PROCESS ENGINEERING AND SCALE-UP OF AUTOTROPHIC *CLOSTRIDIUM* STRAIN P11 SYNGAS FERMENTATION

By

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PROCESS ENGINEERING AND SCALE-UP OF

AUTOTROPHIC *CLOSTRIDIUM* STRAIN P11

SYNGAS FERMENTATION

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CHAPTER I: Introduction and Literature Review

Background

Energy related innovation cycles are long and challenging. To guarantee a sustainable energy future, it is necessary to continue developing traditional technologies and promote renewable energy processes. The volatility and insecurity in gasoline supply, global warming, depleting petroleum resources and national security have invigorated researchers to focus on alternative methods of ethanol production utilizing low-valued lignocellulosic substrate such as perennial grasses and crop residues. Perennial grasses offer several advantages such as a diversified cropping pattern, wide harvest window, low fixed cost, better crop management (by use of wide variety of feedstock) and ability to grow in marginal land (Thorsell et al., 2004). It has been estimated that the US possesses the necessary potential to produce 1 billion tons of biomass annually, the equivalent of displacing 30% of the current fossil fuel usage by the year 2030 (Perlack, 2005). Oklahoma, with its soil and climatic conditions, offers an ideal environment for the cultivation of perennial grasses such as switchgrass, which has been noted as a potential energy crop (Thorsell et al., 2004).

Ethanol production has gained worldwide attention and rapid expansion driven by rising gas prices, energy security concerns and sustainability and environmental repercussions such as global warming. Ethanol fermentation utilizing lignocellulosic substrates offers several advantages in terms of economic growth, security, lesser environmental impact and sustainability. Lignocellulose is a complex polymer composed of cellulose, hemicellulose, and lignin (Jeffries, 1988). Lignocellulosic biomass is comprised of agricultural and forestry residues (bagasse, corn stover, grasses, woody biomass), industrial waste and dedicated woody poplar. Switchgrass, along with other prairie grasses, is considered as a promising energy crop.

Fuels made from such grasses are “carbon negative” and will lead to long-term removal of 1.2 tons of carbon dioxide per acre per year (Tilman et al., 2006).

Improvements in processes and culture microorganisms will enhance the efficiency and economics of bioconversion of lignocellulose to ethanol. One of the foremost factors dictating the development of the bioconversion process is the compact and complex structure of lignocellulose. The structure consists of fibrous bundles of crystalline cellulose encased in a protective polymeric matrix of hemicellulose and lignin. On average, the lignocellulose fraction consists of 50% cellulose, 25% hemicellulose and 25% lignin (Ingram and Doran, 1995). The conversion of lignocellulosic substrate to ethanol is a two step process involving hydrolysis of cellulose and hemicelluloses to fermentable sugars by acid and/or enzymes and fermentation of sugars to ethanol. Prior to hydrolysis, the biomass must be pretreated to increase the porosity of the substrate, reduce cellulose crystallinity, solublize hemicellulose and disrupt the lignin structure, thereby making cellulose more accessible. Physico-chemical pretreatment procedures such as steam explosion, ammonia fiber expansion, hydrothermolysis, acid and alkali pretreatment, are popular, but results in degrading cellulose and hemicellulose to monomeric sugars such as glucose and xylose, which are further degraded to the microbial inhibitors hydroxymethylfurfural and furfural, respectively.

Acetogens

Acetogens are a group of bacteria that synthesize acetate from CO_2 or other one carbon precursors. Phylogenetically, acetogens consist of 19 genera and have been shown to inhabit diverse ecosystems. Several acetogens were found to have hydrogenase and been shown to grow on CO_2/H_2 as the sole source of carbon and energy and can also be referred to H_2-oxidizing autotrophs (Ljungdahl, 1986). Acetogens are obligate anaerobes that use the acetyl-CoA (Wood-

Ljungdahl) pathway as their predominant pathway for utilizing C_1 compounds such as CO_2, CO or formate (Diekert and Wohlfarth, 1994). The acetyl-CoA pathway is a mechanism for reductive synthesis of acetyl-CoA from CO_2. Formation of CO_2 serves as a terminal electron accepting, energy conserving mechanism, also resulting in the synthesis of cellular carbon. Acetyl-CoA has been identified as one of the key intermediates during the autotrophic fixation of CO_2 in acetogens.

Acetogens such as *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) convert hexoses into acetate (Pezacka and Wood, 1984). During this process, the carbohydrates are first converted to pyruvate and then to acetyl-CoA and CO_2. The CO_2 acts as a terminal electron acceptor during fermentation. The set of reactions are shown below:

$$\text{Glucose} + 2\text{ADP} + \text{Pi} \rightarrow 2CH_3COCOOH + 4e^- + 4H^+ + 2\text{ATP} + 2H_2O \quad [1]$$

$$2CH_3COCOOH + 2\text{CoASH} \rightarrow 2CH_3COSCoA + 2CO_2 + 4e^- + 4H^+ \quad [2]$$

$$\text{ATP} + CO_2 + \text{THF} + 6H^+ + 6e^- \rightarrow CH_3THF^1 + \text{ADP} + \text{Pi} + H_2O \quad [3]$$

$$2H^+ + 2e^- + CO_2 + CH_3THF + \text{CoASH} \rightarrow CH_3COCoA + \text{THF} + H_2O \quad [4]$$

$$3CH_3COSCoA + 3\text{Pi} + 3\text{ ADP} \rightarrow 3CH_3COOH + 3\text{ATP} + 3\text{CoASH} \quad [5]$$

Net Reaction: $\text{Glucose} + 4\text{ADP} + 4\text{Pi} \rightarrow 3CH_3COOH + 4\text{ATP} + 4H_2O$ [6]

Synthesis Gas and Fermentation

Biologically, ethanol can be produced either by direct fermentation of fermentable sugars in sugar cane or sweet sorghum, or by enzymatic and physico-chemical degradation of insoluble cellulosic biomass. One of the major disadvantages with the utilization of straw, wood and other

[1] Tetrahydrofolate

lignocellulosic biomass is the presence of a large proportion of non-fermentable components such as lignin. Gasification of cellulosic biomass can be one approach of overcoming this major hurdle in utilization of biomass. Gasification produces synthesis gas (syngas) in which CO and H_2 are the essential components for subsequent ethanol production. Gasification has been identified to be a significant process in converting low-value biomass, waste and residue substrate for subsequent fermentation (Ragauskas et al., 2006). Compared to catalytic conversion of syngas, fermentation offers several advantages such as higher specificity of biocatalysts, lower energy costs, greater resistance to catalyst poisoning and the requirement for a fixed CO:H_2 ratio (Bredwell and Worden, 1998; Klasson et al., 1992).

Since biological fermentation of syngas is irreversible in nature, it ensures complete conversion of the syngas components. Production of acetate by utilizing CO, CO_2 and H_2 can be summarized by the following reactions (Ljungdahl, 1986).

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \ (\Delta G° = -37.8 \text{ KJ/g mol } CH_3COOH) \quad [7]$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \ (\Delta G° = -18.6 \text{ KJ/g mol } CH_3COOH) \quad [8]$$

Stoichiometry for ethanol production is not available since an autotrophic ethanol producing microorganism has not been identified. Hence based on the reactions [7] and [8] above, the following stoichiometry for ethanol production has been proposed (Vega et al., 1989).

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \ (\Delta G° = -59.9 \text{ KJ/g mol } C_2H_5OH) \quad [9]$$

$$2CO_2 + 6H_2 \rightarrow C_2H_5OH + 3H_2O \ (\Delta G° = -23.2 \text{ KJ/g mol } C_2H_5OH) \quad [10]$$

Ethanol production has been shown to be non-growth related (Klasson et al., 1991). The acetyl-CoA pathway leading to acetate formation has a balanced ATP, and the subsequent reduction of acetate to ethanol results in the net consumption of ATP. This makes ATP not

available for bacterial growth. The autotrophic synthesis of acetate from CO_2 by heterotrophs enables certain bacteria to grow on CO_2 and H_2. The hydrogenase enzyme system in these bacteria generates necessary electrons ($H_2 \rightarrow 2H^+ + 2e^-$) to reduce CO_2 to methyltetrahydrofolate and form acetyl-CoA, which then serves as a substrate for anabolic processes.

Wood-Ljungdahl (Acetyl-CoA) Pathway

Acetogenic bacteria use the reductive Wood-Ljungdahl pathway for the formation of acetate as the primary end product by fermenting organic substrates or by respiring on H_2/CO_2 and/or CO (Figure 1.1) (Henstra et al., 2007). The principle governing the Wood-Ljungdahl pathway is simple, involving the synthesis of two-carbon compound (acetyl-CoA) using one-carbon precursors. The acetyl-CoA pathway can be divided into three main steps (Muller, 2003):

1. CO_2 undergoes sequential reduction to a methyl group by a series of reactions involving tetrahydrofolate dependent enzymes.

2. The methyl group is transferred to the cobalt center of a corrinoid iron sulfur protein (CFeSP).

3. Bifunctional CODH-ACS (carbon monoxide dehydrogenase-acetyl CoA synthase- a key enzyme of the pathway) condenses the methyl group, CoA and CO to form acetyl–CoA. Depending on the acetogenic species, the acetyl-CoA is then reduced to acetate, ethanol, and/or butanol.

Oxidation of H_2 to $2[H^+]$ or of CO with H_2O to CO_2 and $2[H^+]$ produces the reducing equivalents for the conversion of CO_2 to formate (HCOOH), methylene-tetrahydrofolate (CH-THF) to methenyl-tetrahydrofolate (CH_2-THF), CH_2-THF to methyl-tetrahydrofolate (CH_3-THF) and CO_2 to CO. In a non-cyclic pathway, acetyl-CoA synthase/CO dehydrogenase complex

(ACS-CODH) catalyses the formation of acetyl-CoA from bound methyl group, a bound CO group and coenzyme A (CoA). CO dehydrogenase has a bifunctional activity which reduces CO_2 to CO. Nicholls and Ferguson (2002) indicated that organisms growing on H_2 and CO_2 transfer electrons from H_2 via hydrogenase into the ubiquinone of the cyclic electron transport system, which is driven by reversed electron transfer through a rotenone-sensitive NADH.

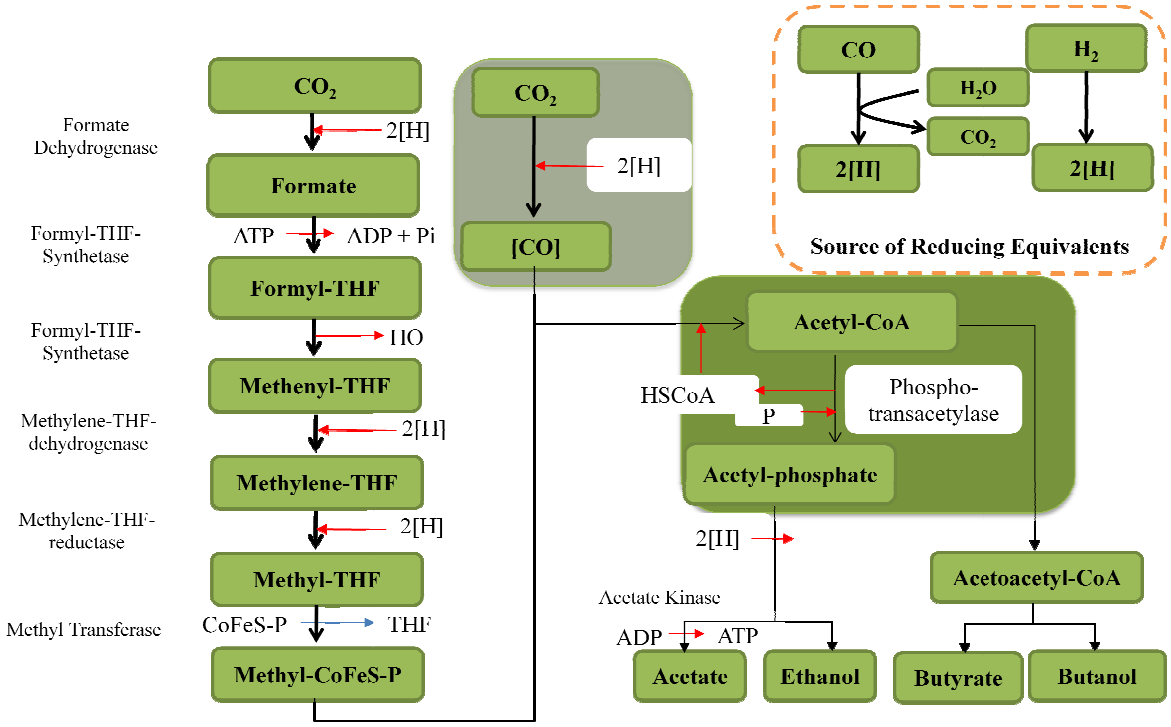

Figure 1.1. Schematic representation of the reductive acetyl-CoA pathway governing the synthesis gas fermentation in acetogenic bacteria. (Adapted from Muller, 2003).

Bioenergetics of Acetogenic Bacteria

Overall theoretical stoichiometries and standard free energy changes during autotrophic growth on H_2 and CO as shown in equations 1 and 2 indicate that both forms of autotrophic acetogenesis require eight reducing equivalents for acetate synthesis (Daniel *et al.*, 1990). The following facts are known about the bioenergetics of acetogens:

- Involvement of electron transport phosphorylation in energy conservation during growth (Ljungdahl, 1986).
- Presence of metalloenzymes and electron carriers (Ljungdahl, 1986).
- Presence of ATPase and other catalysts as components of membrane associated electron transport system.
- Sodium is an important component in energy conservation (Gottwald et al., 1975).

Various acetogens showed comparatively higher growth yield on CO than H_2, and the acetate-to-biomass ratio was lower on CO compared to H_2 (Daniel et al., 1990). Currently, the energy generation mechanism of acetogens is speculated, ascribed to the following observations (Andreesen and Ljungdahl, 1973).

- Increased ATP synthesis per CO derived electron pair.
- Energy was required during the formation of CO from CO_2 during H_2 dependent acetogenesis. However, during CO dependent acetogenesis, no net yield of ATP was observed during substrate level phosphorylation, even though ATP was required for the formation of formyltetrahydrofolate from CO.
- Substrate level phosphorylation is the primary route for energy generation for acetogens growing on sugars.
- Growth on H_2-CO_2 showed no net substrate phosphorylation. ATP generated during the formation of acetyl phosphate was required for the formation of formyltetrahydrofolate from formate and tetrahydrofolate.

The above deliberations indicate that an alternative mechanism involving cytoplasmic-membrane-associated chemiosmosis and the presence of membrane associated electron transfer proteins such as cytochromes and menaquinones may exist in acetogens (Gottwald et al., 1975). Chemiosmotic energy transduction involving H^+-ATPases catalyzing pH dependent ATP synthesis was observed in acetogens (Ivey and Ljungdahl, 1986). It was shown earlier that enzymes involved in acetate synthesis were soluble and were not associated with the cytoplsamic membrane (Ljungdahl, 1986). But in a later study using *Moorella thermoautotrophicum* (formerly *C. thermoautotrophicum*) 701/5, it was shown that the enzymes CODH and methyltetrahydrofolate reductase were associated with the cytoplasmic membranes and were involved in the CO dependent reduction of membrane components including two b-type cytochromes (Hugenholtz and Ljungdahl, 1989).

