtrlB, indicating a possible adsorption of Co onto PLBC. Both absorption of Zn and Co in PLBC treatment could be due to its highest CEC. It was reported that cation exchange in biochar was one mechanism of metal adsorption, among others (surface functional groups, pH-dependent precipitation, electrostatic attraction), as reported by Ahmad et al. (2014). Trace amounts of Ni, Mo, Se and W were added in all media (Table 5.2). These metals were not detected by the ICP due to either their utilization by *C. carboxidivorans* or adsorption onto the

biochar.

5.3.3 Effects of SGBC and PLBC loadings on syngas fermentation

Fig. 5.4 shows the profiles of pH, cell mass and product concentrtations and cummulative uptake of H_2 and CO and CO_2 production by *C. carboxidivorans* at various loadings of SGBC and PLBC. The pH in all treatments decreased due to acetic acid and butyric acid production until 48 to 72 h when pH started to increase. The initial syngas pressure in all treatments at 168 h was reduced from 170.2 to 142.7 kPa to reduce CO inhibition on cell activity. This initial pressure decrease caused pH in PLBC10 and PLBC20 treatments to decrease again, which was accompanied by additional growth and increase in acetic acid, butyric acid, hexanoic acid concentrations and H_2 consumption . However, the recovery of cell mass, increase of carboxylic acids and H_2 consumption were not observed in either PLBC5 treatment or all SGBC treatments. This indicates that the pH buffering capacity (98.6 μmol of H^+ required for 1.0 pH unit drop) and nutrients using 5 g L^{-1} PLBC were not enough to recover cell activity compared to PLBC10 and PLBC20 treatments.

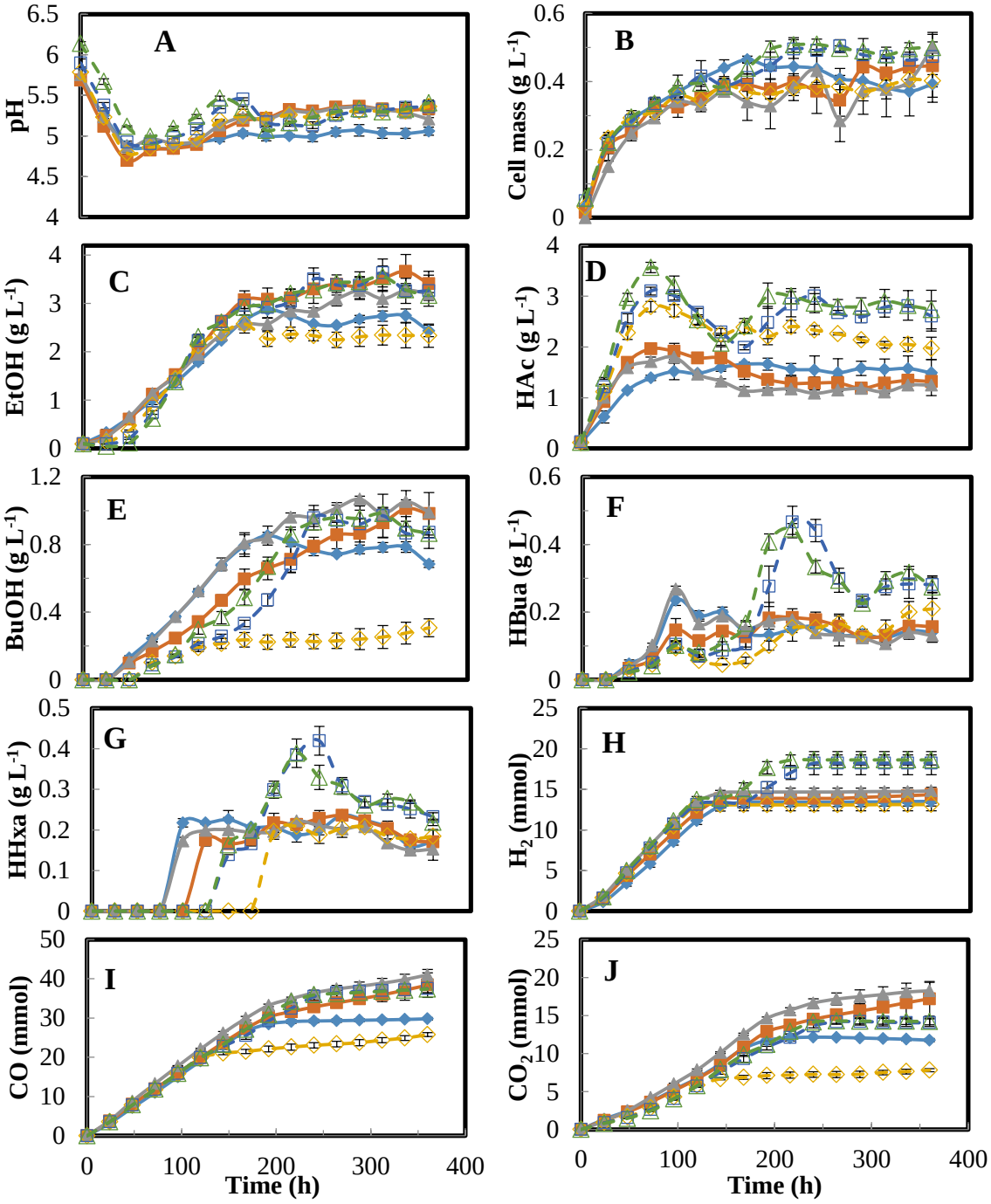

Fig. 5.4. Profiles of (A) pH, (B) cell mass, (C) ethanol (EtOH), (D) acetic acid (HAc), (E) butanol (BuOH), (F) butyric acid (HBua) and (G) hexonoic acid (HHxa) concentrations, cumulative uptake of (H) H_2 and (I) CO and (J) cumulative production of CO_2 during syngas fermentation by *C. carboxidivorans* in treatment using medium with SGBC loading of 5 g L^{-1} (SGBC5, ♦), 10 g L^{-1}(SGBC10, ■), 20 g L^{-1} (SGBC20, ▲), PLBC loading of 5 g L^{-1} (PLBC5, ◊), 10 g L^{-1} (PLBC10, □) and 20 g L^{-1} (PLBC20, Δ).

Ethanol and butanol production in PLBC5 from 192 h to 360 h was significantly lower ($p < 0.05$) than in PLBC10 and PLBC20 treatments. Ethanol and butanol titers in SGBC5 from 240 h to 360 h were lower than in SGBC10 and SGBC20 but the difference was not significant ($p > 0.05$). No significant differences ($p > 0.05$) were measured in ethanol or butanol concetrations in treatments of of SGBC or PLBC. However, ethanol and butanol concentrations in SGBC and PLBC with 5 g L^{-1} loading were significantly lower ($p < 0.05$) than with 10 and 20 g L^{-1} loading. This showed that 10 g L^{-1} loading of SGBC and PLBC was enough to enhance syngas fermentation similar to 20 g L^{-1} loading. Acetic acid production in PLBC treatments regardless of biochar loading rate were significantly higher ($p < 0.05$) than in all SGBC treatments (Fig. 5.4). This was due to the higher ANC, CEC and pH buffering capacity in PLBC than in SGBC (Table 5.3). *C. carboxidivorans* produced more acetic acid with an increase in SGBC and PLBC loadings from 5 to 10 g L^{-1}. However, such effects were not observed during butyric and hexanoic acids production in SGBC treatments. More butyric and hexanoic acids accumulated in 10 and 20 g L^{-1} PLBC treatments from 168 h to 360 h due to recovery in cell activity by the reduction in initial headspace pressure. H_2 consumption in all treatments was similar in the first 168 h. However, *C. carboxidivorans* consumed significantly ($p < 0.05$) more H_2 after 168 h in 10 and 20 g L^{-1} PLBC treatments compared to 5 g L^{-1} PLBC treatment and all SGBC treatments. This again demonstrated the ability of *C. carboxidivorans* to recover H_2 utilization in PLBC medium with a decrease in the initial pressure that reduced CO inhibition on the metabolic acitivty. The treatments with 5 g L^{-1} SGBC and PLBC exhibited significantly ($p < 0.05$) lower level of CO consumption and CO_2 production compared to treatments with 10 and 20 g L^{-1} SGBC

and PLBC. This confirms that 5 g L^{-1} SGBC or PLBC loading was not enough to enhance alocohol production by *C. carboxidivorans*.

No significant differences ($p > 0.05$) were measured in cell mass yield from CO and ethanol yield from CO in all SGBC and PLBC treatments (Table 5.5). Butanol yield from CO in treatment using 5 g L^{-1} of PLBC was significantly lower ($p < 0.05$) than in other treatments. Ethanol to acetic acid ratios in 10 g L^{-1} and 20 g L^{-1} SGBC treatments were significantly higher ($p < 0.05$) than in PLBC treatments. Butanol to butyric acid ratios in SGBC treatments were significantly higher ($p < 0.05$) than in PLBC treatments regardless of biochar loading. There were no significant differences ($p > 0.05$) in total accumulated alcohol at 360 h between SGBC and PLBC treatments at the same loading. More alcohol accumulated in the 10 g L^{-1} and 20 g L^{-1} SGBC and PLBC treatments compared to 5 g L^{-1} SGBC and PLBC treatments. Similar total amounts of alcohol were produced in the 10 g L^{-1} and 20 g L^{-1} SGBC and PLBC treatments. CO utilization in 5 g L^{-1} SGBC and PLBC treatments were significantly lower ($p < 0.05$) than in 10 g L^{-1} and 20 g L^{-1} SGBC and PLBC treatments. The differences in CO utilized by *C. carboxidivorans* in SGBC and PLBC treatments with 10 and 20 g L^{-1} loadings were not significant ($p > 0.05$). H_2 utilization in 10 and 20 g L^{-1} PLBC treatments was significantly higher ($p < 0.05$) than in 5 g L^{-1} PLBC treatment and in all SGBC treatments.

Table 5.5. Parameters of fermentation by *C. carboxidivorans* in treatments with 5, 10 and 20 g L^{-1} of biochar loadings.

Parameters	SGBC5	SGBC10	SGBC20	PLBC5	PLBC10	PLBC20
Cell mass yield (g mol^{-1})[a]	0.62 ± 0.01^{AB}	0.63 ± 0.07^{AB}	0.57 ± 0.02^{AB}	0.68 ± 0.07^{A}	0.48 ± 0.01^{B}	0.52 ± 0.04^{AB}
Ethanol yield (%)[b]	29.45 ± 1.20^{A}	32.47 ± 0.86^{A}	28.35 ± 0.39^{A}	31.11 ± 2.85^{A}	31.44 ± 2.23^{A}	30.61 ± 0.16^{A}
Butanol yield (%)[b]	12.74 ± 0.42^{A}	14.63 ± 0.59^{A}	13.23 ± 0.15^{A}	6.92 ± 1.31^{B}	13.22 ± 1.34^{A}	13.03 ± 0.03^{A}
EtOH/HAc (mmol/mmol)[b]	2.22 ± 0.49^{AB}	3.40 ± 0.44^{A}	3.42 ± 0.43^{A}	1.54 ± 0.01^{B}	1.64 ± 0.09^{B}	1.54 ± 0.25^{B}
BuOH/HBua (mmol/mmol)[b]	6.09 ± 1.44^{AB}	7.52 ± 1.31^{A}	9.21 ± 1.26^{A}	1.77 ± 0.10^{C}	3.69 ± 0.28^{BC}	3.82 ± 0.56^{BC}
Total alcohol (g L^{-1}) [c]	3.12 ± 0.14^{BC}	4.39 ± 0.37^{A}	4.19 ± 0.16^{AB}	2.64 ± 0.29^{C}	4.14 ± 0.41^{AB}	4.03 ± 0.08^{AB}
CO utilization (%)	30.79 ± 1.74^{A}	40.56 ± 5.46^{B}	43.27 ± 2.68^{B}	26.84 ± 1.33^{A}	39.98 ± 3.32^{B}	39.61 ± 1.20^{B}
H_2 utilization (%)	19.49 ± 1.70^{A}	20.79 ± 1.52^{A}	21.43 ± 1.26^{A}	18.98 ± 0.45^{A}	27.45 ± 3.35^{B}	27.72 ± 1.55^{B}

Same capital letter in each row represents no significant differences between treatments ($p > 0.05$).

[a] Based on CO consumed at maximum cell mass concentration for SGBC5 at 168 h, SGBC10 at 144 h, SGBC20 at 144 h, PLBC5 at 144 h, PLBC10 at 216 h and PLBC20 at 192 h.

[b] EtOH/HAc (ethanol/acetic acid); BuOH/HBua (butanol/butyric acid); based on CO measured calculated at 360 h.

[c] Contained ethanol and butanol measured at 360 h.

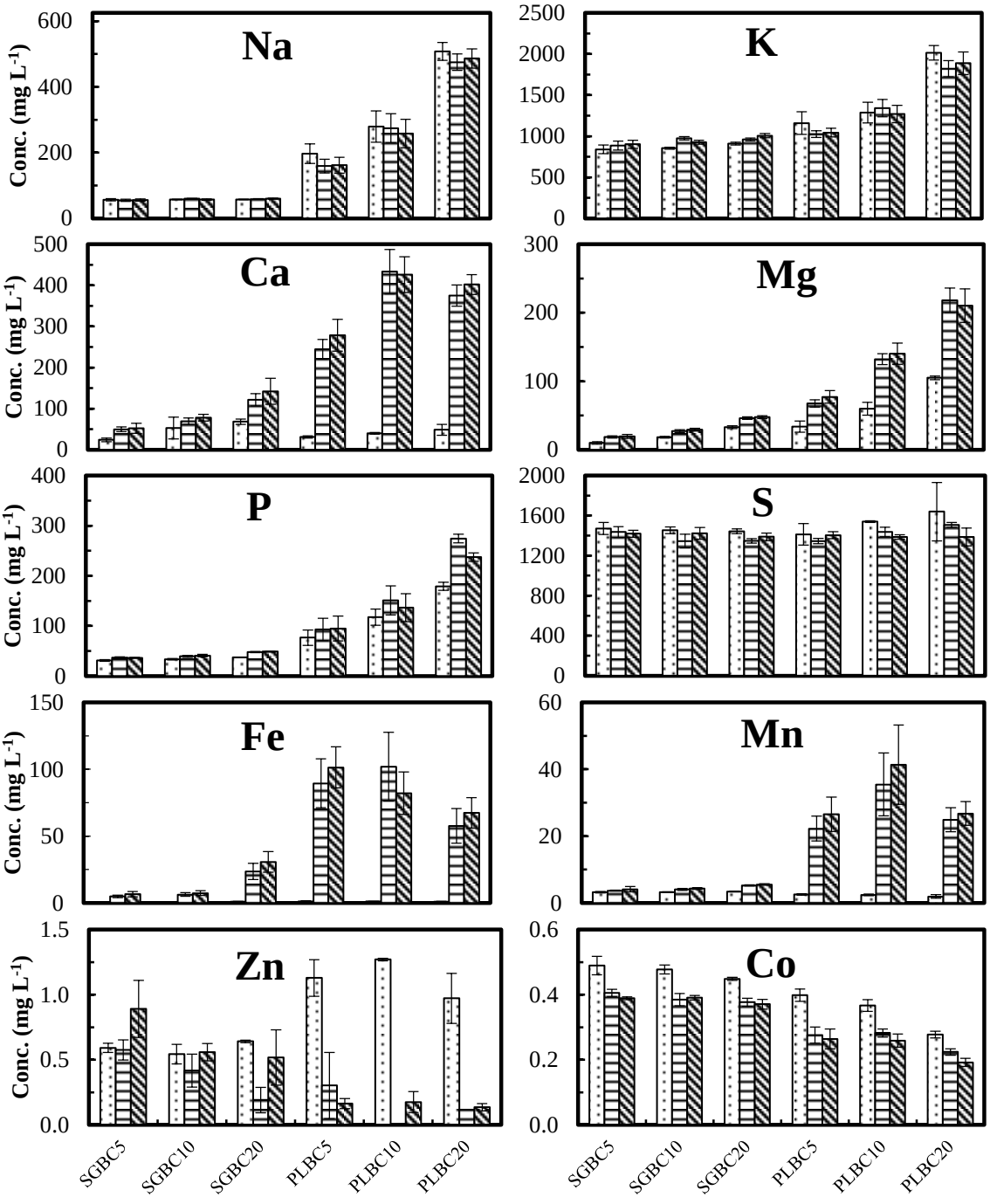

Fig. 5.5. Concentrations of Na, K, Ca, Mg, P, S, Fe, Mn, Zn and Co at 0 d (dots), 10 d (horizontal), and 15 d (diagnal) of fermentation in treatments using SGBC with loading of 5 g L^{-1} (SGBC5), 10 g L^{-1} (SGBC10), 20 g L^{-1} (SGBC20), using PLBC with loading of 5 g L^{-1} (PLBC5), 10 g L^{-1} (PLBC10) and 20 g L^{-1} (PLBC20). The elements Cu, Mo, Ni, Se and W were not detected in all treatments using ICP (detection limit was 0.01 mg $L^{-1}$for Cu, Ni, Mo, and 0.1 mg L^{-1} for Se and W) and were not shown in the figure.

Concentrations of minerals and trace metals in SGBC and PLBC treatments with different loadings are shown in Fig. 5.5. Differences in Na, K, P, S and Mn concentrations in all SGBC treatments at 0 d, 10 d and 15 d were not significant ($p > 0.05$), showing no additional release of these elements in the medium after 0 d. However, Ca, Mg and Fe concentrations in SGBC treatments at 10 d and 15 d increased with an increase in SGBC loading, indicating that these elements were mostly bound or insoluble ions (Table 5.3) and were released during acid production. Na, Ca, Mg and P concentrations in PLBC treatments increased with increased loading. However, Ca, Fe and Mn concentrations at 10 d and 15 d in the treatment with 20 g L^{-1} PLBC were lower than with 10 g L^{-1} PLBC indicating possible adsorptions of Ca, Fe and Mn or more uptake by cells in the 20 g L^{-1} PLBC. Zn and Co concentrations in SGBC treatments were at a similar level as their concentrations (0.36 and 0.38 mg L^{-1}, respectively) in deficient medium (CtrlB) (Fig. 5.3). PLBC treatments released Zn at 0 d, which was then adsorbed later in the process. Co concentrations in PLBC treatments also decreased with increased biochar loading from 5 to 20 g L^{-1} (Fig. 5.5). The drop in Zn and Co concentrations at 10 d and 15 d is possibly due to adsorption of these elements on PLBC (Ahmad et al., 2014). Ni, Mo, Se and W were not detected by the ICP due to either utilization by *C. carboxidivorans* or adsorption onto the biochar.