Energy Conservation

The energy metabolism for acetogenic bacteria is obscure. As shown in Figure 1.1, during the fermentation of H_2 and CO_2 to acetate, one ATP is hydrolyzed in the formyltetrahydrofolate synthetase reaction, while one ATP is generated during the final step of acetogenesis, resulting in no net ATP generation during substrate level phosphorylation (Ljungdahl, 1986). This means that an alternative mechanism for energy generation should exist. Discovery of a Na^+/H^+ antiporter (exchanger) in *M. thermoacetica* along with following two methylene-group reductions, indicated that a Na^+ gradient across cytoplasm was also involved in the energy conservation mechanism of the acetogens (Gottschalk, 1989).

$$\text{Methylene-}H_4\text{methanopterin} + H_2 \rightarrow \text{methyl-}H_4\text{pterin} \ (\Delta G^\circ = -20 \text{ KJ/reaction}) \quad [11]$$

$$\text{Methylene-}H_4F + H_2 \rightarrow \text{methyl-}H_4F \ (\Delta G^\circ = -57.3 \text{ KJ/reaction}) \quad [12]$$

Based on the energy conserving mechanism, the acetogens can be divided into two groups (Figure 1.2).

- Na^+ dependent organisms, such as *Acetobacterium woodii*, that lack cytochromes, but have membrane bound corrinoids and couple the acetyl-CoA pathway to primary and electrogenic translocation of Na+ (Hugenholtz et al., 1987; Ivey and Ljungdahl, 1986; Yang and Drake, 1990) (Figure 1.2A).

- H^+ dependent organisms, such as *M. thermoacetica*, that contain cytochromes and a membrane bound H^+ motive electron transport chain (Heise et al., 1989; Yang and Drake, 1990) (Figure 1.2B).

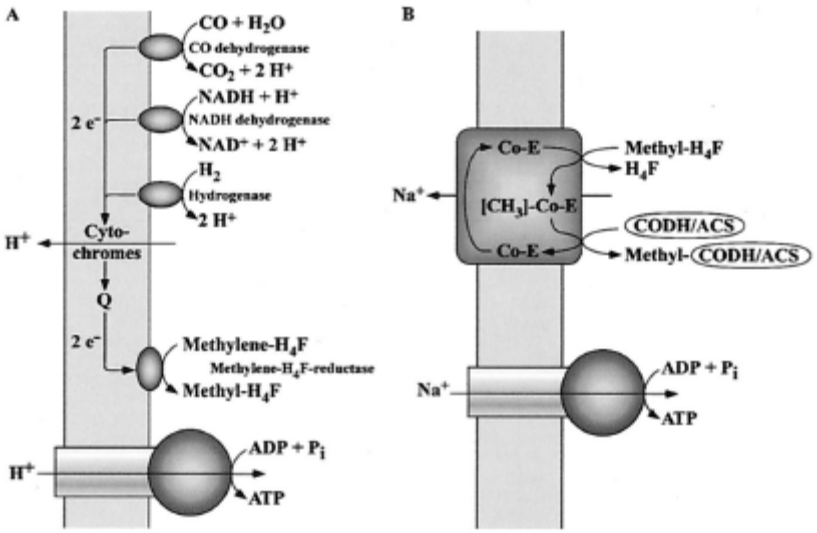

Figure 1.2. Hypothetical model of the energy conservation in acetogens. (A) H+ organisms; (B) Na+ organisms (Adapted from Muller, 2003).

Das et al. (1989) identified several membrane integral electron carriers in the H^+ dependent acetogens were involved in the electron transport processes such as, menaquinone MK-7 (Eo' = -74 mV), two b-type cytochromes (cytochrome b_{559}, Eo' = -215 mV; cytochrome b_{554}, Eo' = -57 mV) and a flavoprotein co-purified with cytochrome b_{559}. Figure 1.2 shows a

hypothetical model for energy conservation in Na^+ and H^+ dependent acetogens. Thauer et al., (1977) suggested that methyl-H_4F reductase catalyzes the last step of the membrane bound electron transport chain from electron donors such as CODH, hydrogenase and NADH hydrogenase. This electron transport mechanism can generate the necessary transmembrane electrochemical potential ($\Delta\mu_{H^+}$) for ATP synthesis; however, sufficient evidence has not been presented (Hugenholtz and Ljungdahl, 1989).

Acetogens use CO as an electron sink and convert it to acetate by means of the complicated acetyl-CoA pathway (Ljungdahl, 1986). H_2 is an obligatory intermediate formed during the fermentation and is recycled for the generation of ATP. The H_2-cycling system in *A. woodii* consists of cytoplasmic and periplasmic hydrogenases, membrane electron transport and H^- translocating ATPase. When sugars such as fructose and glucose are fermented by acetogens, the reducing equivalents are converted to H_2 inside the cell by cytoplasmic dehydrogenase. The molecular H_2 diffuses outside the membrane and is oxidized by periplasmic hydrogenase. The electrons generated during the oxidation are used by the electron transport system, while the protons are used by the H^+-translocating ATPase to generate ATP. However, acetogens growing on CO_2/H_2 as the substrate possibly generate ATP by the mechanism depicted in Figure 1.3 (Ljungdahl, 1986).

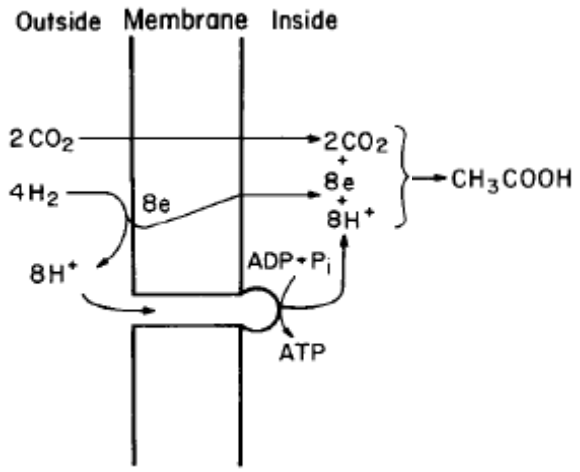

Figure 1.3. A simple schematic for electron transport and H^+ translocating ATPase systems in acetogens growing on H_2/CO_2. (Adapted from Ljungdahl, L.G., 1986).

Enzyme System in Acetogens

The different enzymes involved in acetogenesis (Figure 1.1) have been purified and the properties of some of the key enzymes are elucidated below (Ljungdahl, 1986; Ragsdale, 1991; Ragsdale, 2008; Ragsdale and Pierce, 2008).

Step1: H_4Folate Dependent Synthesis of CH_3-H_4Folate

Formate Dehydrogenase (FDH)

Presence of FDH is unique to acetogens. In general, FDH catalyzes the reversible oxidation of formate to CO_2, and in such organisms, ferredoxin is used as the electron acceptor. Conversely, FDH in acetogenic bacteria catalyzes the first step of the two-electron reduction of CO_2 to formate. Tungsten- and selenocysteine-containing FDH catalyze the thermodynamically unfavorable reduction of CO_2 to formate (E_o = -420 mV). The electrons are provided by

NADPH with a half cell potential of -340 mV. With CO as the substrate, CODH first oxidizes CO to CO_2 followed by the reduction of CO_2 to formate facilitated by FDH.

10-Formyl-H_4Folate Synthetase

This enzyme facilitates the activation of formate from the previous step via an ATP dependent condensation reaction with H_4folate. The enzyme is oxygen stable and is monofunctional in nature.

5,10-Methenyl-H_4Folate Cyclohydrolase and 5,10-Methylene-H_4Folate Dehydrogenase

The cyclohydrolases and dehydrogenases are present as part of a bifunctional protein in *M. thermoacetica* while they are monofunctional in other acetogens such as *A. woodii* and *Clostridium formicoaceticum*. In general, cyclohydrolases favor cyclization and catalyze the formation of 5,10-methenyl-H_4folate. Subsequently, 5,10-methylene-H_4folate dehydrogenase catalyzes the NADP(H) dependent reduction of 5,10-methenyl-H_4folate to 5,10-methylene-H_4folate. It has been shown that the bifunctional nature of the enzyme protects the labile methenyl-H_4folate form hydrolysis, and channels it to the dehydrogenase active site, where it is converted to the more stable methylene-H_4folate form.

5,10-Methylene-H_4Folate Reductase

5,10-methylene-H_4folate reductase catalyzes the final step reduction of 5,10-methylene-H_4folate to 5-methyl-H_4folate in the methyl branch of the acetyl-CoA pathway. The enzyme is oxygen sensitive, and in contrast to other reductases, the acetogenic reductase contains a [4Fe-4S] cluster and uses reduced ferredoxin as an electron acceptor.

Step2: Methylation of C/Fe-SP by CFeSP Methyltransferase (MeTr)

MeTr catalyzes the second step in the acetyl-CoA pathway involving the transfer of the methyl group of methyl-H_4folate to the cobalt center of corrinoid iron-sulfur protein (CFeSP) by displacing the tertiary amine. The bond strength of CH_3-N is much higher compared to the bond strength of the product CH_3-Co that is formed. Further, the 2^+ and 3^+ oxidation states of cobalt are inactive, and the subsequent reduction of Co^{2+} to Co^{1+} state requires a reduction potential of -610 mV. The above conditions exert a difficult reduction reaction, and the acetogen such as *M. thermoacetica* circumvent the situation by protonating the pterin ring thereby lowering the activation barrier for the nucleophilic displacement of the methyl group by Co^{1+} nucelophile.

Step3: Assembly of Acetyl-CoA by CODH

Corrinoid iron sulfur protein (CFeSP) accepts the methyl group from methyl-H4folate, and in presence of with CO, CoA and protein fraction containing CODH, forms acetyl-CoA. This reaction is catalyzed by the bifunctional CODH/ACS enzyme. The coordination state of Co(II) is essential in facilitating the reduction potential favorable for the reaction to proceed. CODH is a key enzyme in the acetyl-CoA pathway that is involved in the reduction of a second molecule of CO_2 to CO, which forms the carbonyl group of acetyl-CoA. The CODH reaction follows a ping-pong mechanism, where in CO reduces CODH in the “ping” step, and the reduced enzyme transfers electrons to an external mediator, such as ferredoxin, in the “pong” step. The reaction mechanism involves a hydrophobic channel for CO and a hydrophilic water channel, which connects the C-cluster with the substrates. The reaction steps shown are analogous to the water-gas shift reaction involving metal-bound carbonyl groups and hydroxide ions and metal-carboxylate intermediates. The key difference between enzyme-catalyzed reaction and water-gas shift reaction is that the non-enzymatic reaction produces H_2 while the CODH catalyzed reaction

produces electrons and protons. The evolution of electrons and protons during the CODH reaction is much more rapid than H_2 evolution.

CODH has been shown to play a fundamental role in the synthesis of acetate from CO. In acetogens, CODH appears to control the flow of electrons in the tetrahydrofolate-corrinoid pathway involved during the reduction of one carbon compounds such as CO and CO_2 to acetate (Diekert & Ritter, 1982; Ljungdahl & Wood, 1982). Oxidation of CO by CODH is given by following reaction

$$CO + H_2O \Leftrightarrow CO_2 + 2H^+ + 2e- \quad [13]$$

CODH generates the required electrons for growth on CO (Kerby and Zeikus, 1983). Ragsdale et al. (1983) purified CODH from *Acetobacterium woodii* and observed that it contained Ni, Z, and Fe-S clusters and have presented evidence that a Ni-C complex is formed during the reaction of CODH with CO or CO_2. CODH plays a functional role in the formation of C_1 intermediate with CO or pyruvate as the substrate and is also required for the reduction of cobalt in the corrinoid protein.

Redox active Ni has been found in several of the acetogenic *Clostridium* species and has been found necessary for the synthesis of CODH (Diekert and Ritter, 1982; Ragsdale et al., 1983). Ni was also observed to be necessary for acetate formation from CO_2. In the absence of Ni, hydrogen formed using protons was used as the electron sink rather than acetate formed from CO_2. Presence of Ni also affected the specific activity of CODH. In *Clostridium* species CODH demonstrated properties similar to corrinoid enzymes (Diekert and Thauer, 1978). When added to the growth medium, Ni was found to stimulate the formation of CODH in various *Clostridium* species (Ragsdale et al., 1983). Drake (1982) observed that oxidation of CODH resulted in inactivation of the enzyme and the dissociation of Ni from the enzyme.

Conversion of acetyl-CoA to acetate by phosphotransacetylase

The conversion of acetyl-CoA from the previous step to acetate is facilitated by phosphotransacetylase and acetate kinase. The reaction is associated with the generation of ATP. Acetyl-CoA formed in the previous step serves as the source of macromolecules and ATP for acetogens. The cleavage of high energy bonds in acetyl-CoA is coupled to ATP synthesis. The activity of phosphotransacetylase was observed to be substantially stimulated by divalent Mn^{2+}. The activity was neither effected by monovalent cations NH_4^+, Na^+, K^+, and Li^+ nor by divalent cations Mg^{2+}, Ca^{2+} and Fe^{2+} (Drake et al., 1981). Phosphotransacetylase demonstrated maximum activity at 75°C and at pH 4.6 (Drake et al., 1981). Acetate kinase subsequently converts the acetylphosphate to acetate and ATP.

Compounds Influencing Acetyl-CoA Pathway

CO oxidation was found to be inhibited by carbon tetrachloride, propyl iodide and arsenate. Arsenate inhibits ATP synthesis, which results in the inhibition of acetate production from CO_2 (Ljungdahl and Wood, 1969).

Synthesis of acetate from CO_2 was shown to be dependent on pyruvate as pyruvate serves as a reducing agent and also is the source of the carboxyl group in acetate (Schulman et al., 1973). Addition of pyruvate was found to increase the rate of CO oxidation. Decrease in the CO concentration decreased the specific rate of CO oxidation, but the lag phase was prolonged at high CO concentration. Presence of arsenate and alkyl halides such as carbon tetrachloride and propyl iodide inhibited CO oxidation (Diekert and Thauer, 1978). Pyruvate was found to stimulate CO oxidation 10-fold , stimulate reductive transcarboxylation of CO_2 to acetate, and serve as an electron source for the reduction of CO_2 to acetate (Schulman et al., 1973).

Addition of reducing agents favors solvent formation by directing the electron flow towards NADH formation; thereby, promoting ethanol formation (Rao and Mutharasan, 1988). In *C. ljungdahlii* fermentation, addition of benzyl viologen at 30 ppm levels produced 3.7 mM ethanol compared to basal medium with 2.5% cysteine HCl which produced 1.40 mM ethanol (Klasson et al., 1991).

Acetogens also show different responses to metabolic inhibitors. Na supplementation was necessary when the growth of *Acetobacterium kivui* and *A. woodii* was dependent on H_2, but not when growing on glucose (Terracciano et al., 1987). However, *M. thermoacetica* showed no dependence on Na supplementation, but was found to be sensitive to metal ionophores and the Na^+/H^+ antiporter inhibitor amiloride (Yang and Drake, 1990).

Economics

In spite of growing consensus to increase the consumption of green fuels, biofuels account for only 3% of the total energy consumed worldwide in 2008 (EIA, 2009). Aided by the Energy Independence and Investment Act, biofuel consumption in the US is expected to increase from 3.58 x 10^7 L d^{-1} in 2006 to 2.27 x 10^8 L d^{-1} by the year 2030 (EIA, 2009). In 2007, the US consumption of ethanol was only 4.5% of the daily gasoline consumption of 1.48 x 10^9 L (Source: Ethanol Producer Magazine, 2007). The key to ensuring a sustainable and secure energy supply is the development of next generation production technologies. To meet these objectives several obstacles must be overcome:

- Identifying and developing new variants of feedstock.
- Optimizing and perfecting new production technologies for commercial ethanol and other hydrocarbon fuel production technologies.

- Developing infrastructure to utilize and consume green fuels.

It is projected that the long term US economic growth will average about 2.5% per year until 2030, and that crude oil prices will peak at \$1.88 L^{-1} in 2035 (nominal dollars) (EIA, 2009). However, non-fossil fuel sources will contribute only 21% towards meeting energy demand with room for development and incorporation of different renewable energy technologies. Two hundred biorefineries produced 10.6 billion gallons of renewable ethanol, supported 400,000 jobs across different sectors and added \$53.3 billion to the nation's Gross Domestic Product (RFA, 2010). Current ethanol industries in the US utilize corn as the primary feed stock. Corn ethanol accounts for a 20% reduction in Green House Gas (GHG) emissions, and has been the center of debate concerning net energy value and its conflict with global food supply. The Renewable Fuel Standard (RFS2) that is part of the Energy Independence and Security Act of 2007 (EISA) mandates a production volume of 136.3×10^9 L yr^{-1} of renewable fuel by the year 2022 with advanced biofuels contributing 79.5×10^9 L yr^{-1} and corn ethanol capped at 56.8×10^9 L yr^{-1} (EPA, 2010). Currently, 28 advanced biofuel plants are under development and construction with a combined production capacity of 6.44×10^8 L yr^{-1}.

Developing commercially viable processes will be key in evaluating and setting meaningful energy policy in this important arena. One of the major considerations for the success of an energy crop is its efficiency in displacing fossil fuels. Scientific findings have suggested a net energy and carbon savings with forage energy crops such as switchgrass (McLaughlin and Walsh, 1998). To compete in the current biofuel market, the energy crop needs to be competitive both as a fuel and as a crop. Other factors such as low management intensity, positive effect on soil quality and a stable income for farmers will be other factors determining the success of the energy crop. Graham (1994) used the national crop production

statistics to estimate that approximately 131 x 10^{10} m^2 of land in the US can be used for growing herbaceous crops such as switchgrass.

Research Initiative

Utilization of lignocellulosic feedstock for the production of renewable energy offers a secure and sustainable source of energy. Lignocellulosic feedstock grows abundantly and is largely available as agricultural and forestry residues. Lignocellulose to liquid fuel conversion processes are currently expensive and will become competitive at a crude oil price above $100/bbl (Lange, 2007). Conversion processes are still immature and require large investments. Process cost reduction will be ensured by continuous improvement in conversion technologies and improved and efficient infrastructure for biomass cultivation and supply. These developments will make the process simpler and less energy intensive, thereby lowering the overall production cost.

A biomass gasification and fermentation process has been identified as a potential process for meeting the biofuel production volume demand for the nation, and several initiatives are currently focused on developing the technology (DOE, 2006). Several challenges need to be addressed to ensure that the technology is scaled from the current pilot-scale to commercial production scale. Some of the broad challenges will include:

- Identifying suitable enzymatic and microbial agents capable of biochemical conversion with increased efficiencies.
- Understanding the protein structure-function relationships for the development of improved microbial strains (DOE, 2006).
- Altering the process conditions and improved fermentor design for higher product yields.

- Regulation of the fermentation pathway by altering environmental conditions such as pH, temperature and medium composition to achieve higher production efficiencies and improved product yields.

- Improvement in mass transfer coefficients for CO and H_2, which have aqueous solubilities of 77% and 68% of O_2 at ambient conditions (Kapic et al., 2006).

Choice of a suitable fermentor design is critical to the development of bio-based fermentation processes and the potential to replace existing chemical-based commodity processes. The choice of fermentor will also determine the development of a commercial fermentation process and the corresponding capital investment.

Fermentors achieving high microbial cell densities and high mass transfer rates are desirable in achieving high syngas conversion rates (Klasson et al., 1991). Mass transfer rates of syngas components have been identified as one of the critical parameters in determining the size of reactor and process capacity. Mass transfer of syngas components during fermentor operation involves three heterogeneous phases: bulk gas phase, the liquid fermentation medium; and the solid microbial cells. For an efficient syngas conversion, the gaseous substrate must be transported across the gas-liquid interface and diffuse into the microbial cells. Due to low solubilities of CO and H_2 in aqueous media, the gas-liquid interface offers the greatest mass transfer resistance and is observed to be the rate limiting step during syngas fermentation (Klasson et al., 1993). According to Henry's law, the solubility of a gas is directly proportional to its partial pressure and indirectly proportional to temperature. Syngas solubility in aqueous media can be increased by improving the volumetric mass transfer coefficient (K_La) which is an important parameter in defining the gas conversion efficiencies (Kapic et al., 2006). Several different strategies have been shown to increase K_La including increasing agitator power per

volume ratio, increasing the superficial gas velocity, increasing the fermentor impeller speed, increasing the partial pressure of gaseous substrate, or by micro-bubble dispersions. Different reactor designs have also been investigated to increase mass transfer rates. In a study comparing a continuous stirred tank reactor (CSTR), packed-bubble column reactor (PCBR) and trickle bed reactor (TBR) utilizing a triculture of *Rhodospirillum rubrum*, *Mathanobacterium formicicum*, and *Methanosarcina barkeri*, it was observed that CO conversion rates at a gas loading rate of 0.4 h^{-1} were 90%, 80% and 100%, respectively (Klasson et al., 1992). The authors attributed the complete conversion of CO in the TBR to operation close to a plug-flow behavior. However, CSTRs are the most common fermentors due to ease of operation and scalability. Scaling a PCBR is a major challenge due to plugging of microbial cells between the packing material and controlling the process parameters since the medium is not-well mixed when scaled-up (Bredwell et al., 1999; Munasinghe and Khanal, 2010). In a TBR, syngas can be purged either in a co-current or counter-current direction to the flow of fermentation media. TBRs offer advantages over other reactors in terms of lower power consumption due to the absence of mechanical agitation (Bredwell et al., 1999) and high mass transfer rates (Klasson et al., 1992). Some of the disadvantages include poor gas-liquid distribution over the packed bed (Maiti and Nigam, 2007) and reduced reactor performance due to excessive biomass growth over the packed bed (Weber and Hartmans, 1996).

Strain selection, enhancement and adaptation and process optimization are important steps in bioprocess development and commercialization. These steps are generally conducted in the laboratory using shake flasks or serum bottle fermentations. Aided by statistically designed experiments, several process and test variables can be screened and evaluated simultaneously, including the interaction effect between the tested variables. This strategy further helps in

reducing material costs and when done precisely, can help in accurately translating the parameters during scale-up (Garcia-Ochoa and Gomez, 2009).

Once the optimal process variables have been identified, the bioprocess can be scaled up in bench- and pilot-scale fermentors that are known to imitate the hydrodynamic and operational conditions closely (Garcia-Ochoa and Gomez, 2009). Garcia-Ochoa and Gomez (2009) also recommend a typical scale-up ratio of 1:10, but recommend that using conservatively lower scale-up ratios would address unexpected fermentor behavior. Four different approaches have been recognized to be helpful during scale-up.

a) Fundamental Method: Using mathematical models to define the influence of operational parameter and geometrical design on the flow pattern in the fermentor. These methods are complicated and frequently require simplifications (Nedeltchev et al., 1999). Recent advances include using computational fluid dynamics to study hydrodynamic characteristics during scale-up (Dhanasekharan et al., 2005).

b) Semi-fundamental Method: Using simplified equations and modeling techniques to practically approximate fermentor operation. The equations are still substantially complex (Garcia-Ochoa and Gomez, 2009).

c) Dimensional Analysis: In this approach, dimensionless groups are kept constant during scale-up (Garcia-Ochoa and Gomez, 2009).

d) Rule of Thumb: Scale-up criterion and their corresponding percentages used commonly in a fermentation industry include: specific power input, P/V, (30%); K_La (30%); and constant impeller tip speed (20%) (Margaritis and Zajic, 1978). This method usually results in different parameters when scaled up since it is impossible to maintain the same ratios of the

criteria in relation to each other (Garcia-Ochoa and Gomez, 2009). Shukla et al. (2001) identified maintaining power input per unit volume or volumetric mass transfer coefficient constant as the best approach during scale-up. The 'rule of thumb' approach is simple but care should be taken to identify the limiting range of process parameters.

In general, a combination of the above methods will prove beneficial in approximating the optimal process conditions. Similar to aerobic fermentation, K_La for syngas fermentation can be considered a significant factor. During scale-up, optimal performance can be realized by keeping K_La constant and determining the other operational parameters (Liu et al., 2006).

The details of the mechanism and strategy involved with selective product formation during anaerobic fermentation are obscure. One determining factor regulating the process is the balance between energy conservation and entropy generation (Thauer et al., 1977). This translates to the mechanism where in metabolic fluxes are favored or regulated depending on the ATP requirement. The mechanism was confirmed by limiting nitrogen availability during *Clostridium acetobutylicum* utilization of glucose in batch and continuous fermentations. It was observed that the fermentation pH indirectly affected the bacterial growth rate and uptake of nitrogen thereby influencing the molar growth rate on ATP (Roos et al., 1985). In general, nutrient limitation was observed to result in a decrease of cell yield in batch, continuous and immobilized cell fermentations (Karkare et al., 1986; Meyer and Papoutsakis, 1989; Monot et al., 1984). In batch fermentation, limiting mineral nutrients resulted in a decrease of cell yield and the specific growth rate reduced to zero, while in continuous fermentation with phosphate limitation, cell yield, product concentration and ATP demand decreased (Hill et al., 1993). Under iron limitation (8 μM) with methyl viologen added at 1 mM, a metabolic shift favoring butanol production was observed in *C. acetobutylicum* in batch fermentation (Peguin and

Soucaille, 1995). In fermentation with *Clostridium ljungdahlii*, limiting calcium panthothenate and cobalt chloride resulted in the decrease of the acetyl-CoA cycle, which caused an increase in the NADP(H) to NAD(P) ratio that favored ethanol production (Gaddy et al., 2007).

Several process parameters such as pH, temperature, buffer addition and agitation have been shown to affect the growth and metabolic activity of acetogens. During acetogenic fermentations two distinct phases are displayed: 1) an acidogenic phase corresponding with production of fatty acids such as acetate and butyrate and a decrease of medium pH to below pH 5.0, and 2) a solventogenic phase that occurs as undissociated acetate and butyrate ion accumulated in the fermentation medium during the first phase gradually pass through the cell membrane and are converted to ethanol and butanol, respectively (Huang et al., 1986; Klasson et al., 1991; Kundiyana et al., 2010). The concentration of undissociated acids in the medium has been found to be one of the critical factors in the initiation of solventogenesis (Worden et al., 1991).

Addition of reducing agents such as methyl viologen, sodium sulfide, and cysteine has been found to alter electron flow towards solvent production (Klasson et al., 1991; Rao and Mutharasan, 1988; Younesi et al., 2005). The presence of alternate electron acceptors, such as nitrates, have been found to repress the acetyl-CoA pathway (Imkamp and Muller, 2002).

The cost of nutrients used in a fermentation medium must be reduced while increasing product concentrations and fermentation productivity. Alternative nutrient sources should also be cheaper, reduce media complexity and be environmentally safe to handle and dispose. Lignocellulose feedstocks are nutritionally poor and must be fortified with common nutrients such as yeast extract, tryptone and peptone, which make the process cost prohibitory on a commercial scale. In a study directed towards developing low-cost fermentation medium, found

that by replacing yeast extract and peptone with corn steep liquor (CSL) reduced the media cost 50-fold in *Saccahromyces cerevisiae* fermentations (Kadam and Newman, 1997). Other complex nutrient sources such as cotton seed proteins and soy products also have been used in pharmaceutical industries for antibiotic production and in food and agriculture applications (Kokoczka and Stevenson, 1976; Yu et al., 1997; Zabriskie et al., 1980).

From the above background, it can be observed that syngas fermentation efficiency can be improved by employing several process strategies. Research reported presented herein addresses some of these strategies. The broad objectives for the research were to improve ethanol yields during syngas fermentation and to design an economical fermentation process. The specific objectives for the study were to:

1. Screen alternative and inexpensive fermentation media components and determine the potential for improving ethanol yields and lower media costs
2. Determine the effect of process parameters such as pH, temperature and buffer on syngas fermentation.
3. Determine the effect of key limiting nutrients on the acetyl-CoA pathway in a continuous series reactor design.
4. Scale up syngas fermentation in a pilot-scale fermentor.

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CHAPTER II: Feasibility of Incorporating Cotton Seed Extract in *Clostridium* strain P11 Fermentation Medium during Synthesis Gas Fermentation

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Abstract

Biomass gasification followed by fermentation of syngas to ethanol is a potential process to produce bioenergy. To make this process more economical, the complexity of media should be reduced while using less costly components. In this study, the feasibility of incorporating cotton seed extract (CSE) as a media component for syngas fermentation to produce ethanol using Clostridium strain P11 was evaluated. A factorial experiment was conducted to screen and evaluate the effect of different media components, in relation to CSE, on ethanol production. Also, different CSE concentrations as well as the presence of MES buffer were tested to determine their effect on ethanol production. Bottle fermentations with media containing only 1.0 g L^{-1} CSE produced more ethanol after 15 d (1.17 g L^{-1}) than fermentation using any other media. Further bottle experiments showed that media containing only 0.5 g L^{-1} CSE produced more ethanol after 15 days (2.67 g L^{-1}) than a control media (0.6 g L^{-1}) and media containing only 1.0 g L^{-1} CSE (2.16 g L^{-1}). Fermentations in 3 L and 7.5 L stirred fermentors with 0.5 g L^{-1} CSE media achieved similar ethanol concentrations to what was observed in bottle studies. These results indicate that CSE can replace all the vitamin and mineral media components generally used for fermentation of syngas to ethanol by Clostridium strain P11, thereby improving the process economics.

Keywords: *Ethanol, Syngas, Clostridium, Cotton Seed Extract, Acetogenic bacteria*

Introduction

Dwindling fossil fuel reserves, energy security and rapidly changing global climate is putting immense pressure on nations to switch from fossil based energy to renewable energy sources. A goal has been set by the US Department of Transportation to replace 30 % of liquid transportation fuel with biofuels (Chum and Overend, 2003). Gasification of cellulosic biomass followed by microbial fermentation can be one approach in meeting the liquid fuel demand for the future. Gasification produces synthesis gas (syngas) in which CO and H_2 are the essential components for subsequent ethanol production. Fermentation of syngas offers several advantages such as high specificity of biocatalysts, lower energy costs, greater resistance to catalyst poisoning and no requirement for a fixed CO:H_2 ratio (Bredwell et al., 1999; Klasson et al., 1992). Acetogens are a class of anaerobic bacteria able to reduce syngas components to acetate via the acetyl-CoA or the Wood-Ljungdahl pathway (Wood et al., 1982).

Ethanol production is a non-growth related product formed during anaerobic syngas fermentation. Several acetogenic organisms have been shown to produce ethanol from syngas. Prominent among these are *Clostridium ljungdahlii*, which produced up to 48 g L^{-1} ethanol in a continuous stirred tank reactor (CSTR) with cell recycle after 560 h, and *Clostridium carboxidivorans* P7, which produced 25 g L^{-1} ethanol after 59 d in a CSTR (Kundiyana et al., 2010; Phillips et al., 1993). Recently *Clostridium* strain P11 was reported to produce 9.2 g L^{-1} ethanol using CO, and is currently the organism of choice at our research facility for syngas to ethanol conversion (Saxena and Tanner, 2006).