The results showed that biochar can be incorporated into medium and enhanced ethanol and butanol production by *C. carboxidivorans*. Biochar from different sources have different properties and effects on syngas fermentation. *C. carboxidivorans* entered solventogenic phase in SGBC treatment earlier than other biochar treatments, resulting in the accumulation of more ethanol and butanol (Fig. 5.2 C,E). Most media used for syngas

fermentation with *C. carboxidivorans* contained ATCC 1754 medium or its modifications as shown in Table 5.6. The present study is the first reoprted for the examination for various types of biochar with *C. carboxidivorans*. Ethanol, butanol and hexanol production depends on syngas fermentation parameters incluing bioreactor type, medium and gaseous substrate conpositions. Ethanol, butanol and hexanol concentrations in 250 ml bioreactor bottles with a medium supplemented with W and Ni were 3.0, 1.0 and 0.3 g L^{-1} (Phillips et al., 2015). The present study showed that SGBC and PLBC enhanced *C. carboxidivorans'* ability to make more alcohols. This is different than *C. ragsdaiei* that produced more ethanol in RCBC and PLBC treatments compared to SGBC treatment (Sun et al., 2018a). Biochar from various sources has different mineral and metal contents, pH buffering capacity, CEC and ANC. The four types of biochar used in the present study with *C. carboxidivorans* produced more alcohol compared to the media without biochar (Table 5.4). This shows the potential for use of biochar as a nutrient suplement with its pH buffering capacity that can neutralize undissociated carboxylic acids, reduce acid stress on acetogenic bacteria and enhance alcohol production from syngas.

Table 5.6 Comparison of syngas fermentations by *C. carboxidivorans* in various fermentation strategies.

Fermentation stragegy	Medium	Gas $CO:H_2:CO_2:N_2$	Products (g L^{-1})[a]	Reference
250 mL bottle	CtrlA	40:30:30:0	EtOH: 2.0, BuOH: 0.2, HAc: 3.5, HBua: 0.3, HHxa: 0.1	This study
	CtrlB		EtOH: 1.8, BuOH: 0.2, HAc: 2.5, HBua: 0.3, HHxa: 0.1	
	SGBC		EtOH: 3.7, BuOH: 1.0, HAc: 1.3, HBua: 0.2, HHxa: 0.2	
	FSBC		EtOH: 2.4, BuOH: 0.6, HAc: 2.7, HBua: 0.6, HHxa: 0.2	
	RCBC		EtOH: 2.0, BuOH: 0.3, HAc: 2.2, HBua: 0.3, HHxa: 0.1	
	PLBC		EtOH: 3.6, BuOH: 1.0, HAc: 2.8, HBua: 0.3, HHxa: 0.3	
7.5 L STR[b]	Modified ATCC 1754[f]	20:5:15:60	EtOH: 2.8, BuOH: 0.5, HAc: 2.5, HBua: 0.1	(Ukpong et al., 2012)
HFM-BR[c]	ATCC 1754 PETC[g]	20:5:15:60	EtOH: 23.9, HAc: 6.5	(Shen et al., 2014b)
250 mL bottle	P7MtWNi	70:20:10:0	EtOH: 3.0, BuOH: 1.0, HxOH: 0.3, HAc: 0.4, HBua: 0.1, HHxa: 0.1	(Phillips et al., 2015)
2 L CSTR[d]	Modified ATCC 1754	100:0:0:0	EtOH: 5.6, BuOH: 2.7, HAc: 1.0, HBua: 0.3	(Fernández-Naveira et al., 2016b)
2 L STR	Modified ATCC 1754	20:10:20:50	EtOH: 2.7, BuOH: 1.9, HxOH: 0.8, HAc: 1.9, HBua: 0.7, HHxa: 0.4	(Fernández-Naveira et al., 2017b)
125 mL bottle	Modified ATCC 1754	56:9:20:15	EtOH: 4.0, BuOH: 1.7, HxOH: 1.3, HAc: 0.2, HBua: 0.1, HHxa: 0.1	(Shen et al., 2017a)
h-RPB[e]	ATCC 1754 PETC	20:5:15:60	EtOH: 7.0, HAc: 6.0	(Shen et al., 2017b)

[a] Alcohols and carboxylic acids are represented as: EtOH: ethanol; HAc: acetic acid; BuOH: butanol; HBua: butyric acid; HxOH: hexanol; HHxa: hexanoic acid.
[b] STR: batch fermentation in stirred tank reactor.
[c] HFM-BR: hollow fiber membrane biofilm reactor.
[d] CSTR: continuous fermentation in stirred tank reactor.
[e] h-RPB: horizontal rotating packed bed biofilm reactor.
[f] ATCC 1754 medium (Tanner et al., 1993).
[g] ATCC 1754 PETC medium has fructose compared to ATCC 1754 medium.

5.4 Conclusion

PLBC and SGBC allowed *C. carboxidivorans* to make more ethanol and butanol compared to FSBC, RCBC and standard media without biochar. SGBC initiated solventogenesis faster than PLBC due to fast pH drop. PLBC has the highest alkalinity, pH buffering capacity and CEC, which contributed to neutralization of more undissociated carboxylic acids and reduced acid stress on *C. carboxidivorans*. SGBC and PLBC loadings of 10 or 20 g L^{-1} were required to enhance alcohol production. This study showed the potential for biochar use to provide buffering capacity and nutrients to enhance biofuel production via syngas fermentation.

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CHAPTER VI

ENHANCED ETHANOL PRODUCTION FROM SYNGAS BY *CLOSTRIDIUM RAGSDALEI* IN STIRRED TANK BIOREACTOR USING MEDIUM WITH POULTRY LITTER BIOCHAR

ABSTRACT

Microbial production of ethanol from syngas is an alternative to carbohydrate-based fermentations. Syngas fermenting microorganisms require appropriate nutrients to grow and convert syngas into products. Essential nutrients can be provided from biochar. Poultry litter biochar (PLBC) contains high amount of minerals, trace metals and high pH buffering capacity, making it a suitable component for syngas fermentation medium. In this study, the effects of PLBC loadings from 1 to 20 g L^{-1} and PLBC leachate on syngas fermentation were evaluated using 250 mL bottle reactors. Results showed that 10 and 20 g L^{-1} PLBC loading enhanced ethanol production compared to standard yeast extract (YE), or PLBC leachate media. The 10 g L^{-1} PLBC loading was then applied in fed-batch fermentation in a 3-L continuous stirred tank reactor (CSTR). Fermentation with 10 g L^{-1} PLBC with and without 2-(N-morpholino)ethanesulfonic acid (MES) showed 64% and 36% more ethanol production, respectively, than in standard YE medium. Maximum amounts of acetic acid accumulated in standard YE medium without MES, PLBC media without and with MES were 0.6, 1.2, and 2.0 g L^{-1}, respectively, which was due to the high pH buffering capacity of PLBC. Besides, the acetic acid accumulated at beginning of fermentation was completely converted into ethanol in all tested media in the CSTR. This research demonstrated the feasibility of incorporating PLBC in medium in the CSTR to enhance ethanol production from syngas, a step further towards commercialization.

6.1 Introduction

The hybrid gasification-syngas fermentation process is an alternative and flexible platform to biochemical or thermochemical platforms for biofuel production (Daniell et

al., 2012). The hybrid conversion process produces biofuels from syngas containing CO, H_2 and CO_2 (Kundiyana et al., 2010b). These gaseous substrates are obtained via gasification of biomass, coal, animal wastes, municipal solid wastes (Kumar et al., 2009), and are present in industrial CO-rich off gases (Molitor et al., 2016). Syngas fermenting acetogens convert gaseous substrates to acetic acid, butyric acid, hexanoic acid, ethanol, propanol, butanol, and hexanol (Gao et al., 2013; Liu et al., 2014a; Phillips et al., 2015), and 2,3-butanediol (Köpke et al., 2011b). Acetogenic bacteria include *Clostridium ragsdalei*, *C. carboxidivorans*, *C. ljungdahlii*, *C. autoethanogenum*, and *Alkalibaculum bacchi* (Abubackar et al., 2015; Liu et al., 2012; Mohammadi et al., 2012; Phillips et al., 2015; Sun et al., 2018a). These bacteria convert syngas of various compositions into alcohols at ambient pressure and temperature, which is different from Fischer-Tropsch process that requires a specific ratio of H_2:CO (e.g. 1.4:1 to 2:1) (Dyer et al., 1987).

One of the major bottlenecks for syngas fermentation is gas-liquid mass transfer because of low solubility of CO (83% that of O_2) and H_2 (71% that of O_2) on molar basis in the fermentation broth (Phillips et al., 2017a). The gas-liquid mass transfer can be a limiting factor when cell concentration is high (Orgill et al., 2013). Efforts have been made to increase gas-liquid mass transfer using different types of reactors such as CSTR, bubble column reactor (BCR), hollow fiber membrane reactor (HFMR), trickle bed reactor (TBR), and horizontal rotating packed bed biofilm reactor (h-RPB) (Datar et al., 2004; Devarapalli et al., 2016; Riggs & Heindel, 2006; Shen et al., 2014b; Shen et al., 2017b). Different configurations of reactor such as impeller, column diffuser, gas sparger, and hollow fiber membrane modules had also been evaluated in improving gas-liquid mass transfer (Munasinghe & Khanal, 2010b; Ungerman & Heindel, 2007).

However, the CSTR is the most commonly used bioreactor in industrial fermentations because of good mixing and ease of operation, although its gas-liquid mass transfer is lower than HFMR and TBR (Orgill et al., 2013). The relatively low mass transfer in CSTR is typically addressed via increasing agitation speed, using microsparger to create microbubbles, and high gas flow rate (Kundiyana et al., 2010b), which, however, require additional cost associated with power consumption.

Commercial syngas fermentation requires stable operation, high substrate conversion, high product specificity and productivity, and cost-effective medium (Pardo-Planas et al., 2017; Phillips et al., 2017a). However, acetogenic syngas fermenting bacteria are sensitive to pH changes and substrate inhibition, resulting in low substrate conversion and productivity (Fernández-Naveira et al., 2017c). A method of controlling syngas supply based on culture pH, which reflects cells activity has been developed and demonstrated to achieve high alcohol productivity and specificity in continuous fermentation using CSTR (Atiyeh et al., 2018). PLBC produced by pyrolysis of poultry litter (mainly chicken manure and bedding material) had rich alkaline compounds, high concentrations of minerals and metals, pH buffering capacity, and cation exchange capacity (CEC) as discussed in Chapters IV and V. These properties of biochar and use in fermentation medium extended acetogenic phase (growth phase) and prevented quick drop in culture pH and acid crash. The incorporation of PLBC in medium in bottle reactors had been demonstrated to enhance syngas utilization and productions of alcohols and organic acids (Sun et al., 2018a). Scale-up of syngas fermentation from bottle reactors to CSTR using biochar-incorporated medium is a step in scale up towards commercialization. Appropriate modifications of the CSTR are required to prevent loss

of biochar particles during fermentation. In addition, the pH of the culture in the CSTR with biochar should be controlled due to the pH sensitivity of acetogen and alkalinity of biochar. Therefore, the main objective of this study was to investigate the feasibility of incorporating PLBC to medium in a 3-L CSTR for ethanol production from syngas by *C. ragsdalei*. The effects of loading and leachate of PLBC were initially evaluated in bottle reactors. The PLBC loading that enhanced ethanol production was then applied in fed-batch syngas fermentation in a modified 3-L CSTR. The effects of MES buffer with PLBC were also investigated in the CSTR to examine medium buffering capacity during ethanol production.

6.2 Materials and methods

6.2.1 Microorganisms

Clostridium ragsdalei (ATCC-PTA-7826) obtained from Dr. Ralph Tanner's Laboratory at the University of Oklahoma was used. *C. ragsdalei* was grown on a standard YE medium and maintained weekly following the procedure discussed in Chapter IV. *C. ragsdalei* inoculum with an optical density at 660 nm (OD_{660}) between 0.5 and 0.6 was obtained after 138 h of weekly culture maintenance and used for inoculation in bottle reactors and CSTR.

6.2.2 Effects of PLBC loading and use of leachate

The PLBC was produced by slow pyrolysis of poultry litter (chicken manure with bedding material) and obtained from Oklahoma State University Soil, Water and Forage Analytical Lab. The PLBC contains (by weight): 26% of particles with size larger than 2 mm, 59% between 1 to 2 mm, 11% between 0.25 to 1 mm, and 5% below 0.25 mm. In

the first experiment, six media formulations were prepared. Control treatment (Ctrl) with standard YE medium and media with five different PLBC loadings (M1: 1 g L^{-1}, M2: 2.5 g L^{-1}, M3: 5.0 g L^{-1}, M4: 10 g L^{-1}, M5: 20 g L^{-1}) were performed in 250 ml serum bottles with 50 ml working volume. Media formulations are shown in Table 6.1. The compositions of stock solutions used are reported in Table 4.1 in Chapter IV. All media components except KOH, cysteine-sulfide and ammonium hydroxide were first dissolved in deionized (DI) water. The pH in M1 to M5 media prior to addition of PLBC was pre-adjusted using 5.0 N KOH solution to various values (e.g. pH was adjusted to higher value in medium with lower PLBC loading and vice versa) so that the final pH values after addition of the respective loading of biochar were between 5.5 and 6.5. The added PLBC to the medium increases medium pH due to its alkalinity as shown in Table 5.3 in Chapter V. The pH in the Ctrl medium was adjusted with 5.0 N KOH to about 6.0.

The media before addition of PLBC and standard YE medium were heated to boiling and then sparged with N_2 to remove dissolved O_2. PLBC as per Table 6.1 was then added to each serum bottle when the medium was cooled. The serum bottles were sealed and sterilized at 121 ℃ for 30 min. After sterilization and cooling of the media, the headspace in the bottles were sparged for 2 min with syngas (mixture of 40% CO, 30% H_2, 30% CO_2, by volume) fed through a 0.2 μm PTFE filter before the addition of 4% cysteine-sulfide solution for medium reduction. *C. ragsdalei* was inoculated at 20% (v/v) rate. The bottles were sparged for 2 min and then pressurized to an initial pressure of 240 kPa. All bottles were incubated at 37 ℃ and 150 rpm for 15 days. Each treatment was performed in triplicate (n=3). Liquid and gas samples were withdrawn for gas and solvent analysis every 24 h. The headspace pressure in each bottle was replenished after sampling

to 240 kPa. A 10% NH_4OH solution was used after sampling to adjust the culture pH to between 5.0 and 5.1 when the pH dropped below 5.0. A volume of 5 ml of liquid sample was withdrawn at 0 d (before inoculation), at 10 d and 15 d of fermentation from each bottle for metal and mineral analysis using Inductive Coupled Plasma (ICP) spectrometer to monitor changes in Na, K, Ca, Mg, P, S, Fe, Mn, Zn, Ni, Cu, Co, Se, Mo and W concentrations.

Table 6.1. Medium formulation for each treatment using PLBC and leachate in bottle reactors.

Medium Name	Ctrl	M1	M2	M3	M4	M5	M6	M7
				g L^{-1}				
Yeast extract	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MES	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Biochar (PLBC)	0.0	1.0	2.5	5.0	10.0	20.0	0.0	0.0
				ml L^{-1}				
Mineral stocks solution	25.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Trace metal stock solution	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Vitamin stock solution	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Deficient stock solution	0.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
0.1% Resazurin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
4.0% Cysteine-sulfide	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
5.0 N KOH solution[a]	6.7	4.7	4.0	3.3	2.7	2.0	5.3	4.0
10% NH_4OH solution	24.0[b], 30.0[c]	16.0	18.0	16.0	14.0	6.0[b], 12.0[c]	8.0	4.0

[a]5N KOH was used to adjust medium pH before adding biochar to 6.07 for M1, 5.98 for M2, 5.87 for M3, 5.76 for M4, 5.57 for M5, 6.08 for M6, and 5.95 for M7.
[b] Used in Ctrl and M5 media in first experiment on effect of biochar loading.
[c] Used in Ctrl and M5 media in second experiment on use of biochar leachate.

In the second experiment, four media formulations were prepared to compare syngas fermentation in standard YE (Ctrl) and deficient (M6) media with media containing PLBC particles (M5) and PLBC leachate (M7) as shown Table 6.1. The PLBC leachate (M7) was prepared as follows: 3 g of PLBC was measured and suspended with 150 mL DI water in 500 mL serum bottle. 1.0 M HCl solution was slowly added to the

PLBC-water mixture until the pH dropped to around 4.5. The bottle was sealed using rubber stopper and shaken at 200 rpm for 24 h. Then, the mixture was filtered through Whatman #2 filter paper and the filtrate was collected and used as the water leachate matrix for preparation of medium M7. The steps for medium preparation, inoculation, fermentation, and sampling were similar as described in the previous experiment.

6.2.3 Fed-batch fermentations in 3-L CSTR with and without PLBC

Culture maintenance and inoculum preparation of *C. ragsdalei* were performed based on methods discussed in Chapter IV. A 3-L BioFlo 110 fermenter (New Brunswick Scientific Co., Inc., Edison, NJ, USA) with 2.4 L working volume (80% of total volume) was used. The fermentation setup is shown in Fig. 6.1. The CSTR was equipped with two Rushton impellers, four-blade baffle, micro-bubble sparger, pH and ORP probes, thermometer, heating jacket, and acid/base peristatic pumps. Two pressure transducers were installed to inlet and outlet gas lines to obtain real-time pressure values of inlet and outlet gases. Media, acid and base were prepared in separate bottles and fed to the CSTR using peristatic pumps. The feeding of medium was started after inoculation and stopped when the working volume reached 2.4 L. Syngas was continuously fed during fermentation. During fermentation, acid (2 M H_2SO_4) and base (50 g L^{-1} $NaHCO_3$) were automatically added by the BioFlo 110 pH controller when needed to prevent pH drop below 4.6 or increase above 5.9. The decrease in pH below 4.6 results in acid crash and cell death. However, the increase in pH above 5.9 results from the continuous release of alkaline compounds from PLBC during fermentation. The syngas flow rate was adjusted automatically within a defined range between 20 sccm and 60 sccm by a mass flow

controller (MFC) through a feedback pH control loop based on a novel method developed in our laboratory (Atiyeh et al., 2018). The pH controller sends a signal to the MFC to increase syngas flow rate within a defined range if the medium pH dropped below the pH set point, or decrease syngas flow rate if the pH increased above the pH set point. After inoculation and natural drop in pH to 4.8 occurred due to acetic acid formation, the pH controller of the syngas MFC was set to 4.8. A woven mesh basket with mesh size of 0.5 mm was installed at the outlet of sampling port to prevent loss of biochar with liquid sampling. Gas samples were withdrawn from the gas exhaust line of the CSTR.

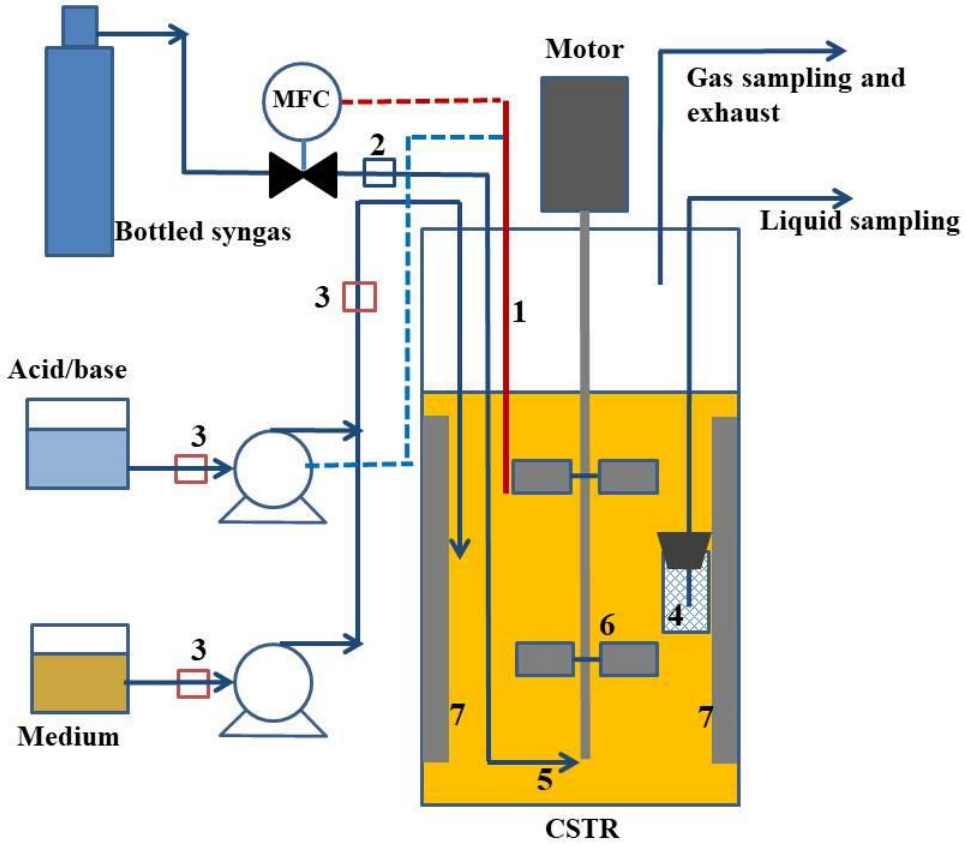

Fig. 6.1 Scheme diagram of the CSTR set-up for fed-batch fermentation. Syngas flow rate and feeding of acid/base were feedback controlled by mass flow controller (MFC) and peristatic pumps, respectively, based on culture pH. Numbers represent: (1) pH probe, (2) 0.2 μm PTFE filter, (3) 0.45 μm cellulose acetate filter, (4) 0.5 mm woven mesh basket, (5) microbubble sparger, (6) Ruston impeller, (7) baffle. Red dashed line represents novel pH feedback control of MFC. Blue dashed line connects the pH probe with the controller of the acid/base pumps.

Based on results of previous experiment with various PLBC loadings, the PLBC loading used in the CSTR was 10 g L^{-1}. Syngas fermentations with and without PLBC and with MES buffer were used to evaluate PLBC and MES buffering effects on growth and product formation. Fermentation using standard YE medium without MES (YE medium) was performed as the control. The media formulations used in the CSTR are

shown in Table 6.2. Components of each medium except PLBC were dissolved in 2.5 L of DI water in a 5-L glass bottle (referred to as "Medium" in Fig. 6.1). The initial pH of the YE medium without MES and PLBC medium with MES was not adjusted before adding biochar. However, the initial pH of the PLBC medium without MES was adjusted from 6.4 to 5.3 using 2 M H_2SO_4 solution before addition of PLBC. A 1.6 L of the medium was transferred to the 3-L CSTR. For PLBC medium, 24 g of PLBC was added into the CSTR (PLBC was added to the medium at a loading of 10 g L^{-1} for a final total volume of 2.4 L). The CSTR containing medium was sterilized at 121 ℃ for 75 min. After sterilization, the medium was allowed to cool and filter-sterilized (0.2 μm PTFE filter) N_2 was purged through the medium at 0.2 L min^{-1} for 12 h to remove dissolved O_2.

Table 6.2 Compositions of YE and PLBC media used in the 3-L CSTR.

Medium	Ctrl- YE medium without MES	PLBC medium without MES	PLBC medium with MES
Components			
YE, g L^{-1}	0.5	0.5	0.5
MES, g L^{-1}	0.0	0.0	10.0
Biochar, g L^{-1}	0.0	10.0	10.0
Mineral stock solution, ml L^{-1}	25.0	0.0	0.0
Trace metal stock solution, ml L^{-1}	10.0	0.0	0.0
Vitamin stock solution, ml L^{-1}	10.0	10.0	10.0
4% cysteine-sulfide, ml L^{-1}	10.0	10.0	10.0
12.6 g of $NaHCO_3$, ml L^{-1}	36.0	0.0	0.0
Deficient stock solution, ml L^{-1}	0.0	10.0	10.0

The CSTR was operated in fed-batch mode (continuous gas feeding with fixed working volume). Syngas (40% CO, 30% H_2, and 30% CO_2) flow rate was set initially at 20 sccm (0.89 mmol min^{-1}). N_2 at fixed 20 sccm was also fed with syngas during fermentation as an internal standard. The 4% cysteine-sulfide solution was added to the

CSTR before inoculation to scavenge any remaining dissolved O_2. Then, *C. ragsdalei* inoculum was added in the CSTR to reach a volume of 2.0 L (inoculation rate was 20% v/v, or 400 ml in 2.0 L). After inoculation, additional sterilized medium from the medium bottle was fed to the CSTR for about 20 h to reach the final liquid volume of 2.4 L. The medium pump was then switched off. The syngas flow rate was maintained initially at 20 sccm and then adjusted by MFC using the patented novel pH controller (Atiyeh et al., 2018). The pH of the medium during fermentation was allowed to decrease naturally but was maintained at 4.8 using the pH feedback control through adjusting syngas flow rate. The fermentation was performed at 37 ℃ with agitation speed initially set at 200 rpm and then increased to 300 rpm after 24 h of fermentation to enhance liquid-gas mass transfer. Labview program (National Instrument Corporation, Austin, Texas, USA) developed in-house was used for data acquisition and with the new pH controller to control syngas flow and other CSTR operating parameters.

Gas and liquid samples were withdrawn from the CSTR every 24 h for analysis of cell mass, gas composition and products concentrations. Additional 5.0 ml of liquid sample was withdrawn every 24 h, filtered and stabilized with 34% (v/v) HNO_3 for elemental analysis (Na, K, Ca, Mg, P, S, Fe, Zn, Mn, Ni, Cu, Co, Se, Mo, and W) using Inductive Coupled Plasma (ICP) spectrometry (EPA, 1996). Syngas fermentation with each media (Table 6.2) in the CSTR was performed in duplicate.

6.2.4 Sampling and analysis

A volume of 100 µL of gas sample from the CSTR exhaust line was withdrawn manually using 100 µL gas tight syringe. The gas composition was analyzed using Agilent 6890N GC (Agilent Technologies, Wilmington, DE, USA) equipped with

thermal conductivity detector (TCD) and Supelco PLOT 1010 column as discussed in Liu et al. (2012). A liquid sample of 1.5 mL from the fermentation broth was withdrawn using a 3 mL BD syringe following gas sampling. The pH of the liquid sample was measured using a pH meter (Thermo Scientific). The cell mass in the Ctrl-YE medium was measured at OD_{660} using UV-1800 spectrophotometer (Shimadzu, Houston, TX, USA) and calculated based on formula: cell mass ($g\ L^{-1}$) = $0.396 \times OD_{660}$ as previously reported (Panneerselvam et al., 2010). The cells were removed by centrifugation at 13,000 g for 10 min using Microfuge 20R centrifuge (Beckman Coulter, Brea, CA, USA). A 0.5 mL of the supernatant was acidified using equal volume of 0.1 M HCl for product analysis using Agilent 6890N GC equipped with flame ionization detector (FID) and DB-FFAP capillary column as discussed in Liu et al. (2012). The cell mass in biochar media was determined by Bradford protein assay (Bradford, 1976). Bradford protein assay was also performed for *C. ragsdalei* culture (without biochar) with known OD_{660} values to establish calibration curve between OD_{660} and measured protein content in biochar media as discussed in Chapter IV.

6.2.5. Data processing and statistical analysis

CO and H_2 conversion efficiencies were estimated based on equations (1) and (2). CO and H_2 utilizations, cell mass yield and ethanol yield from CO were calculated based on method reported in Table 4.4 in Chapter IV. The statistical analysis was performed using Tukey's multiple comparison of means at 95% confidence level in JMP Pro 13.0.0 (SAS Institute Inc., Cary, NC, USA) to determine pairwise statistical differences of final ethanol, maximum acetic acid, final acetic acid concentrations, cell mass yield and

ethanol yield from CO, H_2 and CO utilization between each treatment in bottle reactors and CSTR.

$$CO\ conversion = \frac{Inlet\ CO\ (mmol/min) - Outlet\ CO(mmol/min)}{Inlet\ CO(mmol/min)} \times 100\% \quad (1)$$

$$H_2\ conversion = \frac{Inlet\ H_2\ (mmol/min) - Outlet\ H_2\ (mmol/min)}{Inlet\ H_2\ (mmol/min)} \times 100\% \quad (2)$$

6.3 Results and discussion

6.3.1 Effects of PLBC loading

Syngas fermentation in Ctrl and treatments with various PLBC loadings (M1, M2, M3, M4 and M5) in bottle reactors resulted in no lag phase in cell growth. The pH in all treatments dropped from about 6.2 to between 4.6 and 4.8 at 48 h (Fig. 6.2). Cell mass accumulated in all media from 0 h and slowed down at 72 h. Acetic acid was produced in each treatment and reached to the highest concentration at 72 h. After 72 h, acetic acid concentration started to decrease due to its conversion to ethanol. CO and H_2 are major gaseous substrates for growth and product formation by *C. ragsdalei*. CO provides carbon source for cell mass while both CO and H_2 provide reducing power for acetic acid and ethanol formation via Wood-Ljungdahl pathway (Ljungdhal, 1986; Wood et al., 1986). CO and H_2 consumptions started from the beginning of fermentation and slowed down after 200 h. Total cumulative CO uptake in all treatments was between 51and 74 mmol. The total cumulative H_2 uptake in all treatments was between 19 and 33 mmol (Fig. 6.2).

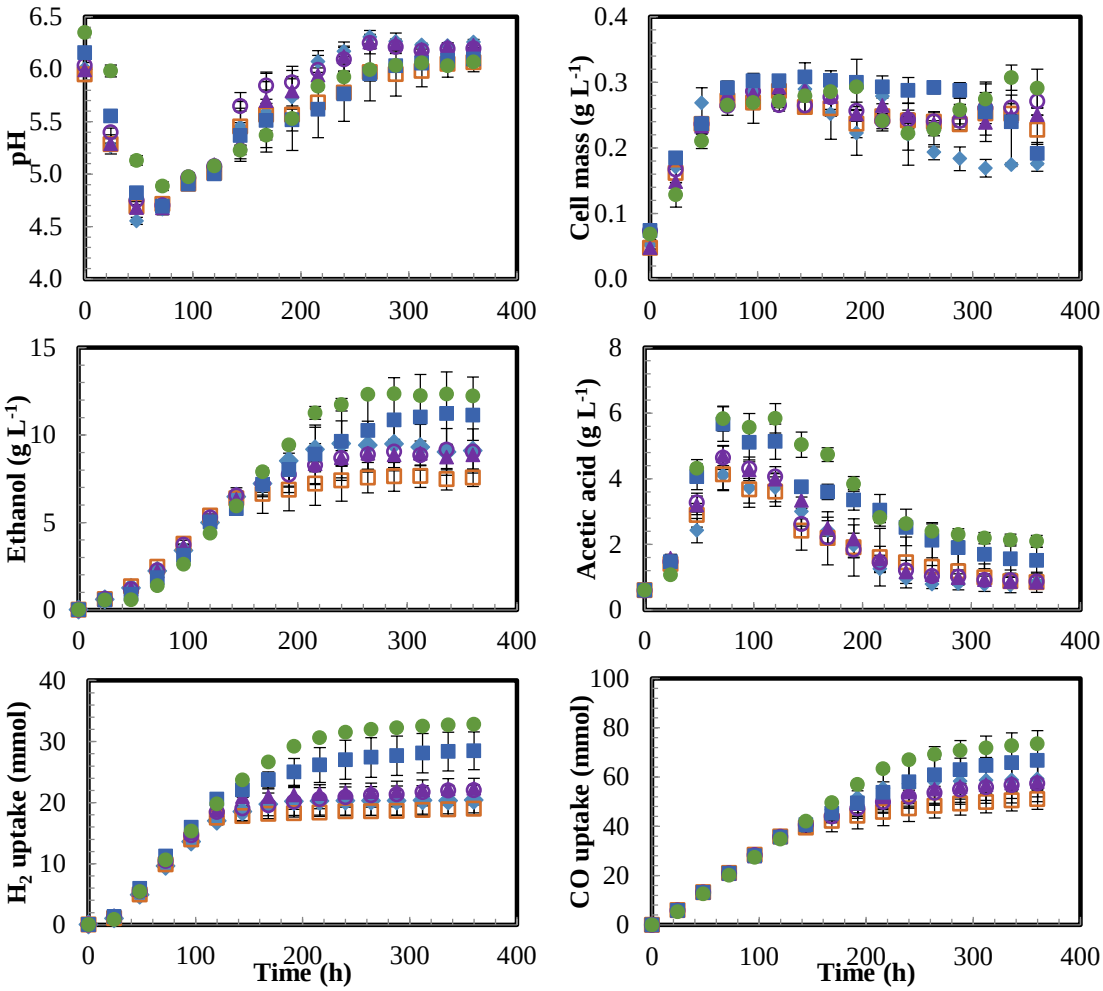

Fig. 6.2 Profiles of pH, cell mass concentration, productions of ethanol and acetic acid, cumulative uptake of H_2 and CO by *C. ragsdalei* at various PLBC loadings (Table 6.1) in bottle reactors, Ctrl (♦), M1 (□), M2 (▲), M3 (○), M4 (■) and M5 (●).

The final ethanol and acetic acid produced, and maximum acetic acid measured in YE medium (Ctrl) and media with PLBC loading of 1.0 (M1), 2.5 (M2), 5.0 (M3), 10.0 (M4), and 20.0 g L^{-1} (M5) are shown in Fig. 6.3A. The final ethanol and maximum acetic acid concentrations in the fermentation medium increased with an increase in PLBC loading. Ethanol production in M5 was significantly higher ($p < 0.05$) than in M1, M2 and M3 media. No significant difference ($p > 0.05$) in the final ethanol produced between M4 and

M5 media, and among Ctrl, M1, M2, and M3 media. Lower ethanol production was due to lower concentrations of mineral and trace metal nutrients (compounds containing Na, Ca, Mg, P, Fe) released from PLBC with loadings below 10.0 g L^{-1} (Fig. 6.4). Fe was considered critical for metalloenzymes such as formate dehydrogenase (FDH), carbon monoxide dehydrogenase (CODH), hydrogenase (H_2ase), and alcohol dehydrogenase (ADH) in *C. ragsdalei* (Saxena & Tanner, 2011). In addition, the depletion of P and Mg resulted in reduced ethanol production in *C. ragsdalei* (Saxena & Tanner, 2012). Besides, maximum acetic acid concentrations in M4 and M5 media were significantly higher ($p < 0.05$) than that in Ctrl and M1 media, indicating an enhanced acetic acid production with 10.0 and 20.0 g L^{-1} PLBC loadings. The enhanced acetic acid was due to extended acetogenic phase with decelerated pH drop, which was caused by high pH buffering capacity of PLBC (Table 5.3 in Chapter V). The results in the present study showed that medium with 10.0 or 20.0 g L^{-1} PLBC enhanced both acetic acid and ethanol production more than in standard YE medium (Ctrl).

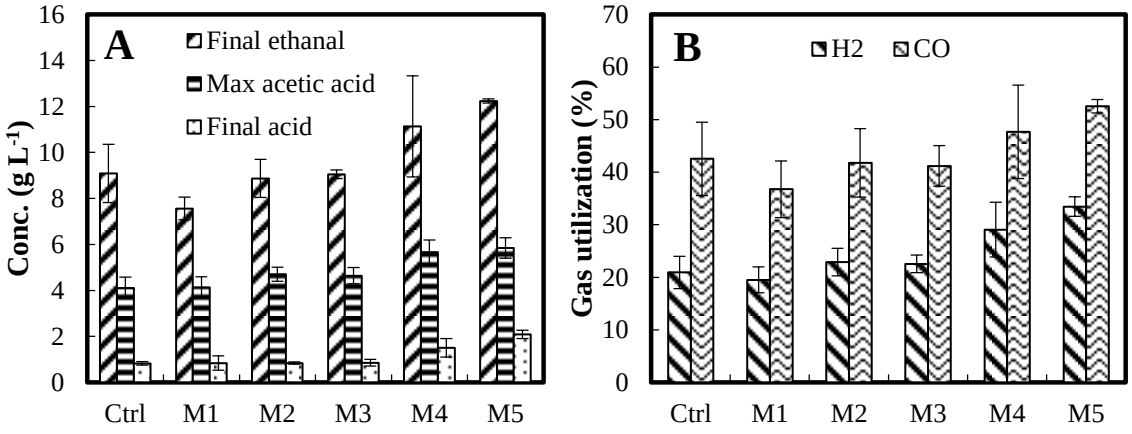

Fig. 6.3 Concentrations of (A) final ethanol, maximum acetic acid, and final acetic acid, (B) final cumulative utilization of H_2 and CO in bottle reactors in Ctrl treatment (YE medium) and treatments with different PLBC loading (M1: 1.0, M2: 2.5, M3: 5.0, M4: 10.0, and M5: 20.0 g L^{-1}). Error bar represents standard deviation of three replications for each treatment (n=3).

The higher utilization of CO and H_2 in M4 and M5 media with PLBC resulted in more product formation (Fig. 6.3 B). Statistical analysis showed that final cumulative H_2 utilization in M4 and M5 media were significantly higher ($p < 0.05$) than in other media. No significant differences ($p > 0.05$) were measured in final cumulative H_2 utilization between Ctrl, M1, M2, and M3 media. Although more CO was utilized in the media with PLBC above 10.0 g L^{-1}, there were no significant ($p > 0.05$) differences in CO utilization among all treatments. This demonstrates that media with PLBC loadings (10.0 and 20.0 g L^{-1}) enhanced H_2 and CO utilization compared to other media.

Fig. 6.4 shows the concentrations of Na, Ca, Mg, K, P, Fe, Mn, Co, and W before inoculation (0 d), at 10 d and 15 d in all six media. The concentrations of Na, Ca, Mg, K, P, Fe, and Mn increased with PLBC loading because these elements were available in PLBC (Table 4.2, Chapter IV) and released during fermentation. No significant changes

($p < 0.05$) in Na, K and P concentrations were measured in PLBC media at 0, 10 and 15 d indicating that fermentation did not influence the release of these elements from PLBC. However, Ca, Mg, Fe, and Mn concentrations significantly increased during fermentation indicating the release of these elements were enhanced by fermentation. This was due to neutralizations of undissociated acetic acid by insoluble compounds containing Ca, Mg, Fe, and Mn during early stage of fermentation (acetogenic phase) as was also shown in Fig. 4.4 in Chapter IV. The compounds released immediately from PLBC in aqueous medium were soluble cations, and those released by acid neutralization were bounded cations (Table 5.3 in Chapter V). The concentrations of Co and W decreased with an increase in PLBC loading. No Co was measured in M5 medium at 10 d and 15 d. W was not observed in either M3 at 10 d and 15 d, or in both M4 and M5 media during fermentation. The reduction or depletion of Co and W with higher PLBC loading could be due to their adsorption on the biochar (Inyang et al., 2016). Similar results were also obtained with *C. ragsdalei* using various types of biochar (Fig. 4.3 in Chapter IV). Although Na, Mg, and K concentrations in M5 medium were significantly higher ($p < 0.05$) than in M4 medium, there were no significant ($p > 0.05$) increases in ethanol production or CO and H_2 utilization by *C. ragsdalei* in M4 and M5 media, which was probably due to excess amounts of these elements in these media.