It is necessary that fermentation media costs are reduced, which is an important criterion in a large scale ethanol production facility. Cotton seed extract (CSE) offers benefits such as lower cost, nutrient stability, extended shelf life, ease in handling and transportation, and ease in

media incorporation. Due to these benefits, CSE has been used extensively as a fermentation media supplement, primarily in the area of industrial antibiotic production and production of xanthan gum (Zabriskie et al., 1980). Steam exploded cotton gin residues were fermented by *Escherichia coli* KO11 with a maximum ethanol yield of 191 L kg^{-1} (Agblevor et al., 2003). Cotton seed flour was also used to isolate clinically important aerobic bacteria (Slifkin and Pouchet, 1975).

CSE at a cost of $0.91/kg (Traders Protein, USA) offers considerable economical benefits compared to yeast extract ($183/kg, Sigma Aldrich, USA) as a fermentation media supplement. A detailed compositional analysis for different cotton seed products has been published previously (Zabriskie et al., 1980). Comparison of the chemical composition shows that mineral and vitamin content in CSE are very similar to the standard *Clostridium* strain P11 fermentation medium (Kundiyana et al., 2010). CSE also contains eighteen amino acids. Some amino acids such as glycine, valine and methionine have been shown to serve as chemoorganotrophic energy substrate in acetogens. Amino acids also have been shown to support the growth of mesophilic *Eubacterium acidaminophilum* (Zindel et al., 1988). Table 2.1 shows the proximate analysis for Proflo cotton seed extract (Traders Protein, USA) used in the present study. CSE has shown varied effects of its incorporation in fermentations. In one study it was shown that the growth of *Clostridium pefringens* ATCC 3624 was possibly inhibited by the presence of different carbohydrate fractions, such as glucose, xylose, mannose, ribose and rhamnose, and gossypol (Kokoczka and Stevenson, 1976). In another study it was observed that the biological availability of amino acid contained in CSE was better compared to the delivery of amino acid in crystalline form (El-Sayed, 1990). No literature has been cited for the use of CSE for ethanol fermentation studies involving anaerobic fermentation. The main objective of the present study

was to determine the effect of CSE on ethanol production by *Clostridium* strain P11 in a syngas fermentation.

Table 2.1. Compositional analysis of Proflo cotton seed extract supplied from Traders Protein, USA. Detailed Proflo composition included in Appendix 1.

Composition Analysis	Percentage, % (dry matter basis)
Total Solids	98.33
Protein (N x 6.25)	59.41
Carbohydrates	23.18
Reducing Sugars	1.17
Non reducing Sugars	1.30
Fat (Oleic & Free Fatty Acids)	4.18
Ash	6.73
Fiber	3.19
Moisture	1.67
Free Gossypol	0.043
pH (aqueous solution)	6.5

Materials and Methods

Bacterial Culture Maintenance

Clostridium strain P11^T originally was obtained from Dr. Ralph Tanner, University of Oklahoma. All subculture fermentations were conducted in 500 mL serum bottles each with a 100 mL final working volume. *Clostridium* strain P11 subculture currently maintained in our laboratory was used as the starting inoculum at the rate of 10 % of the working volume. The bacterium was maintained under strictly anaerobic conditions in a standard medium containing following components (per Liter): 30 mL mineral stock solution, 10 mL trace metal solution, 10 mL vitamin solution, 10 g corn steep liquor (CSL), 10 g morpholinoethanesulfonic acid (MES), 10 mL of 4 % cysteine sulfide solution, and 1 mL of 0.1 % resazurin indicator (Tanner, 2002). CSL was centrifuged at 20,000 g for 10 min, and the supernatant was added to the fermentation

bottles. Table 2.2 shows the detailed composition of the stock mineral, trace metal and vitamin solutions (Huhnke et al., 2008). All fermentation serum bottles were incubated at 37 °C with a constant agitation of 150 rpm on an orbital shaker for 15 d. The treatments were purged with bottled syngas for 4 min (5 % H_2, 15 % CO_2, 20 % CO, 60 % N_2) to a pressure of 239 kPa every 24 h.

Table 2.2. Detailed media composition of vitamin, minerals and trace metal stock solutions used in the *Clostridium* strain P11 standard media formulation (Huhnke et al., 2008**).**

Trace metal stock solution	g/L
$CoCl_2*6H_2O$	0.20
$Fe(NH_4)_{2(}SO_4)_2*6H_2O$	0.80
$MnSO_4*H_2O$	1.00
$NiCl_2*6H_2O$	0.20
Nitrilotriacetic Acid	2.00
$NaMoO_4*2H_2O$	0.02
$NaSeO_4$	0.10
$NaWO_4$	0.20
$ZnSO_4*2H_2O$	1.00

Mineral stock solution	g/L
NH_4Cl	100
$CaCl_2*2H_2O$	4
$MgSO_4*7H_2O$	20
KCl	10
KH_2PO_4	10
NaCl	80

Vitamin stock solution	g/L
p-(4)-Aminobenzoic Acid	0.005
d-Biotin	0.002
Calcium Pantothenate	0.005
Folic Acid	0.002
Mercaptoethanesulfonic acid	0.010
Nicotinic Acid	0.005
Pyridoxine	0.010
Riboflavin	0.005
Thiamine	0.005
Vitamin B-12	0.005

Effect of Incorporating CSE as a Media Supplement

To understand the effect of CSE as a potential media supplement, a Plackett-Burman design was constructed to understand the effect of CSE and other media components on ethanol and acetate production. The *Clostridium* strain P11 media contains stock solutions of minerals, trace metals and vitamins, and yeast extract or corn steep liquor. A classical experimental design with six factors at two levels would require 64 sets of experiments (2^6), which was not feasible.

A Plackett-Burman design is a two level multi factor experimental design to test *N-1* variables in N experimental runs. The runs, also referred to as assemblies, must be a multiple of 4. The design rationale represents a balanced incomplete design (Stanbury et al., 1986). Table 2.3 shows the detailed assembly designed for the CSE experiment. The dummy variable in the table represents the factor not studied as only six factors were observed to be important for the current study. The dummy variable also increases the degrees of freedom in the experiment. "-1" and "+1" refers to the lower (not present) and upper (standard media concentration) levels of individual components, respectively. For CSE "-1" and "+1"equaled 1.0 g L^{-1} and 3.0 g L^{-1}, respectively. All runs were conducted in triplicate.

Optimization of CSE Concentration as a Media Supplement

Following Plackett-Burman experimental design to evaluate the effect of different fermentation media factors, a second series of experiments were conducted to determine the effect of CSE concentration on ethanol and acetic acid production by *Clostridium* strain P11. The experiments were conducted in triplicate in 250 mL serum bottles with 100 mL of working volume. Two levels of CSE concentration, 0.5 g L^{-1} and 1 g L^{-1}, were tested against standard *Clostridium* strain P11 fermentation medium. The CSE fermentation media was prepared only with CSE and no other media components were added. Incubation of the test bottles were similar to the conditions described earlier.

Table 2.3. A Plackett-Burman design to evaluate the affect of CSE incorporation in conjunction with other *Clostridium* strain P11 components. The factors included in the design are: Yeast Extract (A), CSL (B), Minerals (C), Trace metals (D), Vitamin (E), CSE (F) and Dummy variable (G).

Factors →		**A**	**B**	**C**	**D**	**E**	**F**	**G**
Treatment (Trt)	1	1	-1	-1	1	-1	1	1
	2	1	1	-1	-1	1	-1	1
	3	1	1	1	-1	-1	1	-1
	4	-1	1	1	1	-1	-1	1
	5	1	-1	1	1	1	-1	-1
	6	-1	1	-1	1	1	1	-1
	7	-1	-1	1	-1	1	1	1
	8	-1	-1	-1	-1	-1	-1	-1

Effect of MES Buffer on Media Supplement

A third set of experiments was conducted to study the effect of addition of MES buffer when using CSE as a fermentation media component. The purpose of this experiment was to determine the effect of presence or absence of buffer on fermentation and product formation. In the present study, CSE was the only component in the fermentation media at a concentration of 0.5 g L^{-1}. Treatment with buffer was added with MES at a standard rate of 10 g L^{-1}. Elimination of buffer also offers improved process economics since MES accounts for approximately 97 % of the standard *Clostridium* strain P11media cost. All the treatments were conducted in triplicate, and were incubated under conditions described earlier.

Effect of Scale-Up on CSE Incorporation

The final step in identifying the feasibility of CSE was to determine the effect of CSE incorporation in laboratory scale fermentors. Scale-up is important from a commercialization

perspective because the complexity changes from cellular kinetics controlled system response to transport limitations controlled system responses. This becomes even more critical in gas-liquid mass transfer systems, such as syngas fermentations, where solubility of carbon monoxide and hydrogen becomes a challenging operational limitation (Ungerman and Heindel, 2007).

Fermentations were conducted in 3 L and 7.5 L Bioflo 110 New Brunswick Scientific fermentors with 2 L and 5 L working volume, respectively (New Brunswick, NJ). The fermentation media contained only 0.5 g L^{-1} CSE and 1 % MES buffer. It was decided to add the buffer because it was observed in preliminary experiments that presence of buffer gave a better pH control during the growth stages of fermentation. Detailed procedure for inoculum preparation and operation of a fermentor has been described previously (Kundiyana et al., 2010). The fermentors were maintained at 37°C under batch conditions with continuous micro-sparging (Mott Corporation, USA) of bottled syngas (5 % H_2, 15 % CO_2, 20 % CO, 60 % N_2). The gas flowrate maintained at 0.1 L min^{-1} was observed to be appropriate to minimize foaming inside the reactor and avoid excessive addition of antifoam reagent.

Media pH was controlled at 5.9 using 1 M KOH solution only in the first 48 h to promote growth in order to achieve high cell densities in the fermentation medium. The pH was controlled automatically using the New Brunswick Operator Interface Terminal (OIT) with a pump dosing setting of 25 % and a deadband setpoint of 0.2. The total fermentation time in the fermentor was 21 d. Fermentation broth samples were removed every 24 h for measuring product formation.

Analytical Procedures

Analysis samples were collected every 48 h from the serum bottles and every 24 h from the larger fermentors for determining the time course of product formation. Ethanol and acetic

acid were separated by HPLC with an Aminex-HPX-87H ion exchange column (Biorad, Hercules, CA, USA). The eluent was 0.005 mol L^{-1} H_2SO_4 pumped at 0.6 ml min^{-1} and column temperature was 60 °C (Sluiter et al. 2005). Separated components were detected by a refractive index detector (1100 Series, Agilent, Santa Clara, CA, USA). Peaks were identified by comparing with known standards (Sigma-Aldrich, St. Louis, MO, USA). No cell densities were measured in the fermentation bottles or in the reactor studies because of cell aggregation to the CSE solids.

Overhead gas samples were collected every 24 h from the 5 L reactor headspace and analyzed for syngas component utilization. The total run time for sample analysis was 20 min. A ramped temperature profile was followed in the oven for resolving overlapped peaks (32^{o}C for 12 min → ramped to 236 °C at the rate of 30 °C min^{-1}). The separated components were detected using a thermal conductivity detector (TCD) maintained at inlet and outlet temperatures of 200 °C and 230 °C, respectively. Oxidation-reduction potential (ORP) of the fermentation broth was measured using a Ag/AgCl reference ORP electrode (InPro 3200/SG, Mettler Toledo, USA) attached to a transmitter (M400, Mettler Toledo, USA) and the data acquired using pDAQ 56 (Iotech Corporation, USA). Redox potential (E_h) was calculated vs. standard hydrogen electrode (SHE) using equation 1.

$$E_h = E_c + 222.4 \text{ mV} \quad [1]$$

where, E_c was the measured redox value and +222.4 mV is the standard electrode potential of Ag/AgCl electrode (Kakiuchi et al., 2007).

Analysis of variance of data was performed using the GLM Procedure of SAS Release 9.1 (SAS, Cary, NC, USA) with a compound variance covariance structure and repeated measure design. Level of significance was tested at $\alpha = 0.05$.

Results and Discussion

The present study was conducted to evaluate the feasibility of CSE as a fermentation media component for ethanol production from syngas to replace more expensive media components such as yeast extract, minerals, trace metals and vitamins. Incorporation of CSE offers new avenues for media optimization and cost reduction of fermentation media systems. To the best of our knowledge, no studies of CSE use in an ethanol fermentation process have been mentioned in literature. The lower level of CSE was chosen according to the concentration of yeast extract in the standard medium described earlier (1.0 g L^{-1}). A similar amount of cotton seed meal was added for antibiotic P-3355 production using *Streptomyces* sp (Sumino et al., 1976). In another study it was observed that the presence of (0.029%) gossypol and the (1%) carbohydrate fraction from Proflo inhibited the growth of *Clostridium perfringens* (Kokoczka and Stevenson, 1976). It was assumed that *Clostridium strain* P11 would be similarly inhibited e by CSE and hence, 3.0 g L^{-1} 16 of CSE was chosen as the higher level.

Association of CSE with other Clostridium strain P11 Fermentation Media components

Influence on Ethanol Productivity

Figure 2.1 depicts ethanol production for the eight treatment runs averaged across the triplicate treatments plotted as a function of time. Ethanol production was not observed in the first 7 d of fermentation, with subsequent production observed in later stages. Maximum ethanol concentration of 1.17 g L^{-1} was produced at the end of 15 d in treatment with the lower level of all factors (Treatment 8 contained only 1.0 g L^{-1} CSE as a fermentation medium component). A similar ethanol production profile was observed previously in syngas fermentation studies with *Clostridium carboxidivorans* P7 (Ahmed et al., 2006). When treatments with 1.0 and 3.0 g L^{-1} CSE were compared, ethanol production was similar for both CSE levels. Therefore, a level of 1.0 g L^{-1} was sufficient.

Overall, the presence of the mineral stock solution had a positive effect on ethanol production ($F_{1,17}$ = 9.03, p = 0.0080) in the experiment. However, due to the high ethanol production in treatment 8, which contained 1.0 g L^{-1} CSE and no mineral stock solution, the possibility of replacing all of the media components with CSE was further investigated. CSE contains a complex mixture of amino acids, vitamins and minerals; thus, it may satisfy the nutrient requirement of *Clostridium* strain P11 during syngas fermentation. This may justify the possibility of including CSE as the sole fermentation media component (Zabriskie et al., 1980). The proximate analysis of CSE indicates 23.2 % of carbohydrates. At an incorporation rate of 3.0 g L^{-1}, the carbohydrate in CSE would theoretically yield less than 0.3 g L^{-1} of ethanol. Ethanol yields observed in the present study are considerably higher than 0.3 g L^{-1}, indicating that syngas components were utilized as the main carbon source for ethanol production.

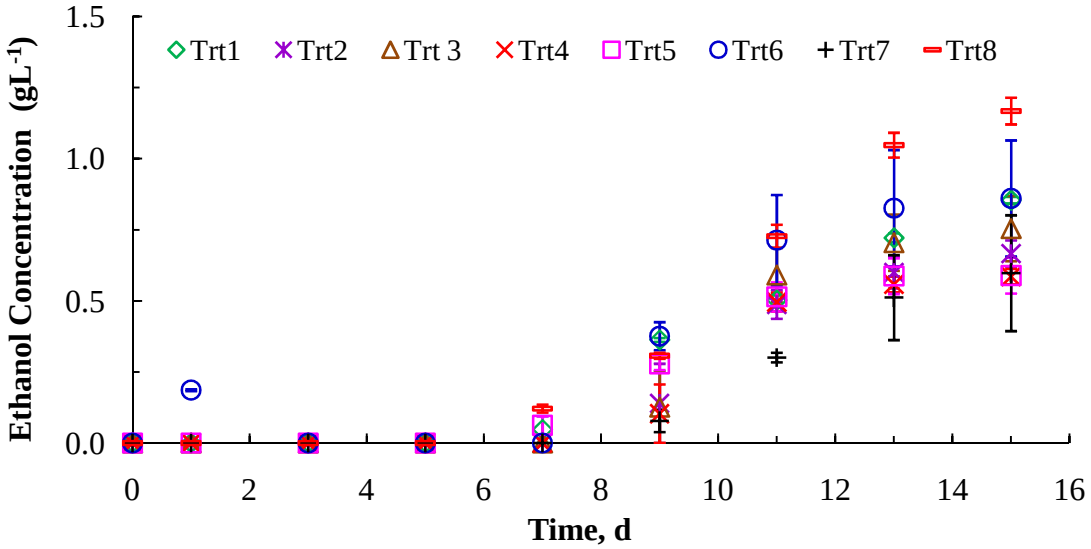

Figure 2.1. *Clostridium* strain P11 ethanol profile in a Plackett-Burman designed experiments to assess the impact of incorporating CSE as a fermentation media component. The error bars represent the standard error (n=3). Details on treatments are given in Table 2.3.

Influence on Acetic Acid Productivity

Figure 2.2 depicts the acetic acid production for the eight treatment runs averaged across the triplicate treatments plotted as a function of time. Acetic acid production showed a linear trend until 7 d, before the *Clostridium* strain P11 switched to ethanol production. A maximum acetic acid concentration of 4.83 g L^{-1} was produced after 7 d in the second treatment of the experimental design. Presence of vitamin and CSE concentration did not affect acetic acid production throughout the fermentation ($p > 0.05$). The presence of yeast extract ($F_{1,17} = 76.70$, $p < 0.0001$) and CSL ($F_{1,17} = 126.10$, $p < 0.0001$) both had a positive effect on acetic acid production .

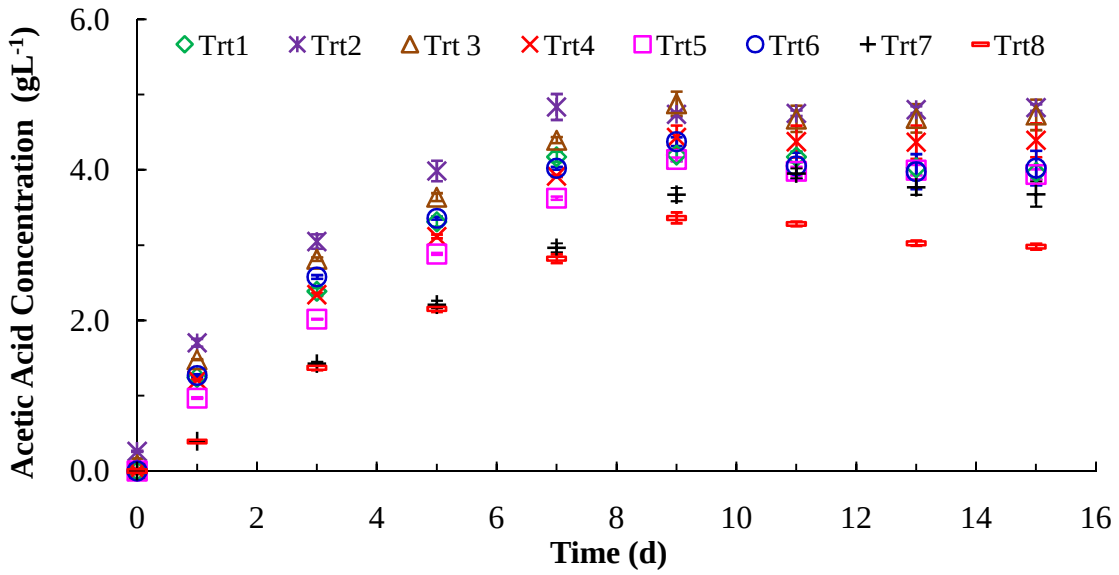

Figure 2.2. *Clostridium* strain P11 acetic acid profile in a Plackett-Burman designed experiments to assess the impact of incorporating CSE as a fermentation media component. The error bars represent the standard error (n=3).

Table 2.4 shows the mean ethanol and acetic acid concentration observed across all the treatments. The above results indicate that production of acetic acid in the initial stages of fermentation is necessary for subsequent production of alcohols in the second stage, and for the

reduction of acids to solvents (Worden et al., 1991). In the present study, the specific reduction of acetic acid to ethanol was not studied and similar interpretation cannot be done from the acetic acid plots, possibly because acetic acid was simultaneously produced along with its reduction to ethanol. The possibility of acetic acid reduction to ethanol previously has been indicated (Hurst and Lewis, 2010). Suitable methodology for determining if acetic acid reduction to ethanol occurs would be to use C14- and C13-isotope labeling studies (Ragsdale and Pierce, 2008), which was beyond the scope for present study.

Table 2.4. Mean ethanol and acetic acid production from treatments containing CS

Time, d	Ethanol		Acetic Acid	
	Mean, g L^{-1}	Standard Error	Mean, g L^{-1}	Standard Error
1	0.02	0.02	1.08	0.17
3	0.00	0.00	2.25	0.22
5	0.00	0.00	3.08	0.23
7	0.03	0.02	3.84	0.24
9	0.22	0.04	4.22	0.18
11	0.54	0.05	4.15	0.16
13	0.70	0.06	4.07	0.20
15	0.76	0.07	4.06	0.21

Effect of CSE concentration on Clostridium Strain P11 fermentation

The objective of this experiment was to determine the effect of different concentrations of CSE on product formation during *Clostridium* strain P11 fermentation. The control for the experiment was the treatment using the standard *Clostridium* strain P11 medium. Figure 2.3 shows the concentration effect of CSE on ethanol production. Maximum ethanol (2.66 g L^{-1}) was produced in the treatment with 0.5 g L^{-1} added CSE. Ethanol production in the control

treatment was associated with a longer lag period, possibly due to the addition of MES buffer in the control medium. Bryant and Blaschek (1988) observed that the buffering effect was dependent on the type of buffer used and also on the fermentation medium composition. They observed that elevated pH was favorable for butanol production by *Clostridium acetobutylicum* (Bryant and Blaschek, 1988). Statistical analysis indicates a significant effect of CSE concentration on ethanol production ($F_{6,2} = 19.62$, $p = 0.0023$). The use of 0.5 g L^{-1} CSE resulted in higher ethanol production than both 1.0 g L^{-1} CSE and the control treatment ($F_{14,42} = 11.72$, $p = 0.0206$).

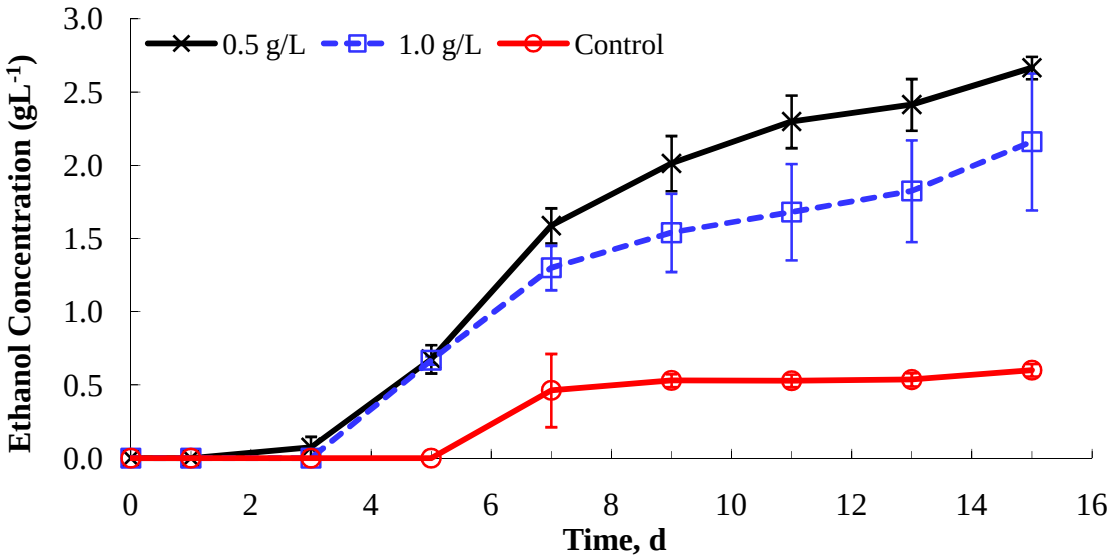

Figure 2.3. Effect of 0.5 g L^{-1} and 1.0 g L^{-1} of CSE concentration on ethanol production with *Clostridium* strain P11. The control refers to fermentation in *Clostridium* strain P11 standard media. The error bars represent the standard error (n=3).

Figure 2.4 shows the acetic acid profile observed during the CSE concentration treatment. The control treatment was associated with comparatively higher yields of acetic acid compared to fermentation using CSE as the only media component. This is encouraging because the fermentations with CSE were more specific to the production of ethanol with low accumulation of acetic acid, which can subsequently improve the process economics by decreasing the product

complexity for the downstream separation process. Statistical analysis indicates a highly significant effect of CSE concentration on ethanol production ($F_{2,6} = 76.65$, $p < 0.0001$). CSE concentration also had a significant effect at different time points when the acetic acid concentration was measured ($F_{14,42} = 3.46$, $p = 0.0009$). The significant effect of the CSE concentration was due to the significant linear interaction between test variables ($F_{2,6} = 44.09$, $p = 0.0003$).

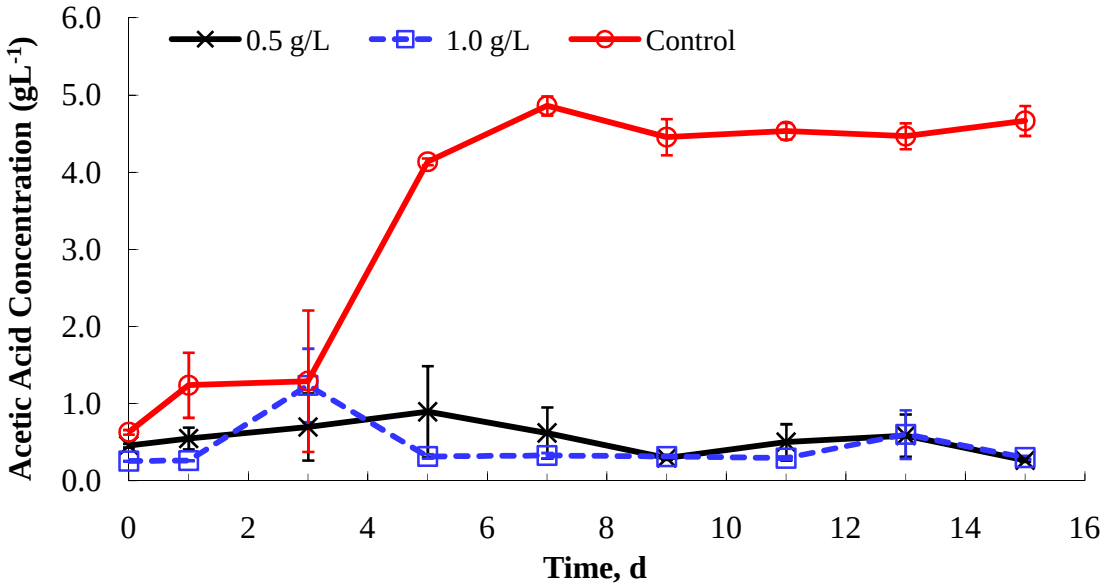

Figure 2.4. Effect of 0.5 g L^{-1} and 1.0 g L^{-1} of CSE concentration on acetic acid production with *Clostridium* strain P11. The control refers to fermentation in *Clostridium* strain P11 standard media. The error bars represent the standard error (n=3).

Effect of MES buffer on media supplement

The objective of this experiment was to determine the effect of MES buffer during *Clostridium* strain P11 syngas fermentation. Figure 2.5 shows the ethanol, acetic acid and pH profiles measured to show the effect of syngas fermentation with- ("Buf") and without buffer ("WO-Buf") treatments on productivity. Statistical analysis indicates that the presence or absence of buffer had a significant effect on ethanol ($F_{1,4} = 30.27$, $p = 0.0053$) and acetic acid production ($F_{1,4} = 575.03$, $p < 0.001$). Treatment with added buffer produced higher amounts of

acetic acid and ethanol compared to treatment without buffer. However, the added buffer treatment also demonstrated a lag in switching from acidogenic to solventogenic phase and also showed a greater accumulation of acetic acid in the fermentation bottles. Accumulation of acetic acid and butyric acid has been found to favor ethanol and butanol production during different *Clostridium* fermentations (Bryant and Blaschek, 1988). The authors also indicate that the pH of the fermentation medium needs to be regulated in order to equilibrate the protonated and ionized forms of acetic acid and butyric acid, which enhances the productivities of ethanol and butanol, respectively (Bryant and Blaschek, 1988). Acetic acid, a weak organic acid, is lipophilic when undissociated and permeates through cell membranes. When diffusing through the cell membrane, they conduct H^+ ions resulting in a decrease of intracellular pH (Overmann, 2006). At low internal pH values, the effect of extracellular pH is magnified, which stresses cells and they counteract the situation by producing solvents (Ahmed et al., 2006).

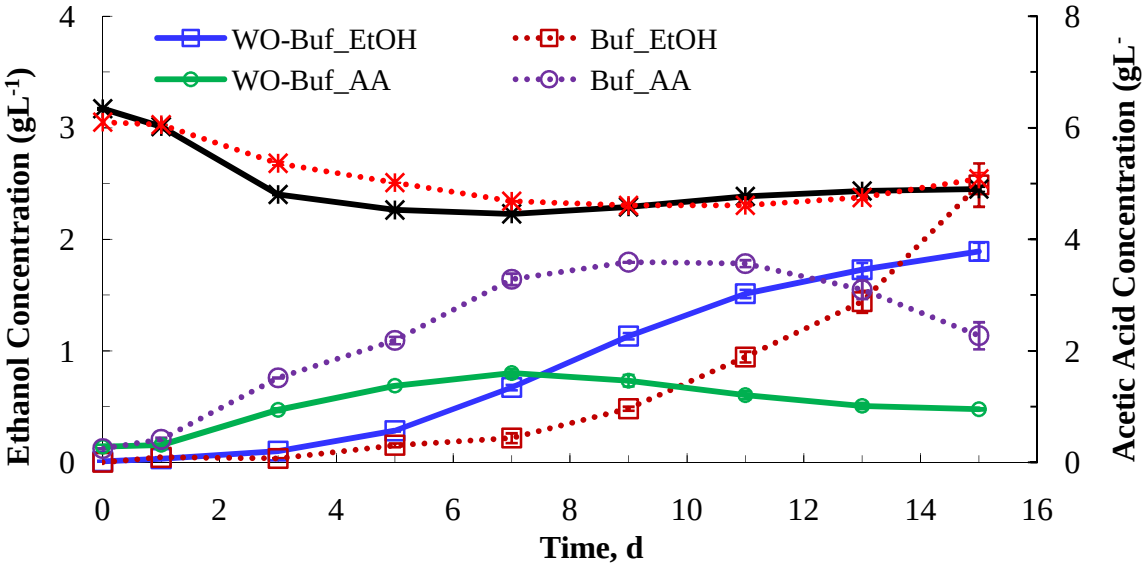

Figure 2.5. Effect of MES buffer addition on ethanol (EtOH), acetic acid (AA) and pH profile during *Clostridium* strain P11fermentations with CSE as the fermentation media component. The error bars represent the standard error (n=3).