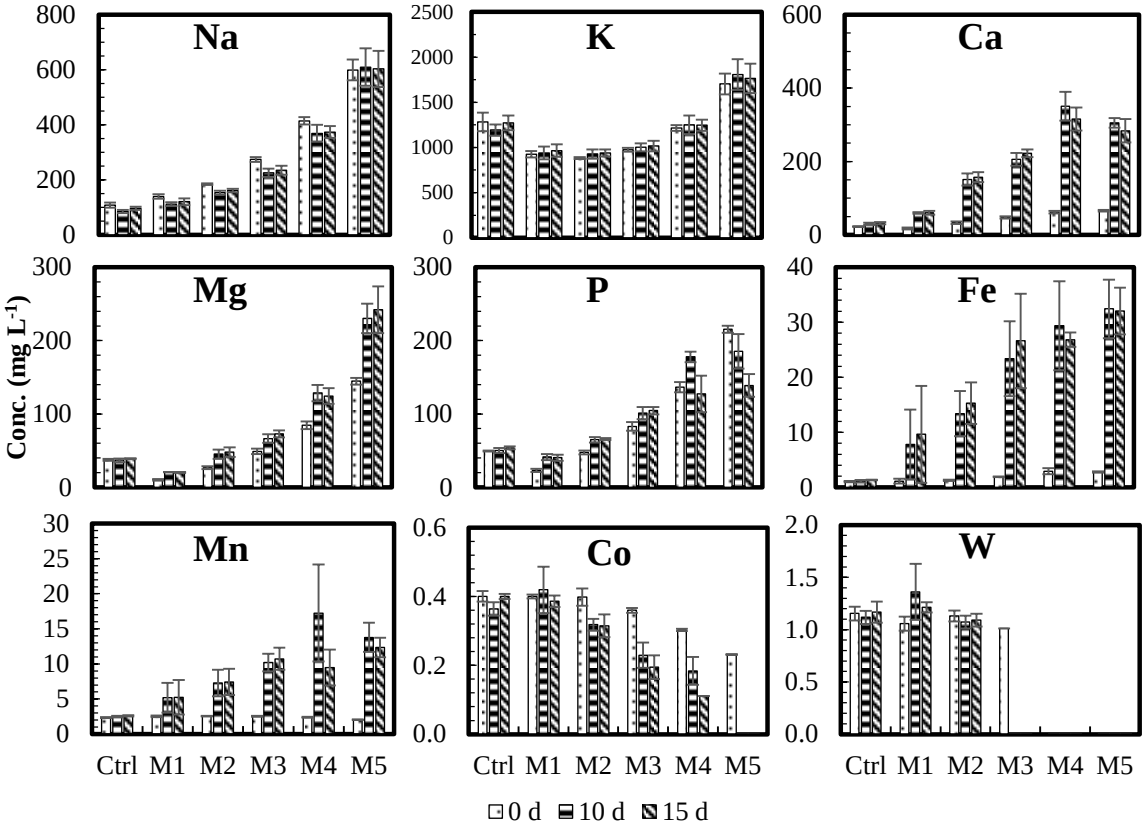

Fig. 6.4 Concentrations of Na, K, Ca, Mg, S, P, Fe, Mn, Co, and W at 0 d, 10 d and 15 d of fermentation in bottle reactors in Ctrl treatment (YE medium) and treatments with different PLBC loading (M1: 1.0, M2: 2.5, M3: 5.0, M4: 10.0, and M5: 20.0 g L^{-1}). Data not shown in the figure was below 0.1 mg L^{-1} detection limit. Cu, Ni, Mo, and Se concentrations were all below detection limit and therefore not shown in this figure. Error bar represents standard deviation of three replications for each treatment (n=3).

6.3.2 Effects of PLBC leachates

Syngas fermentations in four media Ctrl (standard YE), M5 (20 g L^{-1} PLBC), M6 (deficient medium no PLBC), and M7 (PLBC leachates) were examined. No lag phase in cell growth was observed in all treatments (Fig. 6.5). The pH in all media started to decrease and reached their lowest level at 48 h. Cell mass and acetic acid concentrations increased between 0 h to 48 h. The cell mass and acetic acid concentrations in M5

medium were significantly higher ($p < 0.05$) than those in Ctrl, M6 and M7 media. Ethanol production in M5 medium became significantly higher than treatment Ctrl, M6 and M7 after 192 h. Similarly, cumulative CO and H_2 consumption were higher in M5 medium than in Ctrl, M6 and M7 media after 192 h.

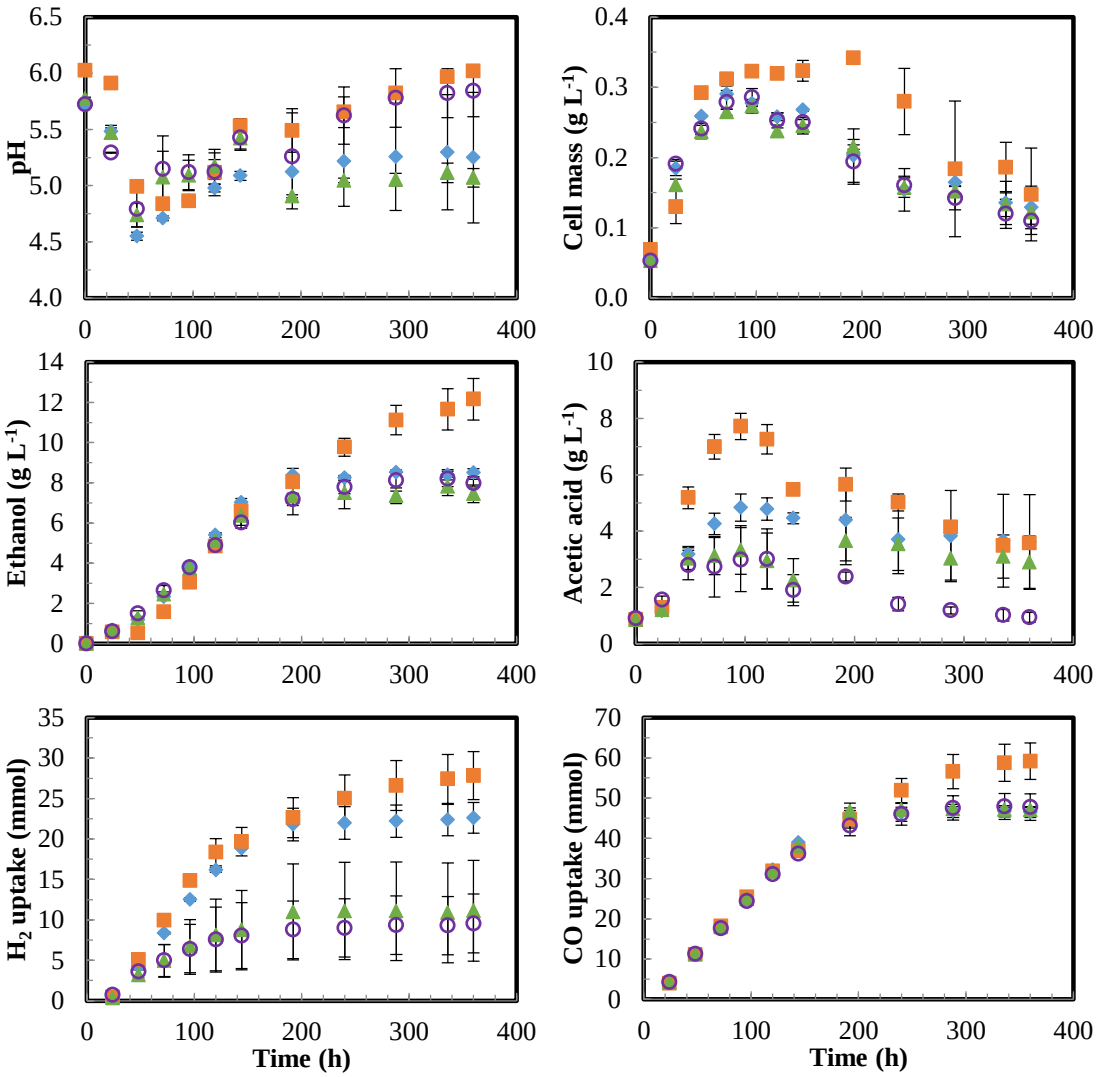

Fig. 6.5 The profiles of pH, cell mass, productions of ethanol and acetic acid, H_2 and CO cumulative uptakes by *C. ragsdalei* in the treatments (Table 6.1) in bottle reactors, Ctrl (♦), M5 (■), M6 (▲), and M7 (○).

Fig. 6.6 showed the final ethanol and acetic acid concentrations, maximum acetic acid concentration and final cumulative CO and H_2 utilization in Ctrl, M5, M6 and M7 media. The final ethanol produced and maximum acetic acid concentration in M5 medium was significantly higher ($p < 0.05$) than in Ctrl, M6, and M7 media as shown in Fig. 6.6A. The final cumulative H_2 utilization in Ctrl and M5 media was significantly ($p < 0.05$) higher than in both M6 and M7 media. However, the final cumulative CO utilization in M5 medium was significantly higher ($p < 0.05$) than in Ctrl and M6 media (Fig. 6.6B). The difference between M5 and M6 media is that M5 contains PLBC particles. The higher ethanol and acetic acid concentrations and CO and H_2 utilization in M5 medium than in M6 medium clearly demonstrated that the addition of PLBC enhanced the syngas fermentation. The difference between M5 and M7 media is that M7 medium only contains leachate prepared from PLBC. The results showed that ethanol can be produced in the medium that contains PLBC leachate in M7 medium. However, no enhancement in ethanol production was measured in M7 medium compared to Ctrl or M6 media. Ethanol production enhancement occurred in M5 medium, in which PLBC particles were added and continue to release mineral and metal nutrients during fermentation.

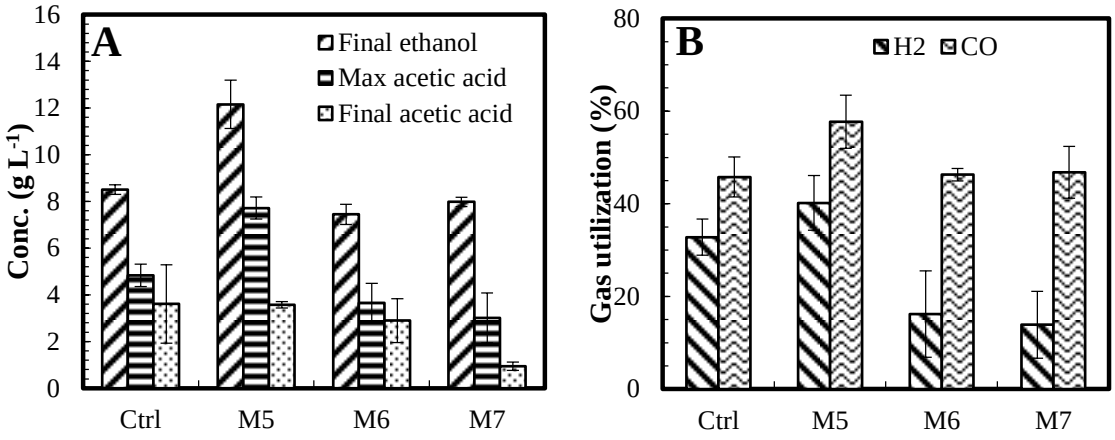

Fig. 6.6 Concentrations of (A) final ethanol, maximum acetic acid and final acetic acid, (B) final cumulative utilization of H_2 and CO in bottle reactors in standard YE medium (Ctrl), medium with 20.0 g L^{-1} PLBC loadings (M5), deficient medium without PLBC (M6), and medium with PLBC leachate (M7). Error bar represents standard deviation of three replications for each treatment (n=3).

The PLBC leachate in M7 medium had higher Na, K, and P concentrations than M6 deficient medium (Fig. 6.7). However, leachate medium M7 had lower Na, Ca, Mg, P, Fe and Mn concentrations than M5 PLBC medium. This indicates PLBC particles in M5 medium continued to release minerals and metal nutrients during fermentation. Therefore, for ethanol production enhancement, the PLBC particles have to be present in medium. The enhanced release of Ca, Mg, Fe and Mn in M5 medium during fermentation was due to neutralization of water-insoluble compounds of Ca, Mg, Fe and Mn by the protons in the undissociated acetic acid produced during *C. ragsdalei* acetogenic phase as was also shown in Fig. 4.4 in Chapter IV. Because of neutralization of the acetic acid produced in M5 medium by the high acid neutralizing capacity (ANC) and pH buffering capacity of PLBC, the drop in the culture pH was decelerated. The slow pH drop extended acetogenic phase and produced more acetic acid, which was converted to ethanol during solventogenic phase. In addition, the slow in pH drop reduced chances of

cells' acid crash. The fact that Co and W were lower in M5 medium than in other media during fermentation (Fig. 6.7) demonstrated the adsorption of Co and W by PLBC particles, which might be caused by the high CEC of PLBC (Table 5.3 in Chapter V) as was previously reported (Inyang et al., 2016).

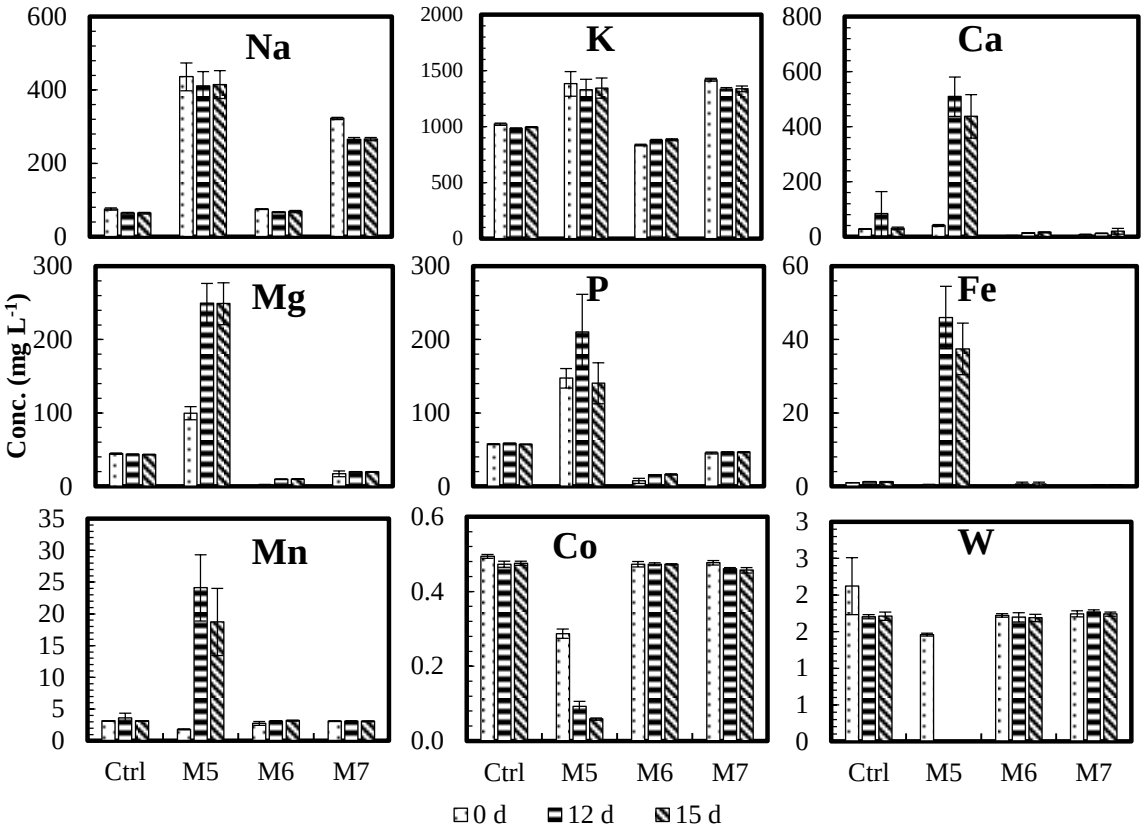

Fig. 6.7 Concentrations of Na, K, Ca, Mg, S, P, Fe, Mn, Co, and W at 0 d, 10 d and 15 d of fermentation in bottle reactors in standard YE medium (Ctrl), medium with 20 g L^{-1}PLBC loadings (M5), deficient medium without PLBC (M6), and medium with PLBC leachate (M7). Data not shown in the figure was below 0.1 mg L^{-1} detection limit. Cu, Ni, Mo, and Se concentrations were all below detection limit and therefore not shown in this figure. Error bar represents standard deviation of three replications for each treatment (n=3).

6.3.3 Fed-batch fermentation using standard YE medium in CSTR

Two fermentation runs in the 3-L CSTR were performed using a standard YE medium without MES as a control for comparison with PLBC media. The pH of the medium in the CSTR before inoculation was about 6.3 after addition of 24 mL of 4% cysteine sulfide to remove any remaining dissolved O_2 (ORP was below -300 mV). The strict anaerobic environment reduced lag phase of *C. ragsdalei* once inoculated. There was a lag phase in cells' growth during the first 6 h (Fig. 6.8 A). After 6 h, cell mass concentration increased with production of acetic acid and decreased pH (Fig. 6.8 B). The pH quickly decreased to 4.8 with acetic acid concentration reaching 0.6 g L^{-1} at 19 h. The culture pH was controlled at set point of 4.8 by automatically adjusting the syngas feeding rate using the developed patented novel pH controller (Atiyeh et al., 2018). When the pH dropped below 4.8 indicating more acetic acid production, the pH controller adjusted the supply of syngas (i.e., increasing reactants CO and H_2) to convert the acetic acid to ethanol as the desired product. From 19 h to 50 h, the syngas feeding rate was automatically adjusted within the range between 0.9 to 2.8 mmol min^{-1}, to provide enough syngas for the available cells to grow and convert acetic acid to ethanol. During this period, the culture pH was maintained around 4.8 (Fig. 6.8 A and B). Cells' activity were the highest between 19 h to 50 h, which resulted in the highest CO and H_2 conversion efficiencies from 41% to 66% and from 35% to 41%, respectively (Fig. 6.8 C). However, the cell growth slowed down after 50 h and cells entered stationary phase due to depletion of nutrients and unfavorable low culture pH for growth. These stresses on the cells can force acetogens to switch from acetogenesis (growth and acid production) to solventogenesis (limited growth and alcohol production) (Gao et al., 2013; Liu et al.,

2012; Phillips et al., 2015). At 80 h, the syngas feeding rate was manually reduced to 2.2 mmol min^{-1} to relieve CO inhibition on H_2ase which creates reducing power for cell mass and products synthesis (Skidmore, 2010). The pH continued to increase from 50 h to 190 h because of no more accumulation of acetic acid in its conversion to ethanol. No acetic acid was detected in the CSTR after 99 h. However, ethanol production continued until 150 h (Fig. 6.8A). After 150 h, cells mass concentration decreased sharply, indicating cells' lysis. During death phase, no ethanol production and low syngas consumption were measured. The highest ethanol concentration (8.1 g L^{-1}) in the YE medium was achieved at 190 h with no accumulation of acetic acid.