Effect of Scale-Up on CSE incorporation

Figure 2.6 depicts the acetic acid and ethanol production profiles observed in the 3 L and 7.5 L fermentors. The 7.5 L fermentor shows comparatively higher ethanol and acetic acid productivity than the 3 L fermentor and the serum bottle fermentations, clearly indicating improved productivity due to scale-up with similar media composition and gas flow rates in the reactors. The measured acetic acid concentration is lower compared to the bottle fermentations, corresponding with a favorable production regime and a lower downstream separation constraint.

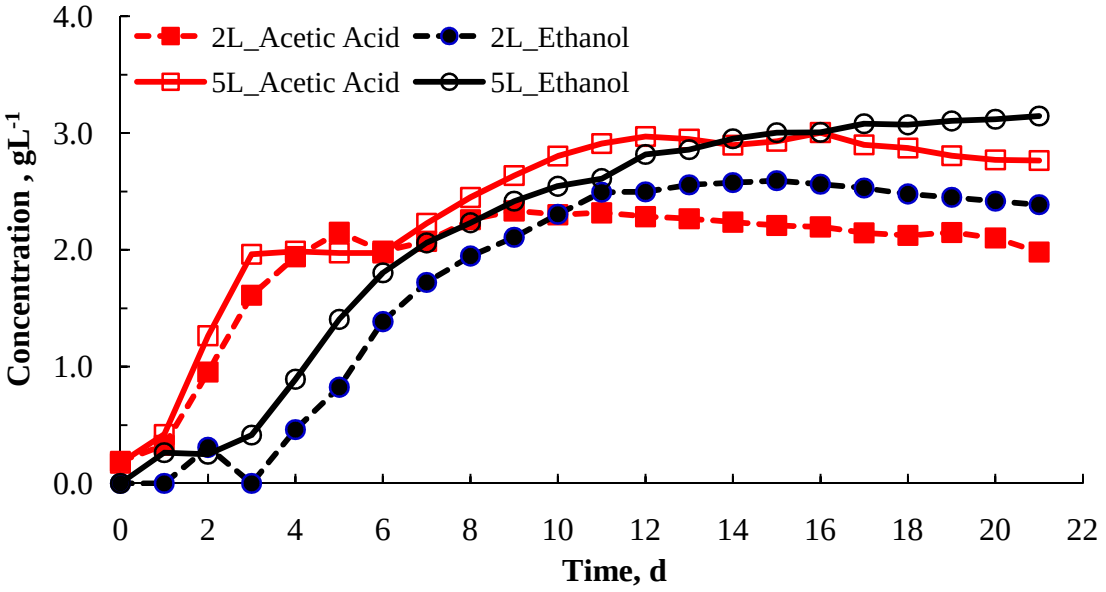

Figure 2.6. Product profiles, acetic acid and ethanol, measured during scale-up of CSE fermentation in 3 L and 7.5 L fermentor.

The Wood-Ljungdahl pathway governing acetogenic bacterial fermentation functions as an electron accepting, energy conservation pathway and as a pathway for autotrophic assimilation of carbon (Ragsdale and Pierce, 2008). Redox measurement values present a unique metric to determine the product distribution in *Clostridium* fermentations. In fermentation with *Clostridium acetobutylicum*, it was observed that the ratio of NADPH to NADH was important in manipulating the fermentation end products (Peguin and Soucaille, 1996). These authors

observed a linear relationship between the fermentation redox potential and NADH concentration, with lower redox potential associated with higher NADH concentration. The redox and pH profile for CSE fermentation in the 7.5 L fermentor is shown (Figure 2.7).

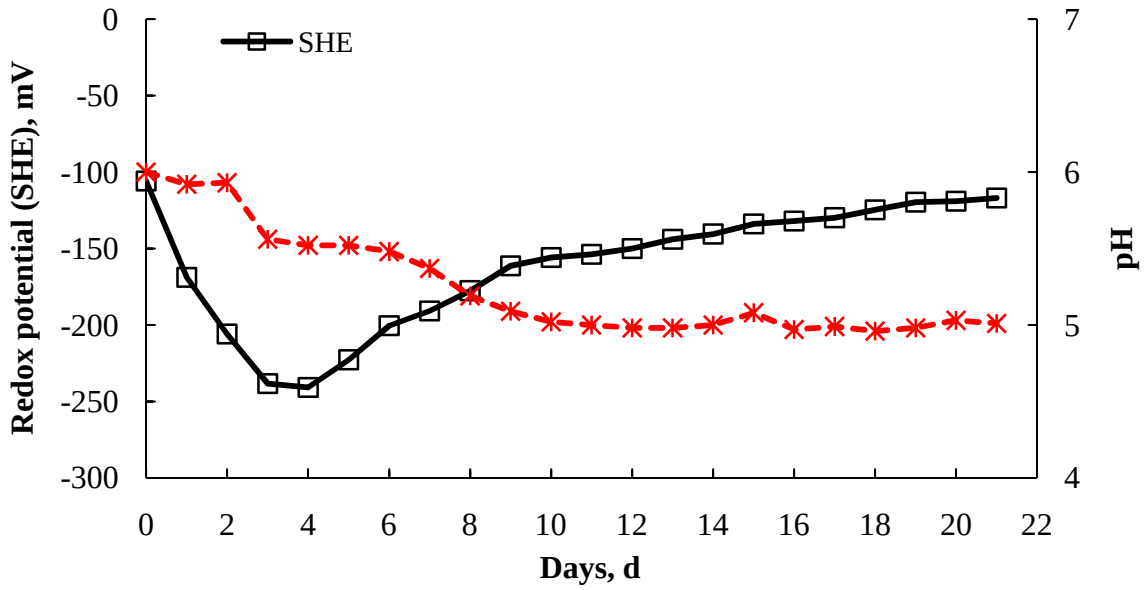

Figure 2.7. Redox and pH profile during the *Clostridium* strain P11 fermentation with CSE as the media component in a 7.5 L fermentor. The redox values were registered every 1 min and the values plotted are averaged over a 24 h time period.

At the beginning of the fermentation, E_h value was -105.9 mV SHE with a corresponding pH of 6.0. With the production of acetic acid, the pH value and the E_h value decreased until the end of 3 d before the bacteria switched from acetogenic to solventogenic phase. This switch was observed to be earlier than the serum bottle fermentations and previous studies with *C. carboxidivorans* P7 (Ahmed et al., 2006). The E_h value started increasing with the onset of ethanol production to a value of -133.9 mV at the end of 15 d before the ethanol production and redox value started plateauing. The redox profile indicates that the *Clostridium* strain P11 cells prefer producing ethanol in the redox range of -240 (4 d) to -130 (15 d) mV SHE. The increase

in redox values observed in the study is possibly due to the fermentation environment becoming less reductive.

Figure 2.8 shows the exhaust gas sample analysis from the 7.5-L fermentor. Day 10 gas analysis was lost due to improper integration of the chromatogram peaks. The overhead gas sample analysis does not show any correlation with the fermentation pH, as observed in *Butyribacterium methylotrophicum* fermentation (Worden et al., 1991). During the acetogenic phase, *Clostridium* strain P11 shows increased utilization of CO corresponding to acetate formation and cell growth. The cells also show utilization of H_2 and CO_2, possibly for cell growth, and minimal amounts of ethanol were produced initially. Ethanol production shows an interesting trend in relation to H_2, CO and CO_2 consumption. During the lag (0 - 3 d) and stationary phase (12 - 17 d) an increased consumption of the syngas components were observed, while the consumption decreased during the log (4 - 11 d) and death phase (18 - 21 d). The trend indicates a higher requirement of reducing equivalents possibly for the maintenance of the cell. Subsequent drop in ethanol productivity resulted in decreased CO consumption (8 - 13 d), which led to the reduced consumption of H_2 possibly due to hydrogenase inhibition by CO (Ragsdale, 2004; Ragsdale and Pierce, 2008). This was further associated with no acetic acid formation, and a very low productivity of ethanol. Reasons for consumption of CO and CO_2 at the end of fermentation can only be speculated. One possible reason could be the utilization of syngas components as a source of maintenance energy under stressed conditions (Younesi et al., 2005).

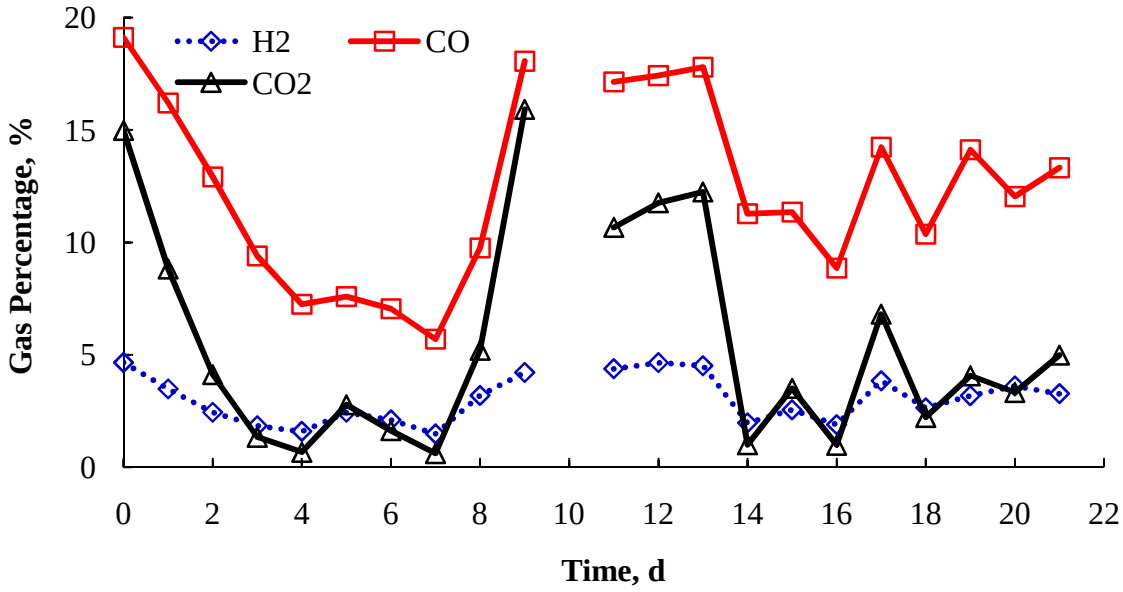

Figure 2.8. Exhaust gas sample analysis from the 7.5 L fermentor (H_2- hydrogen, CO_2- carbon dioxide and CO- carbon monoxide). Inlet syngas had a standard composition of 5 % H_2, 15 % CO_2 and 20 % CO.

Conclusion

CSE can replace the standard media components used in acetogenic syngas fermentation. An average improvement in ethanol productivity of approximately 65% can be achieved during the initial stages of fermentation (between 2 and 9 d) by eliminating MES buffer. Under such buffer deficient fermentations, the fermentation product distribution is also regulated more towards ethanol production rather than acetic acid production.

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CHAPTER III: Effect of Low Temperature, Low pH and Different Corn Steep Liquor Pretreatments on Synthesis Gas Fermentation using *Clostridium* strain P11

Abstract.

The biomass gasification-fermentation process offers a viable approach for the utilization of low cost agricultural raw materials and ensures utilization of lignin for biofuel production. Fermentation pH, incubation temperature, and presence or absence of media buffer can alter the activity of microorganisms. For instance, carbon monoxide and hydrogen components of syngas show decreased solubility with increasing fermentation temperature, Clostridium species preferentially switch from acetogenesis to solventogenesis phase at pH below 5.0, and morpholinoethanesulfonic acid (MES) added as media buffer has been shown to have increased lag time for ethanol production. The objective of the present study was to determine the effects of temperature, pH and MES Buffer on Clostridium strain P11 fermentation. Results indicate that treatment at 32 °C without buffer was associated with higher ethanol concentration and reduced lag time in switching to solventogenesis. Temperature above 40°C and pH below 5.0 were outside the optimal range for growth and metabolism of the bacteria Clostridium strain P11.

Keywords., *Ethanol, Anaerobic, Biomass, Syngas, Acetogens*

Introduction

Biologically, ethanol can be produced either by direct fermentation of fermentable sugars in sugar crops, by enzymatic and physico-chemical degradation of cellulosic biomass to sugar followed by fermentation by yeast or bacteria, or by biomass gasification-fermentation processes. One of the major disadvantages with the utilization of straw, wood and other cellulosic biomass is the presence of a large proportion of non degradable components such as lignin. Gasification of cellulosic biomass can be one approach of overcoming this major hurdle. Gasification produces synthesis gas (syngas) in which CO and H_2 are the essential components for subsequent ethanol production. Syngas fermentation offers several advantages to chemical catalyst conversion of syngas such as the higher substrate specificity of biocatalysts, lower energy costs, greater resistance to catalyst poisoning and the lack of a requirement for a fixed CO:H_2 ratio (Bredwell et al., 1999).

Biological fermentation of syngas is irreversible in nature as it ensures complete conversion of cellulosic feedstock. Production of acetate by utilizing CO, CO_2 and H_2 can be summarized by the following reactions (Ljungdahl, 1986):

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \ (\Delta G° = -37.8 \text{ KJ/g mol } CH_3COOH) \quad [1]$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \ (\Delta G° = -18.6 \text{ KJ/g mol } CH_3COOH) \quad [2]$$

Based on the similarity with respect to equations [1] and [2] above, the following stoichiometry for ethanol production in autotrophic acetogens has been proposed (Vega et al., 1989).

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \ (\Delta G° = -59.9 \text{ KJ/g mol } C_2H_5OH) \quad [3]$$

$$2CO_2 + 6H_2 \rightarrow C_2H_5OH + 3H_2O \ (\Delta G° = -23.2 \text{ KJ/g mol } C_2H_5OH) \quad [4]$$

Organisms that can reduce CO_2 to acetate via the Wood-Ljungdahl pathway (acetyl-CoA pathway), are termed acetogens. Acetogens are strictly anaerobic and produce acetate as the major fermentation end product (Muller, 2003).

Production of ethanol by acetogens follows a path similar to the Wood-Ljungdahl pathway for acetate production by acetogens such as *Clostridium acetobutylicum* (Jones and Woods, 1986). Oxidation of H_2 to $2[H^+]$ or of CO with H_2O to CO_2 and $2[H^+]$ produces the reducing equivalents needed for the conversion of CO_2 and CO to acetate or alcohols (Henstra et al., 2007). Ethanol is a non-growth related product formed during syngas fermentation. Several acetogenic organisms have been shown to produce ethanol. Prominent among these are *Clostridium ljungdahlii* (continuous fermentation) and *Clostridium carboxidivorans* (batch fermentation), which produced up to 460 mM and 175 mM ethanol, respectively (Gaddy et al., 2007; Liou et al., 2005). *Clostridium* strain P11 was reported to produce 200 mM ethanol using CO in a batch process (Saxena and Tanner, 2006).

One potential bottleneck in the utilization of syngas to produce ethanol is the low solubility of CO and H_2 components of syngas in aqueous broths (Riggs and Heindel, 2006). Experiments in a 100-L fermentor in our pilot scale facility have indicated that the conversion efficiency of CO and H_2 is only 20% at a continuous gas flowrate of 0.9 liters per minute (LPM) at 37 °C (Kundiyana et al., 2010). Also, it has been shown that higher temperatures have a negative impact on the solubilities of CO and H_2, which also decreases the mass transfer rate of these gases to fermenting cells (Henstra et al., 2007). Fermentation pH is another factor dictating the switch from acidogenesis to solventogenesis during the anaerobic fermentation of syngas. Media pH values in the range of 4.5 to 4.8 results in the microbial cells switching from acidogenesis to solventogenesis (ethanol production) (Ahmed et al., 2006; Worden et al., 1991).