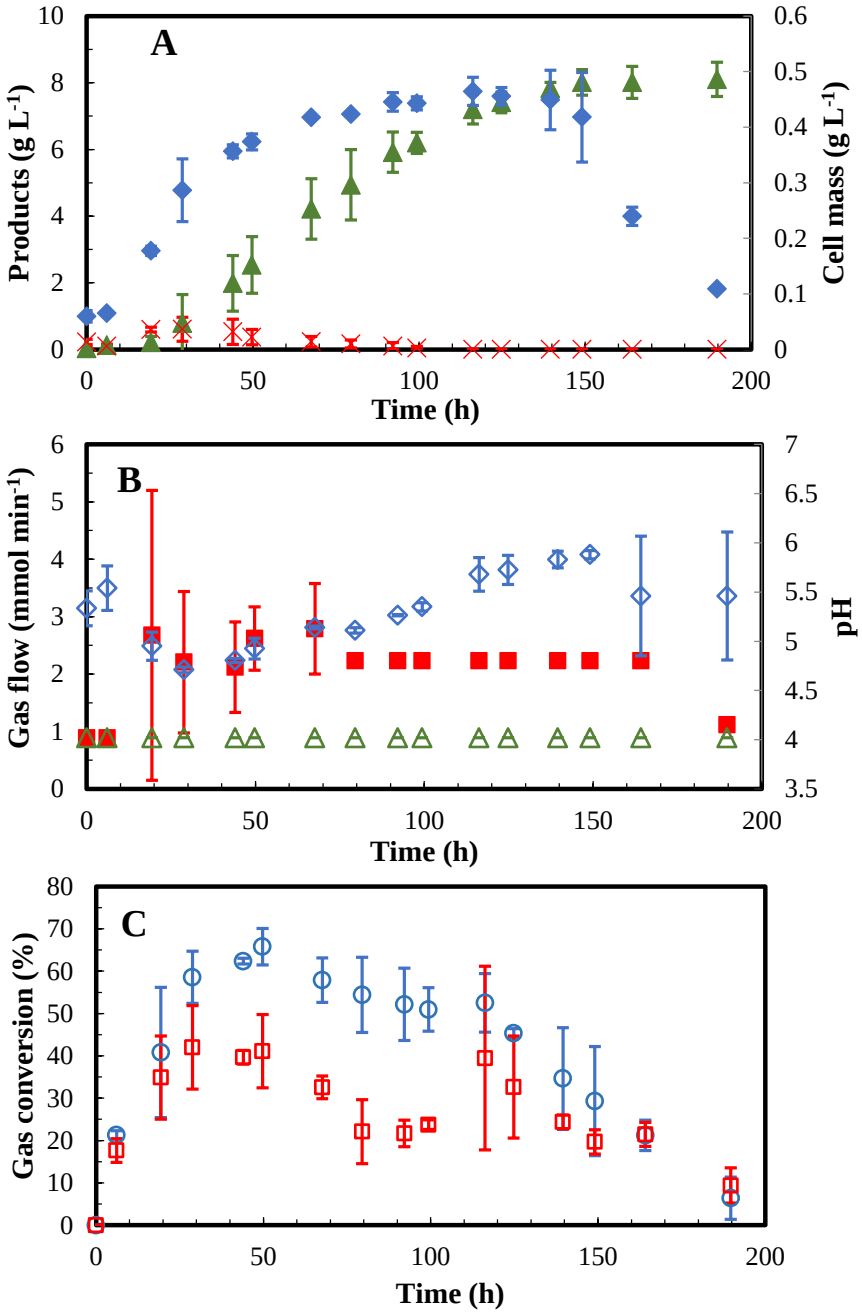

Fig. 6.8 Fed-batch fermentation of *C. ragsdalei* in 3-L CSTR using standard YE medium without MES. (A) profiles of ethanol (▲), acetic acid (×) and cell mass (♦) concentrations, (B) profiles of pH (◊), syngas (■) and N_2 (Δ) flow rate, (C) conversion efficiencies of CO (○) and H_2 (□). Error bar represents standard deviation of two replicate runs (n=2).

Fig. 6.9 shows the changes in various element concentrations in standard YE medium in the CSTR during fermentation. The concentrations of P, Mg, Ca, Mn, Ni, Mo, Co did not change with time. However, Na concentrations increased from 0 h to 19 h due to external additions cysteine sulfide and $NaHCO_3$ solutions at the beginning of fermentation for medium reduction and pH adjustment. No changes in K and Na concentrations were measured after 19 h. Mg, P, Ni, Fe, Co, Mo and W are necessary for synthesis of ethanol by *C. ragsdalei* (Saxena & Tanner, 2011; Saxena & Tanner, 2012). Decreases in S, Fe, Zn, and Cu concentrations were observed at the beginning of fermentation followed by either increased of Zn, stabilization of S or fluctuation of Fe and Cu (Fig. 6.9). This could be due to their utilizations by the cells during cells' growth followed by their release when cells lysed. The slight increase in Se and W concentrations might be due to decreased volume of fermentation broth caused by water vaporization.

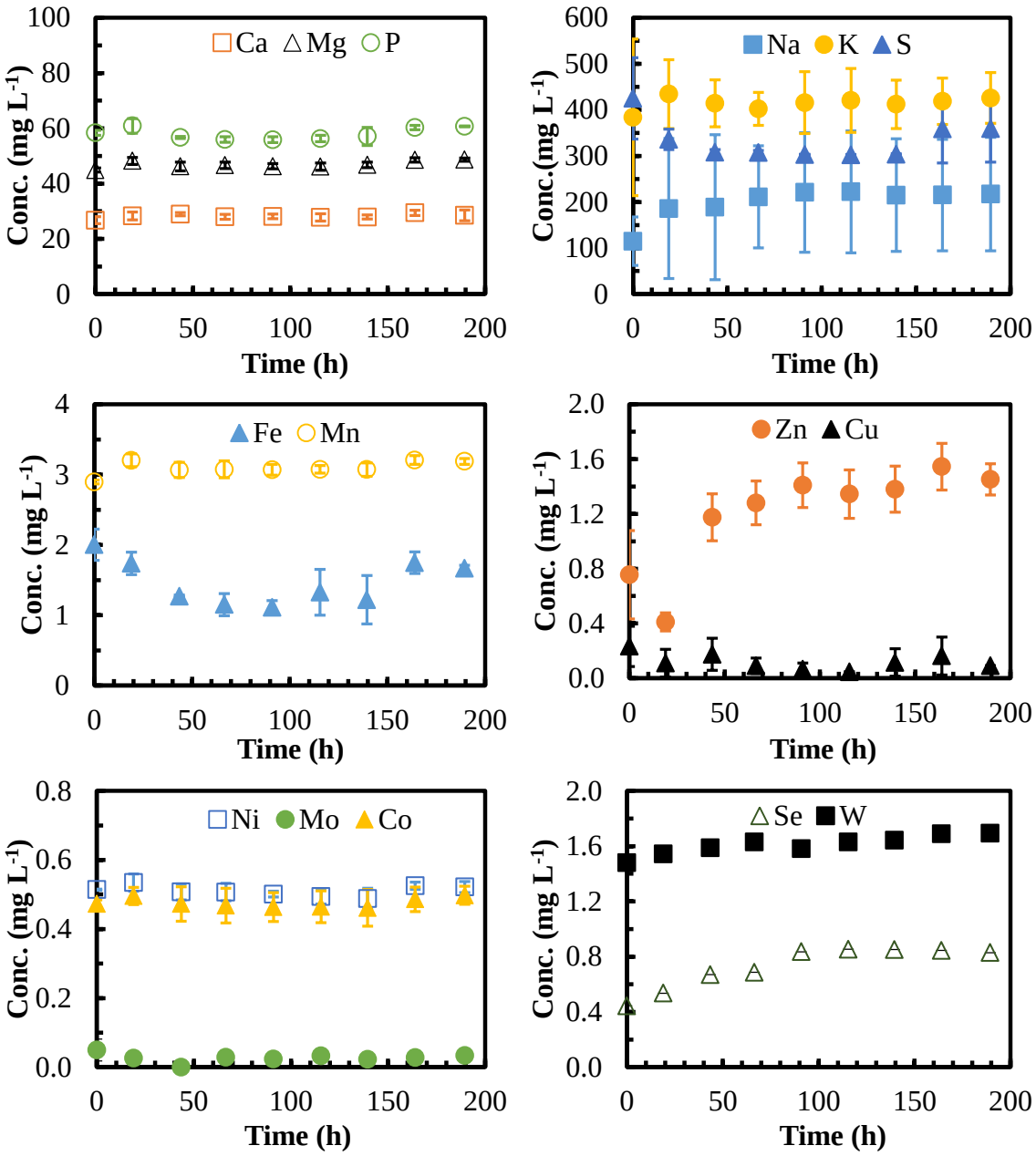

Fig. 6.9 Concentration profiles of Ca, Mg, P, Na, K, S, Fe, Mn, Zn, Cu, Ni, Mo, Co, Se and W during fed-batch fermentation of *C. ragsdalei* in 3-L CSTR using standard YE medium without MES. Se and W only reported for fisrt run because they were below the detection limit in the second run. Error bar represents standard deviation of two replicate runs (n=2).

6.3.4 Fed-batch fermentation using medium with PLBC in CSTR

The pH of the PLBC medium in the CSTR before inoculation with *C. ragsdalei* was 7.2 after the addition of 10 mL of 4% cysteine sulfide to achieve anaerobic environment (ORP below -300 mV). The pH dropped to 6.1 before inoculation using 2 M H_2SO_4 solution. After inoculation, the culture pH dropped to 5.7 due to transfer of acetic acid with the inoculum (Fig. 6.10 A, B). However, the pH in the CSTR increased to 5.8 after about 30 min (data not shown), which was due to the release of alkaline compounds (mainly Ca and Mg) from PLBC with the pH decrease. The pH of the culture was maintained below 5.7 by pumping 2 M H_2SO_4 through the CSTR pH controller until cells started to grow and make acetic acid. This was to provide a suitable pH to initiate cell growth. Preliminary experiment using medium with PLBC without pH control resulted in culture pH increase to around 6.5 after inoculation. The high pH resulted in no cell growth or products formation (data not shown). Therefore, the initial pH in the medium with PLBC was controlled not to increase above 5.7 with the alkalinity of the PLBC.

The cell growth and acetic acid started to accumulate after 7 h of lag phase (Fig. 6.10 A). The acetic acid concentration reached 1.2 g L^{-1} with culture pH of 4.8 at 31 h. After 31 h, ethanol production began to increase during late growth phase and when the pH dropped below 5.0 and acetic acid concentration started to decrease. Ethanol production started after 31 h in PLBC medium compared to 19 h in standard YE medium. Cells required more time to adapt to the PLBC medium. However, the highest acetic acid concentration achieved in PLBC medium (1.2 g L^{-1}) was larger than in standard YE medium (0.6 g L^{-1}). This was due to PLBC pH buffering capacity that slowed culture pH drop and extended acetogenesis. The lowest pH obtained was 4.8 and was maintained for 13 h by pH controller via adjusting syngas feeding rate between 0.89 and 1.73 mmol min^{-}

[1]. The increase in syngas flow rate was to provide more substrate gas as the cells' activity increased. However, the pH increased from 4.8 at 44 h to 5.1 at 50 h. The pH remained around 5.0 from 50 h to 75 h. During this period, ethanol concentration increased from 0.8 to 3.0 g L^{-1}, while acetic acid concentration was about 0.8 g L^{-1}, and cell mass increased to about 0.31 g L^{-1}. The pH increased from 4.9 to 5.2 and ethanol increased from 3.0 to 4.9 g L^{-1} between 75 h and 100 h. At 100 h, the culture pH was reduced to 4.8 by the addition of about 2.0 mL of 2 M H_2SO_4 solution, to enable the release of more minerals and metals nutrients from PLBC and to further shift the fermentation towards solventogenesis. Because of the drop in pH, ethanol concentration increased from 4.2 to 7.2 g L^{-1} and acetic acid concentration decreased from 0.6 to 0.0 g L^{-1}. From 140 h to 244 h, syngas flow rate was maintained between 1.3 and 1.8 mmol min^{-1} (Fig. 6.10 B). The pH was further reduced to 4.8 at 144 h, 165 h, and 189 h by introducing 2 M H_2SO_4 solution to assist in the release of additional mineral and metal nutrients and force the fermentation towards solventogenesis. The cultural pH naturally increased each time after it was manually reduced to around 4.8 by addition of 2 M H_2SO_4. This was due to the neutralization of the added H_2SO_4 by released compounds from PLBC containing bounded cations (Ca and Mg). Each pH reduction to 4.8 made the conditions more favorable to solventogenesis. This was the reason why ethanol production from 140 h to 288 h increased from 8.6 to 11.0 g L^{-1} even with no accumulation of acetic acid (Fig. 6.10). The CO conversion efficiency was maintained above 56% for 133 h in the PLBC medium (Fig. 6.10 C) compared to only 40 h in the standard YE medium (Fig. 6.8 C). The H_2 conversion efficiency was maintained above 26% for about 80 h in the PLBC medium compared to 50 h in the standard YE medium.

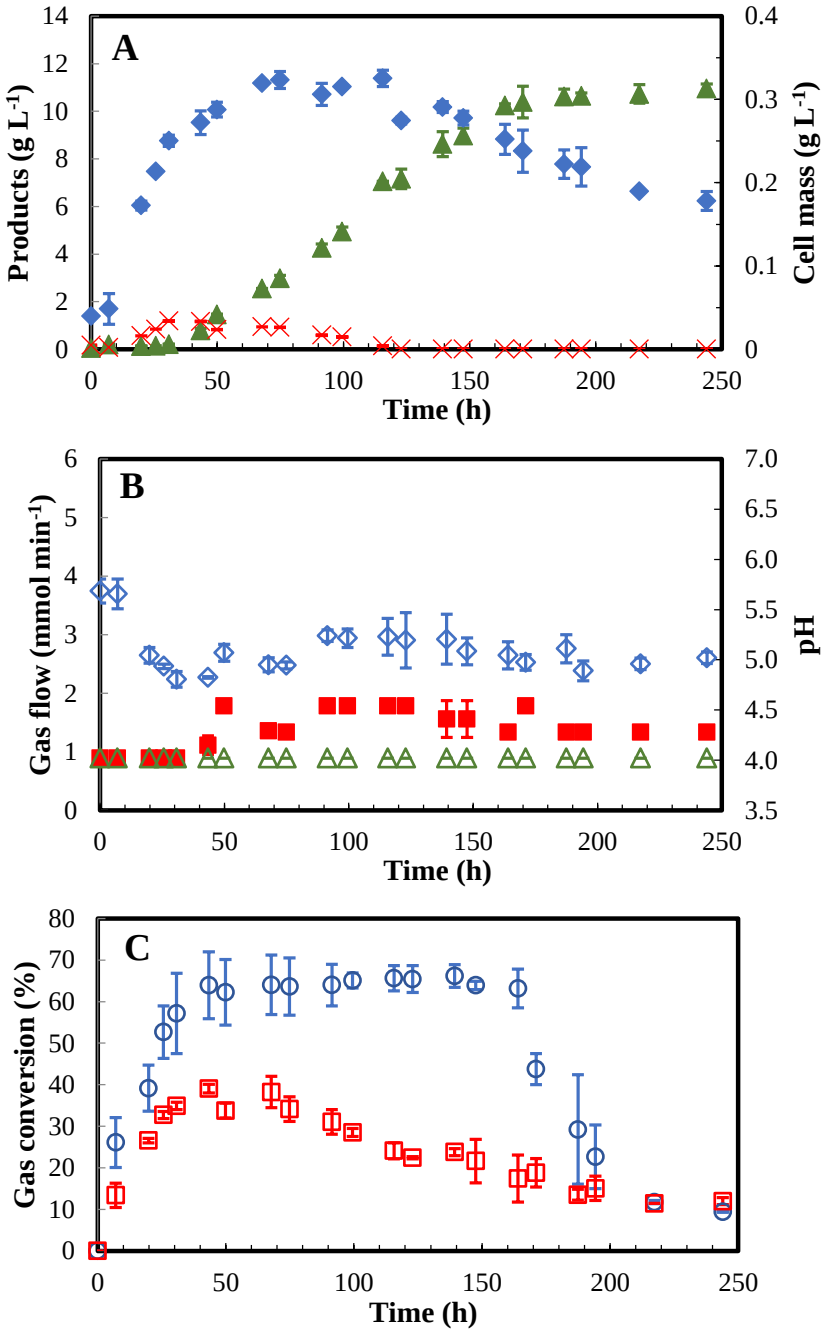

Fig. 6.10 Fed-batch fermentation of *C. ragsdalei* in 3-L CSTR using medium with PLBC without MES. (A) profiles of ethanol (▲), acetic acid (×)and cell mass (♦) concentrations, (B) profiles of pH (◊), syngas (■) and N_2 (Δ) flow rate, (C) conversion efficiencies of CO (○) and H_2 (□). Error bar represents standard deviation of two replicate runs (n=2).

PLBC has basic minerals (Na, K, Ca, Mg, P, S) and trace metals (Fe, Zn, Mn, Ni, Mo, Co, Se) that can be released in the fermentation medium (Fig. 6.11). Soluble cations such as Na and K were released completely in medium before the fermentation started. However, bounded cations such as Ca, Mg with other elements P, Fe, and Mn were mostly released during the fermentation especially when acetic acid accumulated and pH started to decrease as shown in Chapter IV. This property of PLBC enabled it to replace most the minerals and trace metals added with the stocks solutions in the standard YE medium (Table 6.2). Besides, the high pH buffering capacity of PLBC neutralized the undissociated acetic acid produced during fermentation and reduced acid stress on *C. ragsdalei* and extended acetogenic phase as shown in Chapter V. Extended acetogenic phase allowed more acetic acid formation that was converted to ethanol. The change in S concentration during the fermentation was due to the addition of 2 M H_2SO_4 during frequent pH adjustment from 5.3 to 4.9.

Mn concentration increased during the fermentation. It was reported that Mn had no effect on ethanol production with *C. ragsdalei* (Saxena & Tanner, 2011). However, the same study showed that Fe was required by *C. ragsdalei* for ethanol production due to its biological function in hydrogenase (H_2ase), alcohol dehydrogenase (ADH), and carbon monoxide dehydrogenase (CODH) of the Acetyl-CoA pathway. The drop in the Mo and W concentrations was due to adsorption by PLBC biochar. Similar adsorption of Mo and W was observed in bottle reactors using PLBC medium as shown in Chapter IV. It was reported that adsorption of heavy metals by biochar has several mechanisms such as surface complexation, physical sorption on surface sites, surface precipitation, ionic exchange and electrostatic interaction (Inyang et al., 2016).

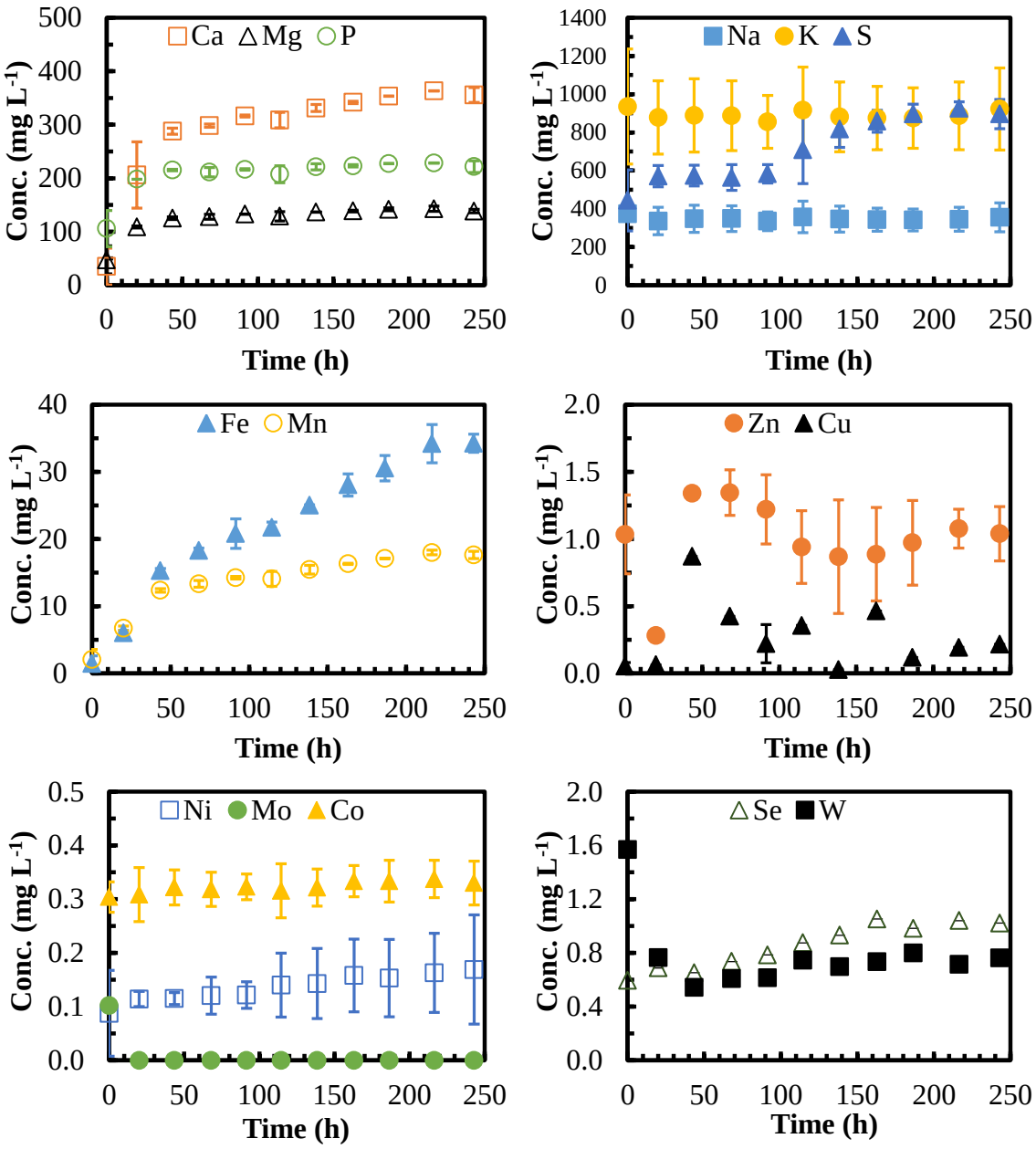

Fig. 6.11 Concentration profiles of Ca, Mg, P, Na, K, S, Fe, Mn, Zn, Cu, Ni, Mo, Co, Se and W during fed-batch fermentation of *C. ragsdalei* in 3-L CSTR using medium with PLBC without MES. Se and W only reported for fisrt run because they were below the detection limit in the second run. Error bar represents standard deviation of two replicate runs (n = 2).

6.3.5 Fed-batch fermention using PLBC medium with MES in CSTR

MES is an expensive buffer that was typically used in standard YE medium for syngas fermentation with pH range from 5.5 to 6.7. However, it was found that the removal of MES from the medium slightly affected syngas fermentation and alcohol production (Gao et al., 2013; Kundiyana et al., 2010a; Phillips et al., 2015). It is difficult to adjust the pH in bottle reactors. This is why MES was typically used to reduce quick changes in pH to lower levels (below 4.6), at which cells start dying. The use of CSTR and efficient pH controller can eliminate the need for MES use. However, in this experiment, the effects of MES in PLBC medium were compared to PLBC medium without MES.

The initial pH of PLBC medium with MES after the addition of 10 mL of 4% cysteine sulfide solution (ORP around -343 mV) and before inoculation was 6.0. The initial pH of PLBC meidum with MES was lower than that in the PLBC medium without MES (pH 7.2). This was due to presence of sulfonic acid group in MES. Therefore, no pH adjustment was required before inoculation. After inoculation and 6 h of lag phase, the pH started to drop from 5.8 with accumualtion of acetic acid. At 53 h, the pH reached the lowest level of 4.8 with accumualtion of 2.0 g L^{-1} of acetic acid. The acetic acid concentration in the PLBC medium with MES was higher than in PLBC medium without MES (1.2 g L^{-1}) . This demonstrated that MES enhanced acetic aicd production as was previously reported (Kundiyana et al., 2010a). Ethanol started to accumulate in the medium after 53 h with the decrease in acetic acid concentration (Fig. 6.12 A). At 53 h, syngas flow rate was manually increased to 1.3 mmol min^{-1} to provide more substrates for cell growth and products formation. From 53 h to 77 h, ethanol concentration

increased from 0.6 to 2.3 g L^{-1} and acetic acid decreased from 2.0 to 1.6 g L^{-1}. Syngas flow rate was further increased to 1.78 mmol min^{-1} at 77h for more supply of substrate (Fig. 6.12 B). This resulted in increase in ethanol concertation from 2.3 to 4.4 g L^{-1} and acetic acid reduction from 1.6 to 1.1 g L^{-1} from 77 h to 99 h. The culture pH increased from 4.8 to 5.2 between 53 h and 99 h. The increased pH was due to conversion of acetic acid to ethanol during solventogenesis which was triggered by lower pH around 4.8. At 99 h, the culture pH was reduced from 5.3 to 4.9 by adding 2 M H_2SO_4. The frequent reduction in pH from 5.3 to 4.9 by additions of 2M H_2SO_4 was performed at 118 h, 142 h, and 168 h. This was done as in previous experiment using PLBC medium without MES, to counteract the alkalinity of the compounds released from PLBC and shift the fermentation towards solventogenesis for more ethanol production. The pH increased each time after it dropped to 4.9 due to the release of alkaline compounds from PLBC but at a slower rate. The increase in pH each time was due to the neutralization of the added H_2SO_4 by PLBC's bounded cations (Ca, Mg, Fe and Mn) (Fig. 6.13). This consecutive pH reduction to 4.9 resulted in enhanced ethanol production from 4.4 to 11.9 g L^{-1} between 99 h and 200 h. At 200 h, the syngas flow rate was reduced to 1.3 mmol min^{-1} to relieve CO inhibition with decrease in cells' activity and reduced CO and H_2 conversion efficiency due to nutrient limitations (Fig. 6.12 C). From 200 h to 290 h, ethanol concentration increased to 13.2 g L^{-1}, which was higher than that in PLBC medium without MES (11.0 g L^{-1}). This indicates that addition of MES to PLBC medium also enhanced ethanol production.

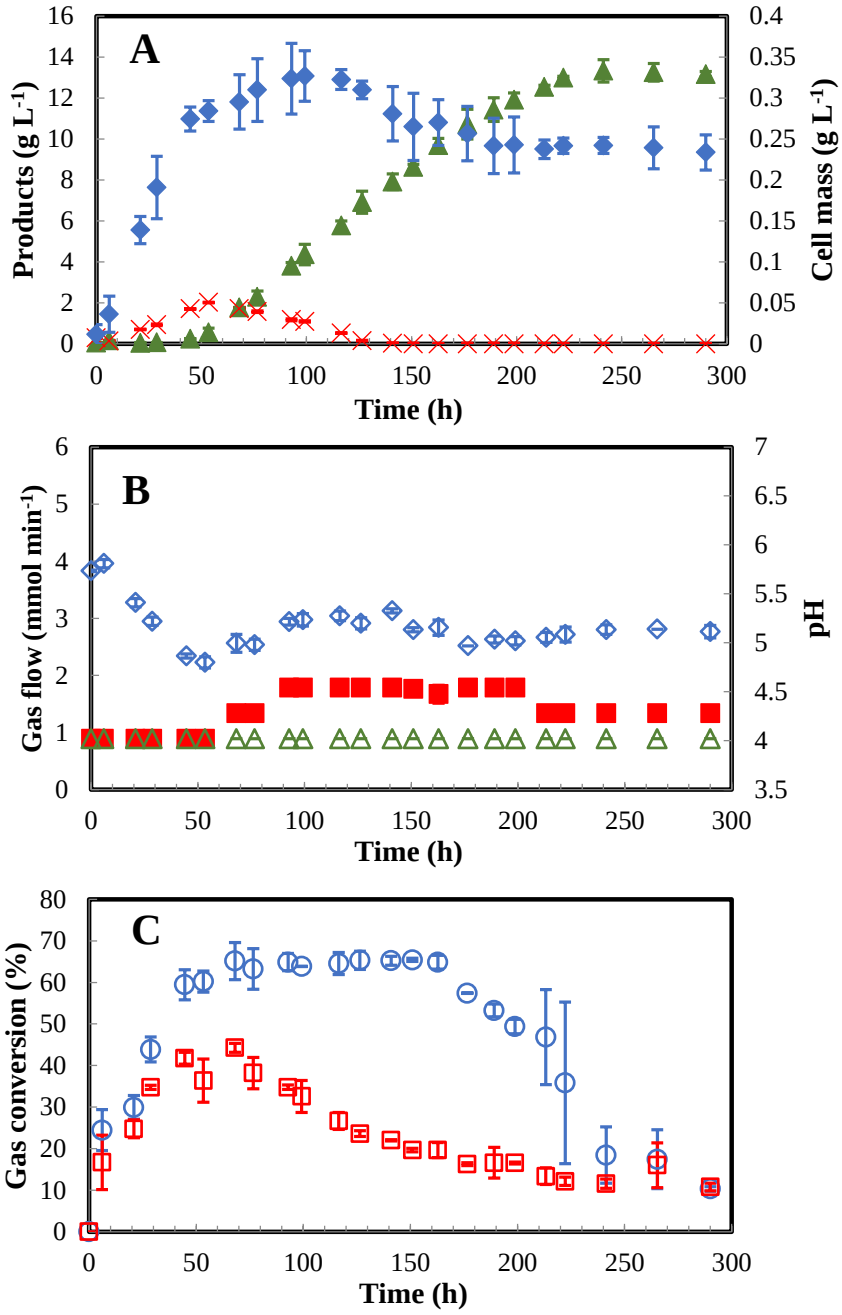

Fig. 6.12 Fed-batch fermentation of *C. ragsdalei* in 3-L CSTR using medium with PLBC and MES. (A) profiles of ethanol (▲), acetic acid (×)and cell mass (♦) concentrations, (B) profiles of pH (◊), syngas (■) and N_2 (Δ) flow rate, (C) conversion efficiencies of CO (○) and H_2 (□). Error bar represents standard deviation of two replicate runs (n=2).

MES contains sulfur and therefore the initial S concentration in the PLBC medium with MES was high (1736 mg L^{-1}) (Fig. 6.13) compared to PLBC medium without MES (444 mg L^{-1}) (Fig. 6.11). The S concentration was later increased due to several additions of H_2SO_4 to adjust the medium pH from 5.3 to 4.9. The Ca, Mg, Fe, and Mn released from the PLBC medium with MES during fermentation were similar to PLBC medium without MES (Fig. 6.13). However, unlike PLBC medium without MES, no significant ($p > 0.05$) increase in Mg and P concentrations were observed and no Se or W were detected in PLBC medium with MES during fermentation. This indicated that Mg and P from PLBC became soluble and Se and W were adsorbed by PBLC before fermentation started which might be due to additional pH buffering effect with the presence of MES in PLBC medium.

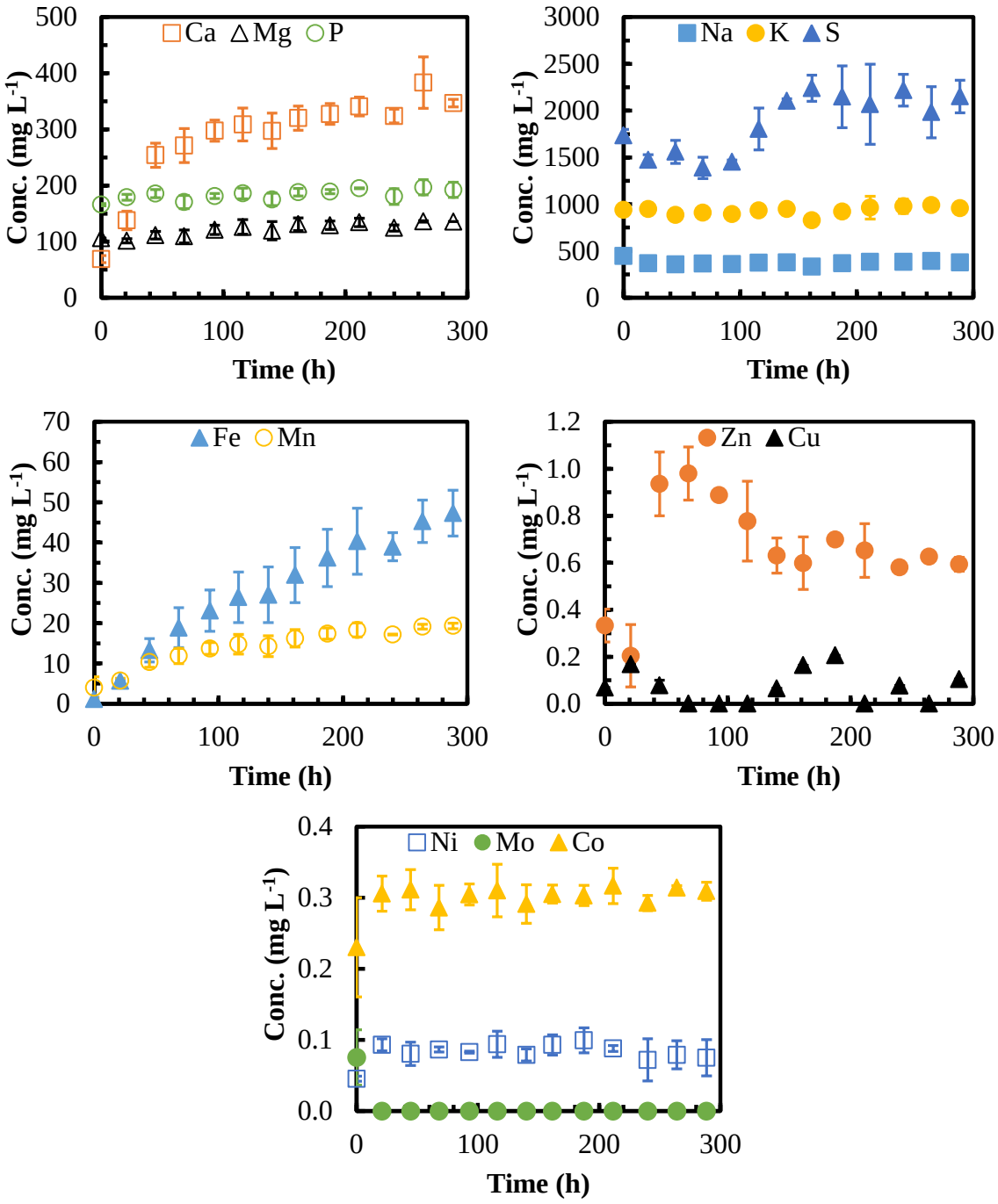

Fig. 6.13 Concentration profiles of Ca, Mg, P, Na, K, S, Fe, Mn, Zn, Cu, Ni, Mo, Co, Se and W during fed-batch fermentation of *C. ragsdalei* in 3-L CSTR using medium with PLBC and MES. Se and W were below detection limit (0.01 mg L^{-1}) in both runs and not shown. Error bar represents standard deviation of two replicate runs (n = 2).

Table 6.3. Parameters of syngas fermentation by *C. ragsdalei* in CSTR using different medium formulations.

Medium name	YE medium without MES	PLBC medium without MES		PLBC medium with MES		
Max cell mass yield from CO (g mol^{-1})[a]	0.34 ± 0.09^{A}	0.72 ± 0.08^{B}		0.60 ± 0.02^{AB}		
Fermentation period	190 h	190 h	244 h	190 h	244 h	290 h
Ethanol yield from CO (%)[b]	51.66 ± 8.49^{A}	84.19 ± 10.16^{B}	78.73 ± 0.80^{a}	92.04 ± 2.14^{B}	85.47 ± 4.38^{a}	71.40 ± 2.72
CO utilization (%)[b]	45.86 ± 0.81^{A}	56.92 ± 2.51^{B}	49.35 ± 2.64^{a}	58.26 ± 1.99^{B}	55.10 ± 1.64^{a}	49.24 ± 1.69
H_2 utilization (%)[b]	29.44 ± 4.59^{A}	26.24 ± 2.01^{A}	23.41 ± 1.96^{a}	27.02 ± 0.53^{A}	24.05 ± 0.71^{a}	22.52 ± 0.86

[a] Based on maximum cell mass concentration for YE medium at 117 h, PLBC medium without MES at 75 h, PLBC medium with MES at 99 h.

[b] Calculated at 190 h for YE medium, at 190h and 244 h for PLBC medium without MES, at 190 h, 244 h and 290 h for PLBC medium with MES.

Same capital letter or lower-case letter in each row represents no significant difference between treatments ($p > 0.05$)

Compared with YE medium without MES, fermentation using medium containing PLBC (with and without MES) led to higher cell mass yields from CO (Table 6.3). The CO utilizations and ethanol yields from CO in medium containing PLBC (with and without MES) were significantly higher ($p < 0.05$) than in YE medium without MES at 190 h. However, no signifiant difference ($p > 0.05$) was found in H_2 utilizations in all three media at 190 h. For PLBC medium with and without MES, no significant differences ($p > 0.05$) were found in ethanol yield from CO, and CO and H_2 utilization at 244 h. These results showed the enhancement in ethanol yield, CO and H_2 utilization using PLBC medium (with and without MES) compared to YE medium without MES.