In addition to the process conditions affecting bacterial metabolism, it is necessary that fermentation media costs are reduced to make the process economical from a commercial perspective. Morpholinoethanesulfonic acid (MES) used as a buffering agent in syngas fermentation media accounts for approximately 97% of the cost of *Clostridium* strain P11 standard media. Removal of the buffer from *Clostridium* strain P11 fermentation medium may offer considerable process cost benefits. The overall objective of the present study was to test different operational parameters during syngas fermentation with *Clostridium* strain P11. The specific objectives for the present study were to determine the effects of: (a) fermentation temperature; (b) medium pH; and (c) buffer concentration, on ethanol production during syngas fermentation.

Materials and Methods

Bacterial Culture Maintenance

Clostridium strain P11^{T} (ATCC PTA-7826) was originally obtained from Dr. Ralph Tanner, University of Oklahoma. The bacterial strain is currently maintained in our laboratory, and was subcultured and used as the starting inoculum at the rate of 10% of the working volume. The species name for this strain has not been officially assigned. All subculture fermentations were conducted in 250 mL serum bottles each with a 100 mL final working volume. The inoculum was prepared by a procedure termed as 'passaging' wherein the inoculum was sub-cultured sequentially with an incubation period of 48 h. The third passage was used as the inoculum for all treatment fermentations. The bacterium was maintained under strictly anaerobic conditions in a standard medium containing the following components (per Liter): 30 mL mineral stock solution, 10 mL trace metal stock solution, 10 mL vitamin stock solution, 10 g corn steep liquor (CSL), 10 g morpholinoethanesulfonic acid (MES), 10 mL of 4% cysteine

sulfide solution, and 0.1 mL 1% resazurin indicator (Tanner, 2002). Detailed composition of the stock solutions has been described previously (Kundiyana et al., 2010). CSL was centrifuged at 20,000 g for 10 min, and the supernatant was added to the fermentation bottles. Removal of solids was found to be necessary to minimize the variability in subculture preparation because of the varying solids concentration in CSL, and to avoid solid precipitation after sterilization which was found to be interfering with cell biomass optical density estimation. The treatment bottles were purged with commercial syngas (Composition: 5% H_2, 15% CO_2, 20% CO, and 60% N_2) every 24 h and incubated at 37 °C with a constant agitation of 150 rpm on an orbital shaker. Analyses samples were collected every 48 h for determining the time course for bacterial cell biomass, substrate, and product concentrations depending on the fermentation protocol. The cell biomass was determined by measuring sample absorbance at a wavelength of 660 nm (A_{660nm}) in 1-cm pathlength cuvettes using a UV-visible spectrophotometer (Cary 50 Bio, Varian, USA). Ethanol and acetic acid was separated by HPLC with an Aminex-HPX-87H ion exchange column (Biorad, Hercules, CA, USA). The eluent was 0.01N H_2SO_4 pumped at 0.6 ml min^{-1} and column temperature was 60 °C (Sluiter et al. 2005). Separated components were detected by a refractive index detector (1100 Series, Agilent, Santa Clara, CA, USA). Peaks were identified by comparing with known standards (Sigma-Aldrich, St. Louis, MO, USA).

Preliminary Screening Experiment

A set of preliminary experiments were conducted to test and compare low temperature treatment (Trt_Temp) (30 °C) and low pH (Trt_pH) (5.0) against control (Trt_Cont) (37 °C and pH 6.0) treatment. A set of preliminary experiments was also conducted to evaluate the effect of pre-treating CSL before adding to the fermentation media. The purpose of this pretreatment was to determine if pretreating CSL had a significant effect on ethanol production. Two different methods for CSL pretreatment were chosen. The first method involved autoclaving CSL

(Trt_AC) at 121 °C for 30 min at 103.4 KPa and adding the whole CSL without separating the solids. In the second method, CSL was centrifuged (Trt_CF) (to separate solids) as described in the previous section and only the supernatant added to the fermentation medium. The two fermentation treatments were compared with the control treatment containing yeast extract (Trt_YE) (1 g L^{-1}) instead of CSL. The incubation time for the first set of screening experiments was 9 d while the second set of screening experiments was incubated for 15 d. All the fermentations treatments were conducted in triplicate for statistical validity.

Effect of Buffer, pH and Temperature on Syngas Fermentation

A 3 by 3 central composite was designed to evaluate the three factors each at three levels (Table 3.1). In the table levels "-1", "0" and "1" represent low, middle and high levels of each factor. Each of the treatments was conducted in triplicate with three central points serving as the control (Level 0). The levels of each factor were determined based on the preliminary tests. Each of the treatment bottles was incubated for 15 d on an orbital shaker at incubation temperature dictated by the experimental design.

Table 3.1. Coded Values for the treatment variables

	Coded Values		
Treatment Variables	**-1**	**0**	**1**
Buffer Concentration	0	1%	2%
pH	5.0	6.0	7.0
Temperature (°C)	32	37	42

Statistical Analysis

For all experiments, analysis of variance (ANOVA) was calculated ($p < 0.05$) using the mixed procedure in SAS (Enterprise Guide version 4.2 SAS, Cary, NC, USA) and differences

among means were calculated using Fisher's protected least significant difference test ($p < 0.05$). Statistical comparison of mean ethanol and acetic acid concentration, and cell optical density were performed using the repeated measure analysis and the means were separated using Fisher's protected least significance difference (LSD) method for comparing multiple treatments at different sample times. Levels of buffer addition, temperature and pH were included as independent variables for determining the main effects, two-way, and three-way interactions. All the treatments were performed in triplicate unless otherwise noted. Level of significance was tested at $\alpha = 0.05$.

Results

Preliminary Experiment

Two different sets of experiments were conducted. In the first set of experiments, the incubation fermentation pH was reduced from 6.0 to 5.0 (Trt_pH), and the incubation temperature decreased from 37 to 30 °C (Trt_Temp). In the second experiment autoclaved CSL (Trt_AC) was compared against centrifuged CSL treatment (Trt_CF) and the control treatment with yeast extract (Trt_YE).

In the first set of experiments, Trt_Temp showed comparatively higher ethanol (3.02 g L^{-1}), acetic acid (4.94 g L^{-1}) and cell biomass (0.68 g L^{-1}) concentrations at the end of 9 d compared to the Trt_pH and the Trt_Cont (plots not shown). At the end of 9 d, the ethanol, acetic acid and cell biomass concentration in the Trt_pH was 1.66 g L^{-1}, 3.96 g L^{-1} and 0.30 g L^{-1} respectively. In the Trt_Cont, ethanol concentration peaked at the end of 7 d (2.41 g L^{-1}) and subsequently decreased to (1.42 g L^{-1}) on the 9^{th} d. The acetic acid and cell biomass concentration at the end of 9 d was 4.75 g L^{-1} and 0.46 g L^{-1} respectively.

In the second set of experiments, it was observed that pretreating CSL either by autoclaving or by centrifugation did not have a significant effect on ethanol and acetic production at the end of 15 d. Maximum ethanol (1.0 g L^{-1}) was produced in the Trt_AC treatment while maximum acetic acid (5.60 g L^{-1}) concentration was produced in the Trt_YE treatment. Statistical analysis indicated that pretreating CSL either by autoclaving or centrifuging (both treatments at 37°C and pH 6.0) was not observed to have a significant effect on ethanol production.

Together these results suggest that lowering the initial pH will not enhance the production of ethanol. Low temperature fermentation may give better ethanol and cell biomass concentration compared to other treatments. Hence it was necessary to test different levels of pH and temperature on syngas fermentation. Since autoclaving or centrifuging CSL did not have a positive impact on ethanol formation, no further testing of CSL pretreatment was conducted.

Effect of Buffer, pH and Temperature on Syngas Fermentation

Preliminary experiments indicated the possibility of improving the syngas conversion by reducing the fermentation temperature. A previous study indicated improvement in ethanol yield and possibility of decreasing the media cost by the removal of buffer (Kundiyana et al., 2010). The ethanol, acetic acid and cell optical density for the different treatment combinations of buffer concentration, incubation temperature and incubation pH levels observed in the 3 by 3 experiment are shown (Table 3.2).

Table 3.2. The experimental data for the tested process variables at the end of 15 d (n=3).

Treatment	Buffer[a]	pH[a]	Temperature[a]	Acetic Acid	Ethanol	Cell Density	pH
1	0	1	1	0.22	0.00	0.08	6.46
2	0	1	-1	6.35	0.58	1.07	4.77
3	0	-1	1	0.34	0.00	0.06	5.19
4	0	-1	-1	3.71	0.52	0.81	4.29
5	1	1	0	7.88	0.30	0.70	5.14
6	-1	1	0	1.60	1.65	0.84	4.50
7	1	-1	0	3.58	0.43	0.62	4.40
8	-1	-1	0	2.41	0.39	0.50	4.34
9	1	0	1	0.21	0.00	0.08	5.98
10	-1	0	1	0.28	0.00	0.08	5.47
11	1	0	-1	3.53	0.73	1.03	4.85
12	-1	0	-1	1.45	1.89	0.95	4.39
13	0	0	0	3.94	0.69	0.74	4.74

[a] See Table 3.1 for code values. Units for acetic acid and ethanol concentration are in g L^{-1} and cell density at A_{660nm}

Effect on Ethanol

Figure 3.1 compares ethanol production in 13 different treatments. Maximum ethanol concentration (1.89 g L^{-1}) was produced in the 12th treatment (No buffer, pH 6.0, 32 °C). In general ethanol concentration in all treatments except treatments 6 (No buffer, pH 7.0, 37 °C) and 12 were lower than 1 g L^{-1}. The control treatment (1% MES, pH 6.0, 37 °C) (treatment 13) was associated with a longer lag time (5 d) compared to the other treatments.

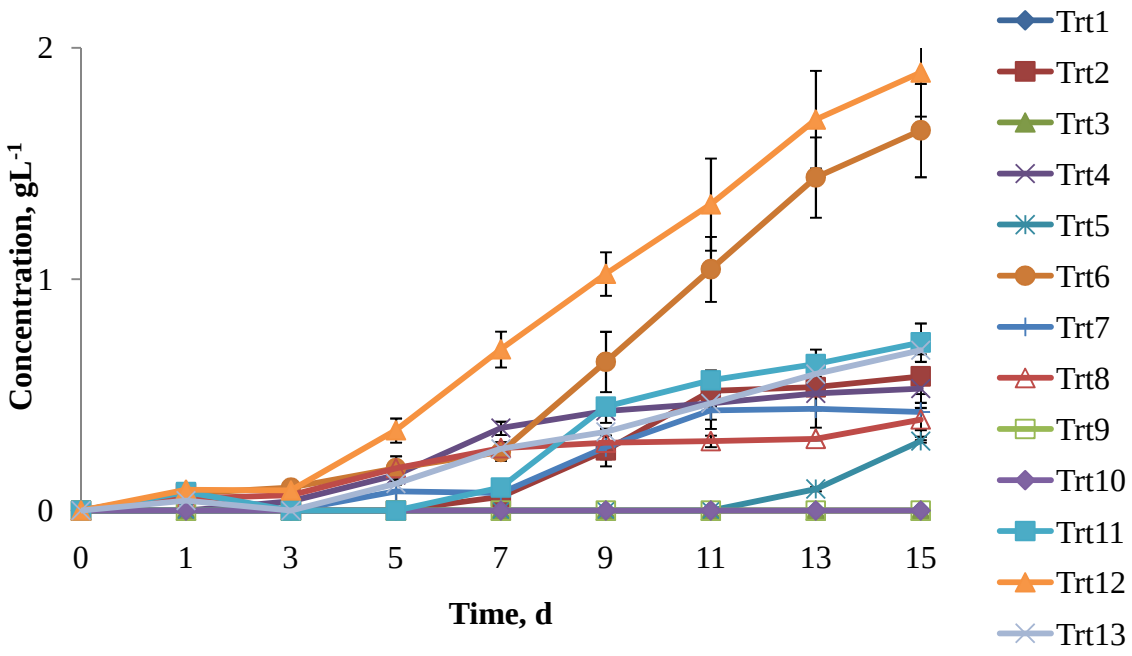

Figure 3.1. *Clostridium* strain P11 ethanol production profile for the thirteen different treatment conditions plotted as a function of time. The error bars represent the standard error (n=3).

The repeated measures ANOVA (within-subject effect, multivariate ANOVA analysis) showed that the main effects of buffer presence ($F_{7,182} = 43.85$, $p < 0.0001$), incubation temperature ($F_{7,182} = 119.58$, $p < 0.0001$) and incubation pH ($F_{7,182} = 16.54$, $p < 0.0001$) were significant during the course of the fermentation. The two way interaction between the temperature and buffer levels ($F_{7,182} = 20$, $p < 0.0001$), and between buffer and pH levels ($F_{7,182} = 47.62$, $p < 0.0001$) were found to be significant while the interaction between temperature and pH levels were not found to be significant ($F_{7,182} = 2.1$, $p = 0.0982$) during the course of fermentation. A lag time of at least 3 d in ethanol production was observed in all treatments. A fermentation temperature of 32 °C and removal of MES buffer were observed to have significantly positive effects on ethanol concentrations after 15 d. The interaction between temperature and presence or absence of buffer determined when the switch from acetogenesis to solventogenesis occurred, as it varied between the different treatments, and was observed to be

significant throughout the incubation period of 15 d. For instance, at 42 °C, no ethanol was produced at any buffer addition levels irrespective of the fermentation pH levels. At 32 and 37 °C, the switch was delayed with increasing buffer concentration. Similarly, comparing the interaction between buffer addition and pH levels, it was observed that irrespective of the fermentation pH, treatments with no buffer addition switched to solventogenesis on the 3rd d. The lag time in switch increased with the increase in buffer addition levels.

Effect on Acetic Acid

Figure 3.2 shows the acetic acid production profile for *Clostridium* strain P11 for the 13 treatments as a function of time. Maximum acetic acid concentration was produced (7.88 g L^{-1}) in the treatment at pH 7.0, 37 °C and with 2 g L^{-1} added buffer. This concentration of acetic acid was higher than previous studies using *Clostridium* stain P11 (Ahmed et al., 2006; Kundiyana et al., 2010).

The main effects of concentration of buffer addition ($F_{1, 26} = 37.18$, $p < 0.0001$), incubation temperature ($F_{1, 26} = 393.8$, $p < 0.0001$), and incubation pH ($F_{1, 26} = 29.09$, $p < 0.0001$) were found to be significant for acetic acid production. Statistical analysis also indicated that the interaction effect between temperature and buffer ($F_{1, 26} = 24.7$, $p < 0.0001$), and temperature and pH ($F_{1, 26} = 24.79$, $p < 0.0001$) was significant. The interaction between buffer and pH ($F_{1, 26} = 2.45$, $p = 0.1293$) was observed to be not significant. An incubation temperature of 32 °C gave a higher concentration of acetic acid compared to 37 and 42 °C. Buffer addition showed a mixed production profile with 1% concentration resulting in a higher acetic acid concentration compared to 2% or no buffer for the first 4 d of fermentation. From the 4th d until the end of fermentation, 2% buffer concentration resulted in a higher concentration of acetic acid than 1% or no buffer. Similar acetic acid production profile was observed with pH 7.0 resulting

in higher acetic acid production compared to other incubation pH levels. The repeated measures ANOVA showed that within the treatment levels (within-subject effects), the main effects of buffer concentration ($F_{7,182} = 12.82$, $p < 0.0001$), pH level ($F_{7,182} = 4.25$, $p = 0.0013$) and incubation temperature ($F_{7,182} = 15.43$, $p < 0.0001$) were significantly at different sampling times. The interaction between temperature and buffer ($F_{7,182} = 2.15$, $p = 0.0627$), and temperature and pH ($F_{7,182} = 1.74$, $p = 0.1286$) at different times was not significant at different sampling time points. Since buffers typically modulate the fermentation pH, a significant two-way interaction was observed between buffer concentration and pH levels ($F_{7,182} = 4.66$, $p = 0.0006$) as the fermentation progressed.