Results in bottle reactors demonstrated that ethanol production was enhanced only in presence of PLBC particles at 10.0 and 20.0 g L^{-1}. Besides, fed-batch fermentations in the 3-L CSTR regardless of media used showed that acetic acid was completely converted to ethanol. This was achieved by the controlling in syngas feeding rate in the

CSTR when culture pH reached around 4.8. In fermentations in the CSTR using YE medium without MES, PLBC medium without MES and in PLBC medium with MES, the culture pH reached to around 4.8 after 29 h, 31 h and 53 h (Fig. 6.8B, 6.10B, 6.12B), respectively, due to cell growth with accumulation of acetic acid (Fig. 6.8A, 6.10A, 6.12A). The acetic acid concentrations reached their highest (YE medium: 0.6 g L^{-1}, PLBC medium: 1.2 g L^{-1}, PLBC medium with MES: 2.0 g L^{-1}) at pH around 4.8. Then, syngas feeding rate was increased automatically to provided more gaseous substrates when cells were highly active, and prevented further pH drop to avoid acid crash. The pH increase was the response for elevated syngas feeding rate and conversion of acetic acid to ethanol, and release of more alkaline compounds from PLBC (in media with PLBC). In media containing PLBC (with and without MES), the addition of H_2SO_4 to reduce the pH from 5.3 to 4.9 during the fermentation released additional metals and minerals from PLBC, served as additional nutrients for cell metabolisms. The pH reduction with H_2SO_4 was required because alkaline and alkali compounds were released from PLBC, which increased culture pH to high values an unfavorable to ethanol production. In addition, lowering the pH to about 4.9 using H_2SO_4 was more favorable for solventogensis. The current study with CSTR for the first time domonstrated that controlling the syngas feeding rate at pH around 4.8 (Atiyeh et al., 2018) in batch fermentation lead to complete conversion of acetic acid to ethanol without maintaining constant culture pH using traditional addition of acid or base.

In addition, media containing PLBC (without and with MES) prolonged the syngas fermentation and enhanced operation stability represented by low standard deviation (error bar) in ethanol production between two replications compared to YE

medium (Fig. 6.8A, 6.10A, 6.12A). The media with PLBC (without and with MES) also accumulated more acetic acid than in YE medium. This was due to high pH buffering capacity of PLBC and high concentrations of soluble and bound alkaline and alkali cations and other elements (Na, K, Ca, Mg, Fe and Mn) (Fig. 6.11, 6.13) as was also reported in Chapters IV and V other studies (Yuan et al., 2011). The benefits of pH buffering on acetogenesis was shown when higher acetic acid accumulation was found in PLBC medium with MES than in PLBC medium without MES (Fig. 6.10, 6.12 A). Compared to other syngas fermentation studies (Table 6.4), the present study demonstrated for the first time the feasibility of using PLBC as medium component in pure culture syngas fermentation in CSTR and developed a novel processing strategy to enhance ethanol production without accumualtion of acetic acid.

Table 6.4. Ethanol production and final ethanol to acetic acid ratio from syngas fermentation in different bioreactors.

Fermentation strategy	Fermentation mode	Biocatalyst	Gaseous substrate ($CO:H_2:CO_2:N_2$)	Final EtOH ($g\ L^{-1}$)	Final EtOH/HAc (mol/mol)	Reference
CSTR using standard YE medium	Semi-continuous	*C. ragsdalei*	40:30:30:0	8.1	No acetic acid	This study
CSTR using PLBC medium				10.9	No acetic acid	
CSTR using PLBC medium with MES				13.2	No acetic acid	
CSTR with cell recycle	Continuous	*C. ljungdahlii*	55:20:10:15	48.0	20.9	(Phillips et al., 1993)
CSTR with activated carbon	Semi-continuous	*C. ragsdalei*	40:30:30:0 or 20:5:15:0	19.0	24.7	(Atiyeh et al., 2016b)
Bubble column bioreactor (4 L working volume)	Continuous	*C. carboxidivorans*	15:4:16:57:4 (CH_4)	5.0	6.5	(Datar et al., 2004)
CSTR (5 L working volume)	Semi-continuous	*C. ragsdalei*	20:5:15:60	3.1	1.6	(Kundiyana et al., 2010a)
CSTR (70 L working volume)	Semi-continuous	*C. ragsdalei*	12-18:7-12:10-17:55-60	15.0	6.9	(Kundiyana et al., 2010b)
CSTR-bubble column reactor (BCR) at two-stage	Continuous	*C. ljungdahlii*	60:35:5:0	20.7	4.0	(Richter et al., 2013)
CSTR (3.3 L working volume)	Continuous	*A. bacchi*	28:60:0:12	7.7	5.6	(Liu et al., 2014a)
Hollow fiber membrane (HFM) reactor coupled with	Continuous	*C. carboxidivorans*	20:5:15:60	23.9	4.4	(Shen et al., 2014b)
CSTR (1.2 L working volume)	Semi-continuous	*C. autoethanogenum*	100:0:0:0	4.3	116.8	(Abubackar et al., 2016)
Trickle bed reactor (TBR) (500 ml working volume)	Semi-continuous	*C. ragsdalei*	38:29:28:5	5.7	0.65	(Devarapalli et al., 2016)
Horizontal rotating packed bed (h-RPB) reactor	Continous	*C. carboxidivorans*	20:5:15:60	7.0	1.6	(Shen et al., 2017b)

6.4 Conclusions

Poultry litter biochar (PLBC) served as a nutrient supplement and provided buffering for syngas fermentation with enhanced ethanol production. The use of the patented novel pH controller to adjust pH resulted in further enhancement in ethanol production. Controlling the syngas feeding rate and pH completely converted acetic acid to ethanol. Because of the buffering capacity of PLBC, a quick drop in pH was avoided, acetogenesis was extended and more acetic acid accumulated during growth. The enhanced ethanol production and complete conversion of acetic acid in the CSTR eliminated the need for separation of acetic acid from ethanol, which reduces overall ethanol production cost.

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CHAPTER VII

CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

The content below is a list of conclusions that were drawn from the study of this research project:

- *Clostridium ragsdalei* can grow and convert syngas (40% CO, 30% H_2, 30% CO_2) into acetic acid and ethanol in media incorporated with poultry litter biochar (PLBC), red cedar biochar (RCBC), forage sorghum biochar (FSBC), and switchgrass biochar (SGBC).
- In syngas fermentation with *C. ragsdalei*, RCBC and PLBC media improved ethanol production by 16.3% and 58.9%, respectively, compared to standard YE medium. Also, 69% more H_2 and 40% more CO were consumed using PLBC medium, and 18% more CO was consumed using RCBC medium, compared to standard YE medium.
- The use of *C. ragsdalei* inoculum pre-cultured for 138 h enhanced ethanol production compared with inoculum pre-cultured for 162 h, 182 h, and 210 h in bottle fermentations.

- Acetic acid accumulation during syngas fermentation with *C. ragsdalei* enhanced the release of Ca, Mg, Fe, and Mn from biochar. Neutralization of the produced acetic acid slowed pH drop during fermentation and reduced acid stress on cells.
- *Clostridium carboxidivorans* grew and converted syngas (40% CO, 30% H_2, 30% CO_2) into acetic acid, butyric acid, hexanoic acid, ethanol and butanol in medium incorporated with PLBC, RCBC, FSBC and SGBC via fermentation in bottle reactors.
- PLBC and SGBC enhanced ethanol production by *C. carboxidivorans* by 90% and 73%, respectively, and butanol production by 3.2 and 3.7 fold compared to standard YE medium (control). CO and H_2 utilization was enhanced by 110% and 15.1% in SGBC medium, and by 94.5% and 60.7% in PLBC medium, compared to standard YE medium.
- PLBC has the highest pH buffering capacity, cation exchange capacity and total soluble and bound cations (Na, K, Ca, and Mg) compared with SGBC, FSBC and RCBC. The high pH buffering capacity of PLBC reduced pH drop, acid stress on cells and extends acetogenesis of *C. carboxidivorans*.
- Loading rates of 10.0 and 20.0 g L^{-1} of PLBC or SGBC significantly enhanced alcohols and acids production by *C. carboxidivorans*. Also, the loading rates of 10.0 and 20.0 g L^{-1} of PLBC also enhanced syngas fermentation by *C. ragsdalei*. PLBC or SGBC loadings below 10.0 g L^{-1} showed no enhancement in syngas fermentation compared to standard YE medium with *C. carboxidivorans*.
- In the 3-L CSTR fed-batch fermentation with *C. ragsdalei*, the final ethanol production using 10 g L^{-1} PLBC was enhanced by 36.4 % compared to standard

YE medium. A further improvement in ethanol production by 26.6% resulted by adding MES to the PLBC medium compared to PLBC medium without MES.

- Medium pH controlled below 5.7 at the beginning of fermentation in fed-batch CSTR was critical to initialize cell growth and metabolisms when PLBC was added.
- In CSTR fed-batch fermentation, the automatic patented control of syngas feeding rate at pH set point of 4.8 resulted in complete conversion of acetic acid to ethanol, reduced acid crash on cells and prolonged a stable syngas fermentation process.

7.2 Future work

The content below is a list of recommended future work based on what had been achieved in this study:

- Biochar with high pH buffering capacity (e.g. biochar from diary, swine and turkey wastes) other than poultry litter biochar should be considered as a potential material and tested for their enhancement of syngas fermentation using acetogenic bacteria.
- Biochar with high pH buffering capacity could also be applied in pure culture and mixed culture fermentation to convert syngas into higher alcohols and more valuable chemicals.
- In CSTR fed-batch fermentation using medium with biochar, acetic acid could be used to replace sulfuric acid for multiple pH reductions. This could improve total

alcohol production even more due to addition of more carboxylic acids besides those accumulated by cells.

- Also, continuous syngas fermentations with biochar should be performed in CSTR and in trickle bed reactors to examine the benefits of biochar in continuous fermentations with various types of reactors.

APPENDICES

APPENDIX A

This appendix contains information about production methods for biochars from switchgrass (SGBC), forage sorghum (FSBC), red cedar (RCBC) and poultry litter (PLBC). SGBC, FSBC and RCBC were produced by gasification using a downdraft gasifier at Oklahoma State University. The gasification was performed at three equivalence ratios (ERs) as shown in Table A1. At the end of each run, biochar was collected.

Table A1 Production method of biochars used in this dissertation.

Feedstock type	Thermo-chemical conversion	ER[a]	Remarks
Switchgrass	Downdraft gasification	0.28	Collected below reactor
Forage sorghum	Down draft gasification	0.22	Collected above reactor
Red cedar	Downdraft gasification	0.20	Collected below reactor

[a] Equivalence ratio, calculated by dividing actual oxygen (or air) to biomass molar ratio to the stoichiometric oxygen (or air) to biomass molar ratio.

The poultry litter used for the biochar was collected from a commercial poultry house in Eastern Oklahoma. The poultry litter biochar (PLBC) was produced by a company named Eprida in Georgia. The poultry litter was fed to a slow pyrolysis unit at

rate of 50 kg per hour. An inert gas generator was used to maintain bed temperature profiles. The unit was filled with cool charcoal as inert media to distribute heat. The PLBC production conditions are shown in Table A2.

Table A2 Production conditions of poultry litter biochar by slow pyrolysis.

Production conditions	Values
Temperature (°C)	497.7 ± 14.4
Pressure (psig)	0.9 ± 0.1
Steam temperature (°C)	510.7 ± 23.4
Off-gas temperature (°C)	532.6 ± 15.8
Steam flow (kg h^{-1})	9.7 ± 0.6
Biomass feed (kg h^{-1})	5.77
Char yield	As received: 37.7 % Dry basis: 57.0 %

APPENDIX B

This appendix discussed continuous fermentation of *C. ragsdalei* in 3-L CSTR using standard YE medium without MES. The fermentation evaluated the effects of dilution rate and syngas composition on cell growth, ethanol and acetate production, and consumption of H_2 and CO.

B1. Background

C. ragsdalei was used in this study. The maintenance of *C. ragsdalei* was discussed in Chapter IV. The fermentation was performed in a 3-L CSTR (2.4 L working volume). The pH was controlled at 4.8 using a novel patented pH controller (Atiyeh et al. U.S. Patent No. 10,017,789) by adjusting the syngas feeding rate. Liquid medium contained (L^{-1}) 0.6 g yeast extract (YE), 30 ml minerals stock solution, 12 ml vitamin stock solution, and 12 ml trace metal stock solution. Syngas composition is shown in Table B1. Supply of liquid media and syngas, and a controlling system which contained BioFlo 110 controller and Labview program was shown in Fig. B1. The LabView program was developed inhouse.

Table B1. Compositions, source and stoichiometry of bottled syngas (I, II, III, IV, V) and gas mixture (VI) used in this study.

Syngas	H_2	N_2	CO	CO_2	CO/ H_2	Source and stoichiometry	ΔG^0
		%					kJ mol^{-1}
I	27.9	5.47	38.17	28.46	1.37	Modeled coal producer gas	-
II	58.94	10.32	30.74	0	0.52	$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O$	-137.1
III	67.21	10.49	0	22.3	-	$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O$	-97.5
IV	4.95	61.03	19.44	14.58	3.93	Modeled switchgrass producer gas	-
V	0	0	50-100	0-50	-	$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$	-217.8

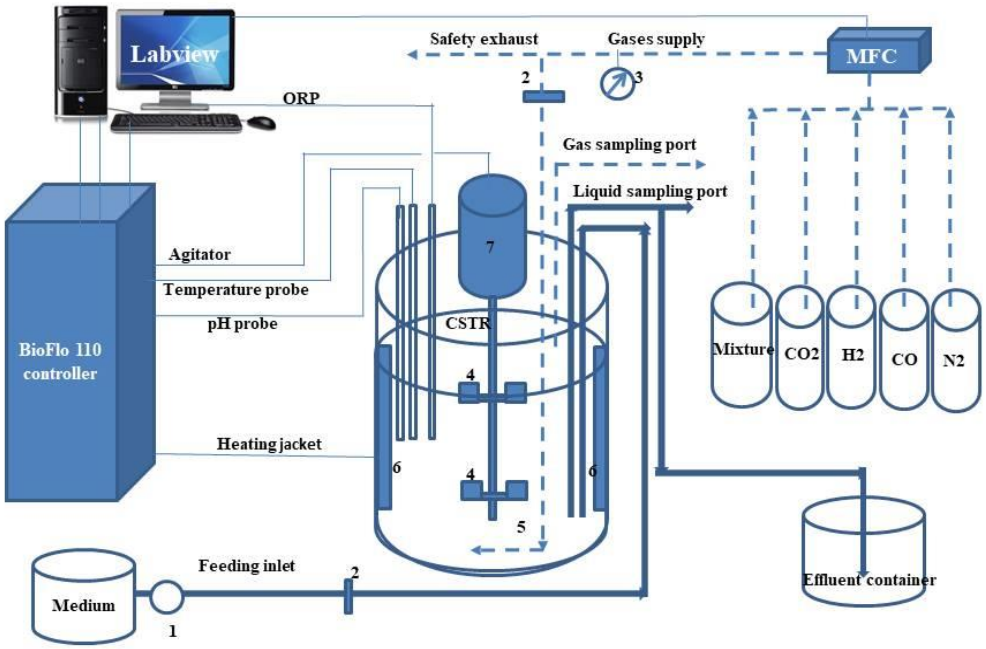

Fig. B1 The CSTR systems with 3-L BioFlo 110 agitated fermenter, gas supply, media supply, BioFlo 110 controller, and Labview program. (1) Peristaltic pump, (2) filter, (3) pressure gauge, (4) six-blade Ruston impeller, (5) microbubble sparger, (6) baffle, (7) agitator. Bold solid lines indicate liquid tubing. Dashed lines indicate gas tubing. Thin solid line indicates power cable.

The effect of dilution rate on syngas fermentation was studied by adjusting medium flow rate. Different dilution rates 0.015 (D_1), 0.013 (D_2), 0.008 (D_3), and 0.004 h^{-1} (D_4) corresponding to residence time of 67, 80, 125, 250 h, respectively, were tested in this experiment.

B2. Results and discussion

Syngas with different compositions were tested in this study. Cells mass concentration reached 0.9 g L^{-1}, ethanol concentration reached 12 g L^{-1}, and acetic acid remained between 1.0 and 2.0 g L^{-1} in the continuous fermentation fed with Syngas I containing 38% CO and 28% H_2 between 0 h and 565 h. However, cell mass concentration decreased and ethanol production increased (reached 14 g L^{-1}) with Syngas II which has higher H_2 composition (59%) and lower CO (31%) between 565 h and 684 h. The cells' activity diminished with Syngas III containing no CO between 684 h and 720 h. Cells started to recover when syngas IV containing 5% H_2 and 19% CO was fed between 720 h and 810 h. Acetic acid was maintained below 2 g L^{-1} throughout the fermentation with the novel pH controller at 4.8 with syngas flow rate (Fig. B2A). The gas-feeding rate depends on cells' activity and pH in the medium. The gas-feeding rate increased when pH was below 4.8, which was caused by an increase in acetic acid production associated with an increase in cell growth and activity. Therefore, cells were more active using Syngas I (Fig. B2 B). Besides, conversion efficiency of H_2 and CO, and ethanol to acetic acid ratio were at high levels when Syngas I was fed (Fig. B3). CO conversion efficiencies were between 70% and 80% with Syngas I and Syngas II. H_2 conversion efficiencies were between 60% and 75% with Syngas I and Syngas II. Ethanol/acetic acid molar ratio was between 6 and 16 during fermentation with Syngas I and Syngas II.

Four different dilution rates were tested with Syngas I mixture. Dilution rate was (0.015 h^{-1}) at beginning of fermentation resulting in above 11 g L^{-1} ethanol accumulation. Then, dilution rate was reduced to 0.013 h^{-1} to increase residence time. This allowed cell mass to increase with slight decrease in ethanol accumulation. The further reduction in dilution rate to 0.008 h^{-1} resulted in a decrease in cell mass and ethanol. This could be due to depletion of some nutrients in medium in the CSTR. At 370 h, 150 ml out of 2.4 L broth was removed from CSTR and 150 ml active culture was inoculated to CSTR. The dilution rate was reduced to 0.004 h^{-1}. Cell grew and cell mass concentration increased to similar level and ethanol concentration reached to around 13 g L^{-1} at a slower rate compared to those at 0.015 h^{-1} dilution rate (Fig. B2 A).

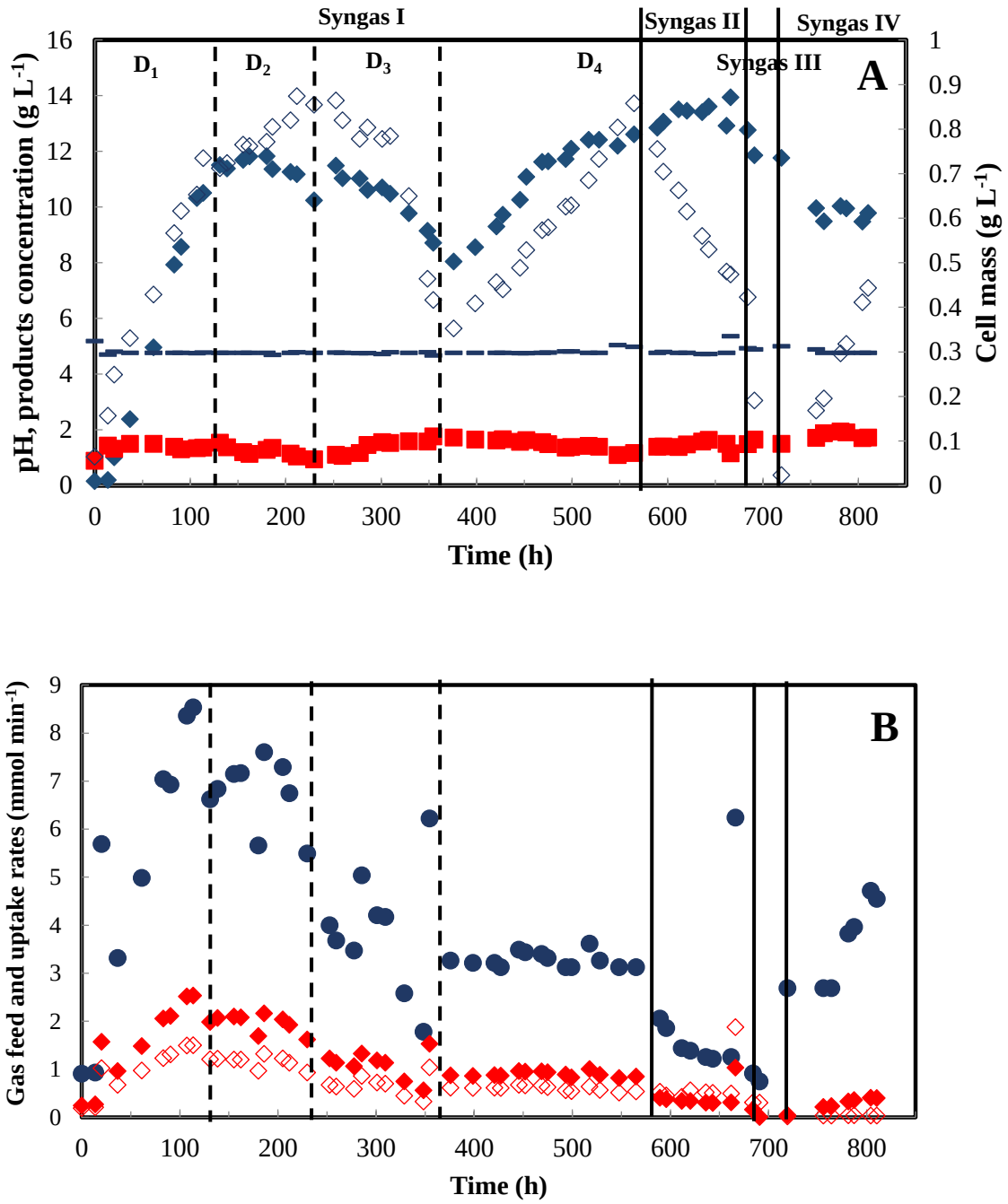

Fig. B2 (A) Profiles of pH (─) and concentrations of ethanol (♦), acetic acid (■), and cell mass (◊), and (B) profiles of syngas feeding rate (●), CO uptake rate (♦), and H_2 uptake rate (◊) during continuous syngas fermentation by *C. ragsdalei* using in 3-L CSTR.

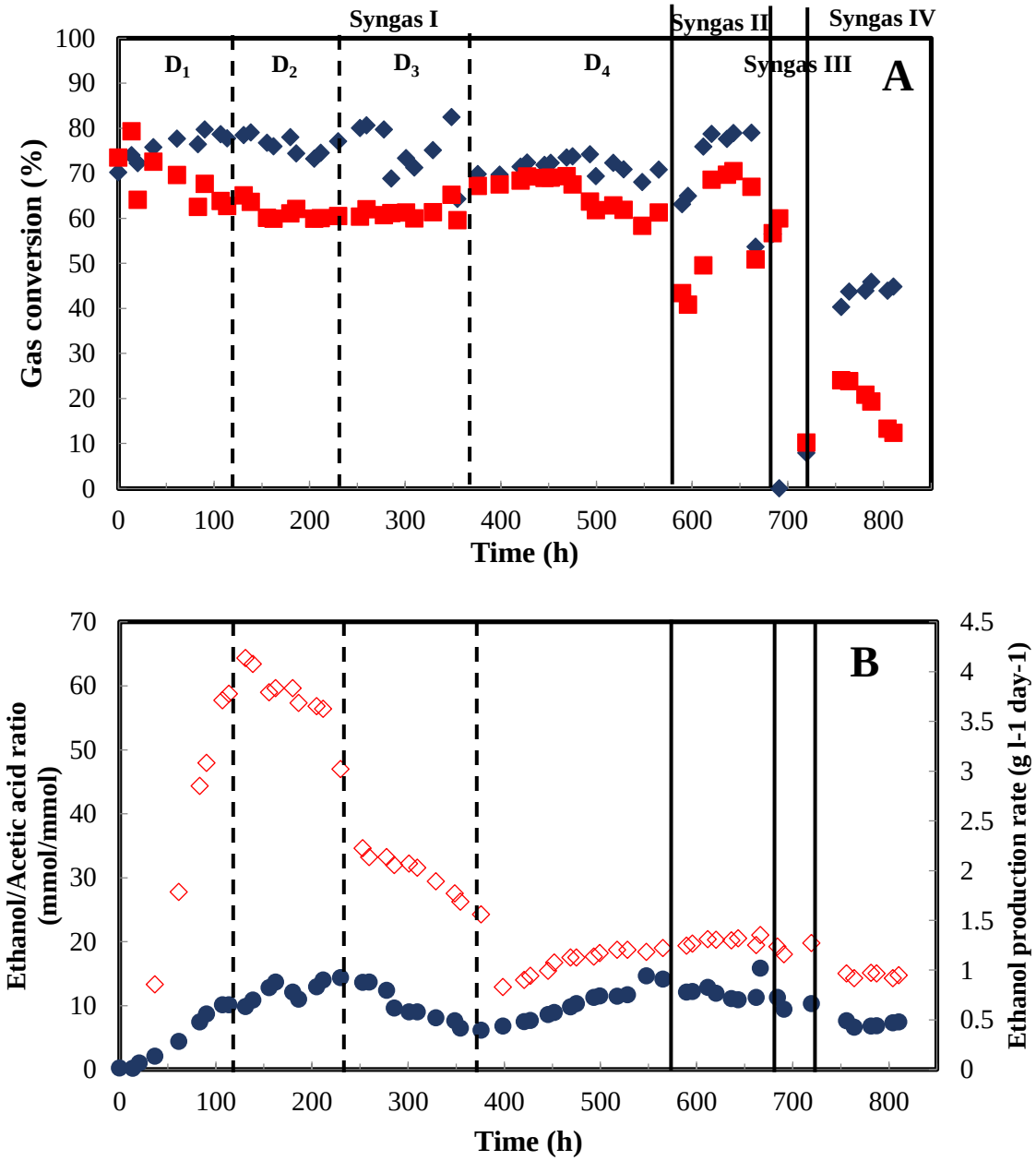

Fig. B3 (A) Conversions of CO (♦) and H_2 (■), and (B) profiles of ethanol/acetic acid ratio (●) and ethanol production rate (◊) during continuous syngas fermentation by *C. ragsdalei* in 3-L CSTR.

APPENDIX C

This appendix discussed continuous fermentation of *C. ragsdalei* in 3-L CSTR using standard YE medium without MES. The fermentation evaluated the effects of addition of fructose and yeast extract on cell growth, ethanol and acetate production, and consumption of H_2 and CO.

C1. Background

The microorganism, setup of CSTR and medium preparation were the same as in previous experiment in Appendix B. Syngas mixture I (Table B1) was used from 0 h to 1609 h. Syngas mixture V (Table B1) was used for Phase V from 1609 h to 1875 h. The continuous fermentation was performed based on various phases as in Table C1.

Table C1 Summary of phases with various parameters during continuous syngas fermentation using CSTR.

Phase	Time range (h)	Description
I	0 - 527	Used dilution rate of about 0.004 h^{-1}, Syngas I
II[a]	527 - 935	Fed medium with 2.5 g L^{-1} fructose, Syngas I
III	935 - 1400	Fed medium with 5 g L^{-1} fructose, Syngas I
IV	1400 - 1609	Fed medium with 1.2 g L^{-1} YE, Syngas I
V	1609 - 1875	Used gas mixture without H_2, Syngas V

[a] Low agitation (50-150 rpm), low dilution rate (0.0009-0.004 h^{-1}), low syngas flow (0.45-0.9 mmol min^{-1})

C2. Results and discussion

Profiles of cell mass, pH, product concentration, syngas feeding and uptake rate are shown in Fig. C1. Dilution rate of 0.004 h^{-1} was used from 0 h to 520 h (Phase I). Cell

mass reached to above 0.8 g L^{-1} at 340 h. Ethanol accumulated to 22 g L^{-1} at 432 h. However, both cell mass and ethanol concentrations declined when reaching to their highest values, which could be due to depletion of nutrients. During Phase II, the fed medium contained 2.5 g L^{-1} of fructose, which was enough to recover cell growth and ethanol production. However, after the increase in fructose concentration to 5.0 g L^{-1} at the beginning of Phase III, cell mass and ethanol increased to maximum levels of 0.5 and 12 g L^{-1} at around 1320 h. However, no more increase in cell mass and ethanol was observed at the end of Phase III from 1320 h to 1400 h. In Phase IV, a medium with increased yeast extract (YE) concentration (1.2 g L^{-1} compared to 0.5 g L^{-1} in standard medium) without fructose was fed in the CSTR, which enhanced ethanol production and cell mass to 17 and 0.9 g L^{-1}, respectively. In Phase V, gaseous substrate containing various ratios of CO and CO_2 without H_2 was used while the fed medium remained the same (with 1.2 g L^{-1} YE). This change resulted in improved cell mass from 0.9 g L^{-1} in previous Phase to 1.1 g L^{-1}. Besides, ethanol production further increased to above 20 g L^{-1}. The use of syngas without H_2 enabled decrease of acetic acid and no acetic acid concentration was detected after 1823 h.

This experiment suggests that lower dilution rate, higher fructose and yeast extract concentrations were favorable for accumulations of higher concentrations of ethanol and cell mass in continuous fermentation of *C. ragsdalei* using CSTR. Besides, syngas containing CO without H_2 improved both cell growth and ethanol production and could lead to no accumulation of acetic acid.

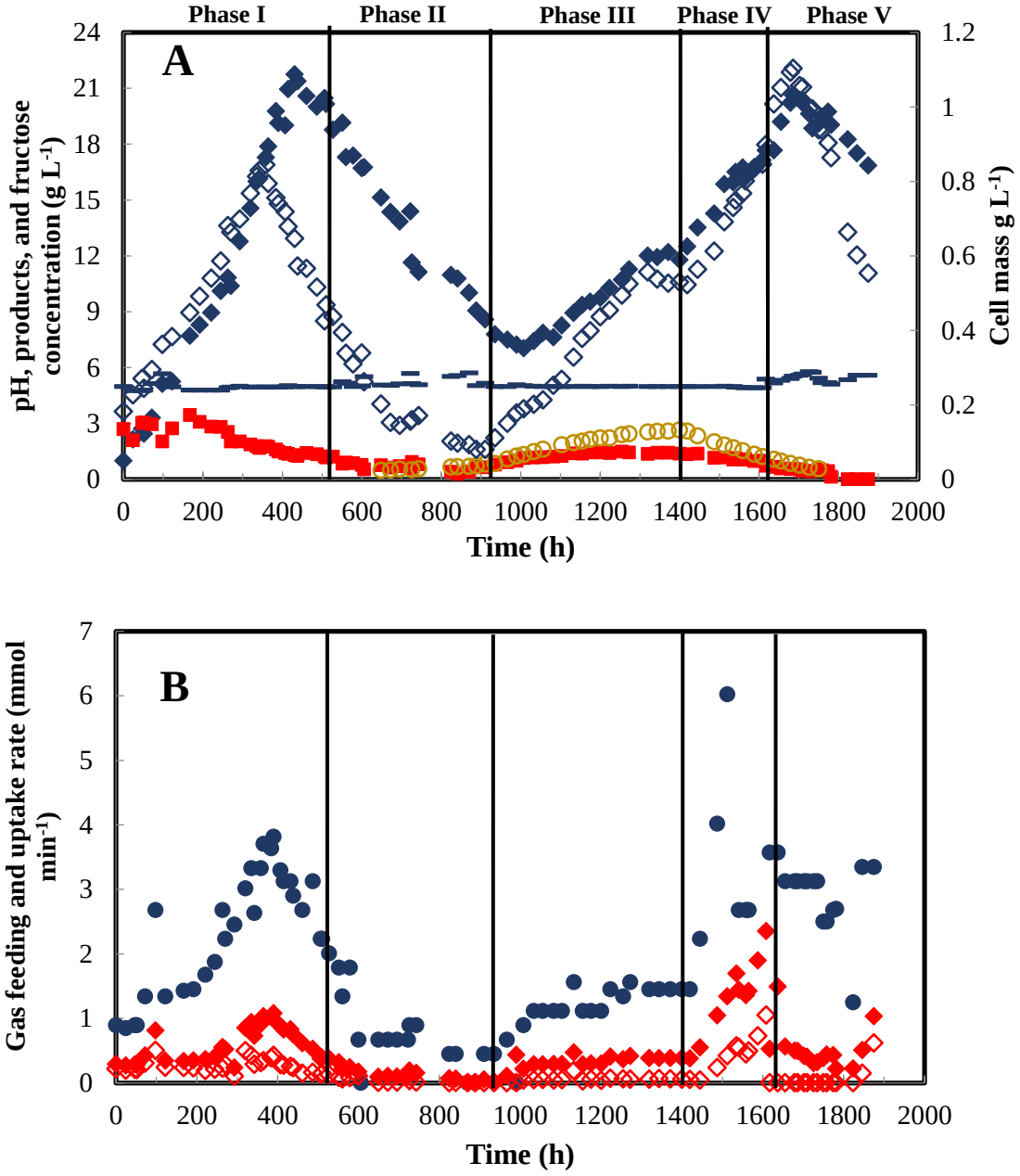

Fig. C1 (A) Profiles of pH (—), concentrations of ethanol (♦), acetic acid (■), cell mass (◊) and fructose (○), and (B) syngas feeding rate (●), profiles of CO uptake rate (♦) and H_2 uptake rate (◊) during continuous syngas fermentation by *C. ragsdalei* in 3-L CSTR using rich YE medium and its modifications (addition of 2.5 g L^{-1} of fructose between 527 h and 935 h, addition of 5 g L^{-1} of fructose between 935 h and 1400 h, addition of 1.2 g L^{-1} yeast extract between 1400 h and 1875 h).

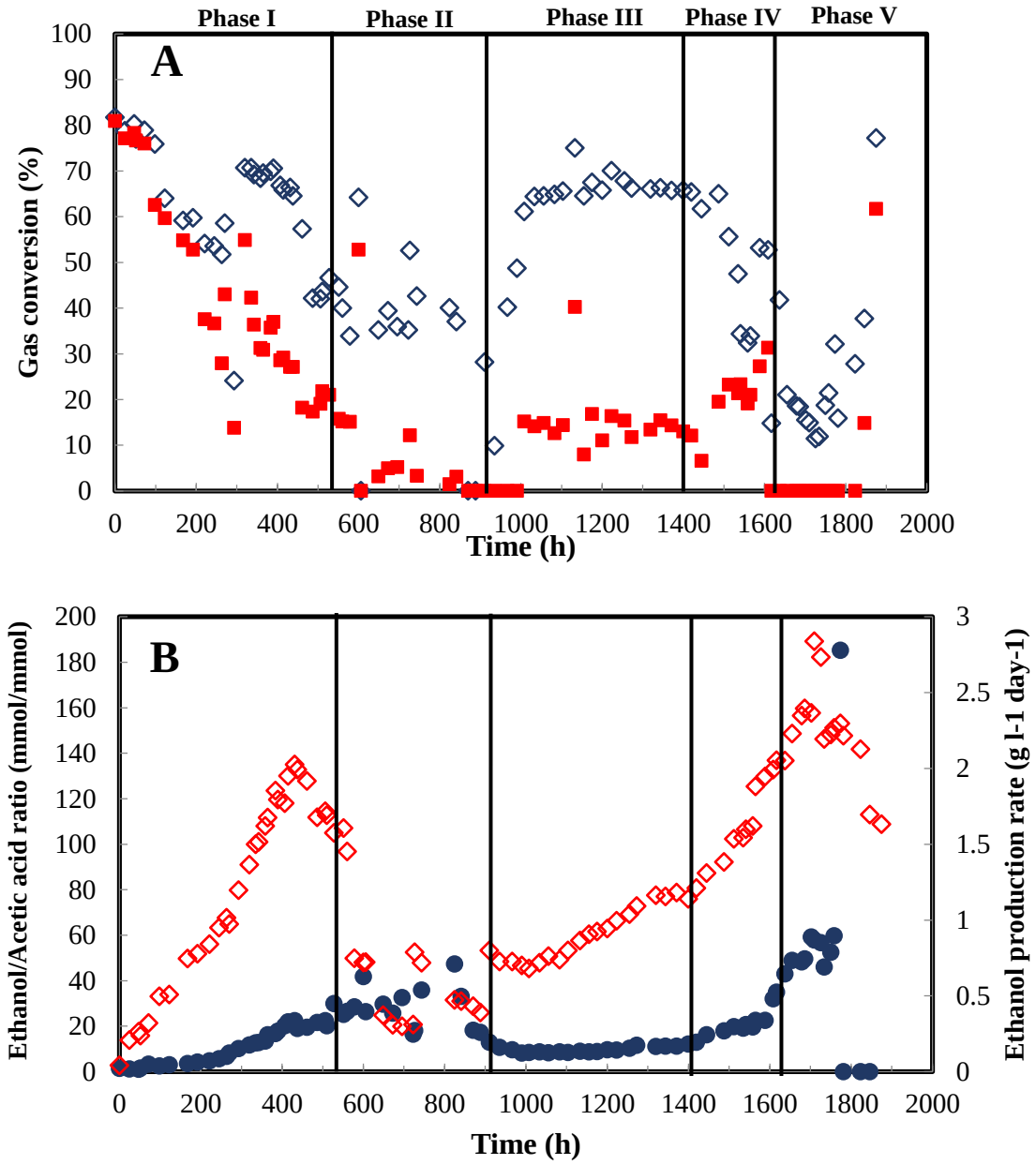

Fig. C2 (A) Gas conversion of CO (◊) and H_2 (■), and (B) profiles of ethanol/acetic acid ratio (●) and ethanol production rate (◊) during continuous syngas fermentation by *C. ragsdalei* using rich YE medium and its modifications (addition of 2.5 g L^{-1} of fructose between 527 h and 935 h, addition of 5 g L^{-1} of fructose between 935 h and 1400 h, addition of 1.2 g L^{-1} yeast extract between 1400 h and 1875 h).

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