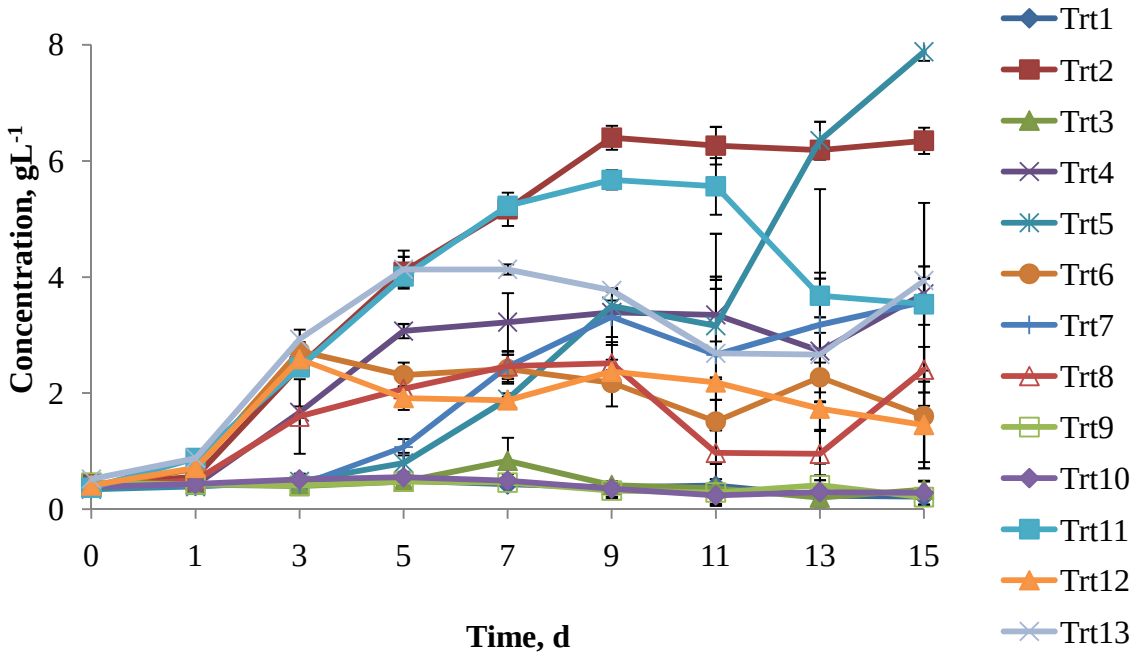

Figure 3.2. *Clostridium* strain P11 acetic acid production profile for the thirteen different treatment conditions plotted as a function of time. The error bars represent the standard error (n=3)

Effect on Cell Biomass

Cell biomass concentration showed an increasing trend in the first 5 d before leveling off (Figure 3.3). In general, the treatments incubated at 42 °C showed minimal or no growth. Fermentations at 32 °C showed higher cell densities than fermentations at 37 °C with maximum cell concentration observed in treatment 2 (Abs_{660nm} = 1.34). This may have been due to the increased solubility of CO and H_2 in the fermentation medium at 32 °C as opposed to the higher temperatures; thereby, providing more available substrate for the fermentation. However, CO and H_2 concentrations in the media were not measured. From the cell density profile, it was also observed that a combination of pH 5.0 and higher fermentation temperature (42 °C) offered an unfavorable environment for cell growth and cell metabolism as was previously noted by the lack of ethanol production in this treatment.

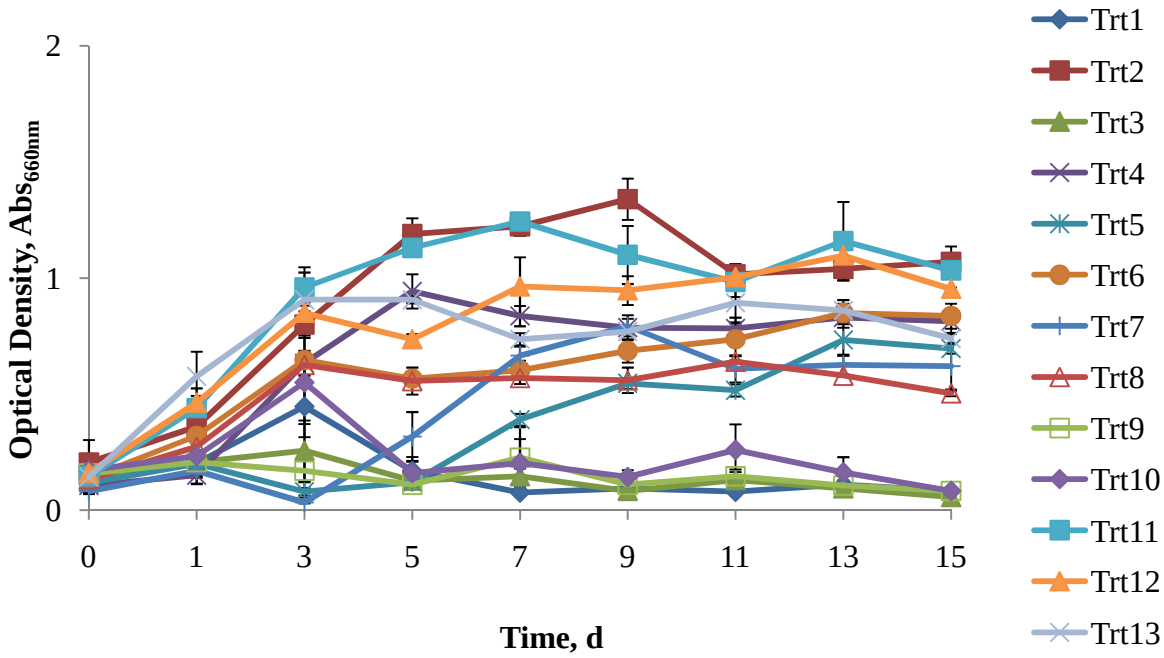

Figure 3.3. *Clostridium* strain P11 cell mass concentration profile for the thirteen different treatment conditions plotted as a function of time. The error bars represent the standard error (n=3).

The cell density plot is further supported by repeated measure ANOVA , which indicates that the two way interactions between the tested variables (between-subjects effect) were significant ($F_{1,26}$, $p< 0.05$). The statistical analysis indicated that at different sampling times, buffer concentration ($F_{7,26} = 27.97$, $p< 0.0001$) and temperature ($F_{7,26} = 5.94$, $p< 0.0001$) was significant. Pair-wise t-tests (Least Square Difference) further indicated that at all sampling times, the treatment containing no buffer and incubation temperature of 32 °C had density higher cell mass concentration than other treatments, thereby substantiating the positive effect of syngas fermentation at 32°C and without buffer.

Discussion

In this study we have observed different times at which acidogenesis shifted to solventogenesis depending on the treatment conditions. Unless the fermentation was conducted at higher temperature and buffer levels, treatments at different levels of buffer, pH and temperature showed production of ethanol along with cell growth and acetate production (Table 3.2). This contradicts an earlier study that stated ethanol production was only produces in non-growth conditions (Ahmed et al., 2006). Concurrent increase in ethanol and acetic acid concentration during cell growth possibly has been cited because the inoculum used for the fermentation may be in different stages of growth (Vega et al., 1989). This means that some of the cells may be in lag phase, some in log phase and some in stationary phase. Due to this reason, cells will exhibit acetic acid and ethanol production along with increase in cell mass concentration.

Process parameters, such as temperature, pH, and media buffers added to regulate fermentation pH interact in a complex manner and are known to affect acetogenic metabolism (Dabrock et al., 1992). In this study we have observed a significant interaction between buffer

and temperature on acetic acid and ethanol formation and on bacterial growth. The influence of high fermentation temperature of 42 °C and starting incubation pH of 5.0 was observed to negatively impact *Clostridium* strain P11 metabolism. Previous studies in our laboratories with cotton seed extract as the only media component in syngas fermentation have shown that buffer addition did not significantly improve ethanol concentration after 15 d of incubation time. However, the presence of buffer was associated with higher accumulation of acetic acid (Kundiyana et al., 2010). Bryant and Blaschek (1988) have indicated that accumulation of acetic acid and butyric acid was necessary for subsequent ethanol and butanol formation during different clostridial fermentations. Similar observations were made in fermentation with *C. acetobutylicum* where it was observed that the level of butyrate needed to switch from acidogenesis to solventogenesis was dependent on the external pH of the fermentation medium (Monot et al., 1984). They also observed that high levels of fatty acids were required to induce solventogenesis. Lowering fermentation pH resulted in the decrease of unsaturated fatty acid content. The "fatty acid effect", which is the ability of weak acids to abolish the transmembrane pH-gradient along with suitable concentration of acetic acid, has been shown to induce solventogenesis in *C. acetobutylicum* strains (Gottschal and Morris, 1981; Holt et al., 1984). In general the majority of prokaryotes are neutrophiles with pH optima falling between pH 5 and 9. During bacterial growth production of extracellular products, such as organic acids, changes the fermentation pH and the cells counteract the situation by displaying metabolic properties not exhibited at their optimal pH range. One such phenomenon is the production of non-acidic products, such as ethanol, resulting in the shift from acidogenesis to solventogenesis. Weak organic acids in their undissociated form are lipophilic and diffuse through cell membranes. During this process they conduct H^+ ions along the transmembrane gradient resulting in the lowering of intercellular pH (Overmann, 2006). At lower intercellular pH, the impact of

extracellular pH is magnified, which imposes a physiological stress on the bacterial cells and the cells possibly counteract the situation by producing solvents (Ahmed et al., 2006). Overmann (2006) suggested the use of a buffer to regulate fermentation pH in the optimal range. In contrast, we observed that absence or lower concentration of buffer may have initiated solvent production earlier than the treatment with 2% buffer addition. In this study when pH dropped below 5.0, un-ionized acetic acid regulated the pH gradient between the medium and the intercellular space by moving passively into the cell, a mechanism which previously was found necessary for production of ethanol with a subsequent cessation of acetic acid production (Baronofsky et al., 1984). On the other hand, fermentation treatment at pH 5.0 may have exhibited an unfavorable environment for cell growth and adaptation leading to lower cell concentrations during fermentation (Huhnke et al., 2008). The results from the present study suggest that lowering the initial pH is not favorable for ethanol production. Production of alcohols in the solventogenic phase also corresponds with the reduction of acids to ethanol. It has been shown that acid concentration produced in the acidogenic stage is necessary for the production of solvent in the solventogenic stage (Worden et al., 1991).

From the *Clostridium* strain P11 growth profile, even though ethanol was produced during the growth phase, the productivity of ethanol was enhanced during the non-growth stage (Ahmed et al., 2006). Formation of ethanol further inhibited bacterial growth resulting in a decrease in cell density. The exact mechanism by which alcohols inhibit bacterial growth is not understood; however, it is speculated that the formation of alcohols interferes with membrane physiology by partitioning the lipid bilayers. Furthermore, they interact with the lipid-lipid and lipid-protein interactions with a net effect of decreasing the membrane viscosity (Lepage et al., 1987).

Syngas fermentation temperature impacts microbial growth and solubility of syngas components. Influence of temperature on ethanol production is two-fold. First, solubility of gases in aqueous media decreases with increasing temperature. Typically, acetogenic bacteria are mesophilic bacteria optimally growing between 37 and 40 °C (Munasinghe and Khanal, 2010). However in this study, we have shown the ability of *Clostridium* strain P11 to grow and produce more ethanol at 32 °C compared to its standard incubation temperature of 37 °C. According to Henry's Law, gas solubility increases with decreasing temperature, hence it is possible that at 32 °C, molar concentrations of CO and H_2 increased in the fermentation medium due to increased solubility, thereby improving the overall gas-liquid mass transfer rates and increased availability of reducing equivalents for the fermentation process. Increased availability of dissolved substrate could have resulted in increased cell densities and higher product yields compared with treatments at 37 and 42 °C. Second, temperature has been shown to affect membrane lipid composition and the compositional adaptation of membrane lipids via 'homeoviscous adaptation' (Sinensky, 1974). The general response of this adaptation leads to decrease in fatty acid chain length and degree of unsaturation at higher fermentation temperatures (Lepage et al., 1987).

Conclusion

Findings from this study indicate that it is a feasible option to conduct syngas fermentation at 32 °C without any buffer addition. This holds promise for the development of an economical syngas fermentation process. Temperatures above 37 °C are above the optimal temperature range for growth and ethanol production by *Clostridium* strain P11. Conducting fermentation at an incubation pH less than 6.0 did not reduce lag time and was not associated with increased ethanol concentrations.

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CHAPTER IV: Effect of Nutrient Limitation and Two-Stage Continuous Fermentor Design during *Clostridium strain* P11 Syngas Fermentation

Abstract

The effect of three limiting nutrients, calcium pantothenate, vitamin B_{12} and cobalt chloride ($CoCl_2$), on syngas fermentation using Clostridium strain P11 was determined using serum bottle fermentation studies. Significant results from the bottle studies were translated into single- and two-stage continuous fermentor designs. Studies indicated that three-way interactions between the three limiting nutrients, and two-way interactions between vitamin B_{12} and $CoCl_2$ had a significant positive effect on ethanol and acetic acid formation. Ethanol and acetic acid production ceased at the end of 9 d. Reactor studies indicated the three-way nutrient limitation in two-stage fermentor showed improved acetic acid and ethanol yield compared to treatments in single-stage fermentors. In the two-stage fermentor during the end of the fermentation, the redox values were positive, resulting in the cessation of solvent production. This was further confirmed by hydrogenase activity, which showed decreasing activity as the fermentation progressed, possibly due to CO inhibition. Results indicate that it is possible to modulate the product formation by limiting key nutrients, while use of continuous fermentation in two-stage fermentor design improves product yields.

Keywords: *Ethanol, Syngas, Clostridium, Nutrient Limitation, Two-stage fermentor*

Introduction

Gasification has been identified as a potential process in converting biomass substrate for subsequent fermentation into secondary metabolites such as ethanol (Ragauskas et al., 2006). Compared to other processes, gasification overcomes the major hurdle in utilizing the recalcitrant lignin, along with hemicelluloses and cellulose fraction of the biomass. The lignin fraction is not directly available for microbial conversion. Biomass gasification produces synthesis gas (syngas) in which CO and H_2 are the essential components for subsequent ethanol production from all organic compounds.

Compared to chemical processes, fermentation of syngas offers several advantages such as higher specificity of biocatalysts, lower energy costs, greater resistance to catalyst poisoning and the lack of need for a fixed CO:H_2 ratio (Bredwell et al., 1999; Klasson et al., 1992). Anaerobic bacteria such as *Clostridium ljungdahlii* and *Clostridium cabroxidivorans* convert biomass generated producer gas composed of CO, CO_2, and H_2 to ethanol and acetic acid (Ahmed et al., 2006; Vega et al., 1990). Organisms able to reduce CO_2 to acetate via the Wood-Ljungdahl pathway (also referred to as acetyl-CoA pathway) are termed acetogens. Acetogens are a group of bacteria which synthesize acetate from CO_2 or other one carbon precursors.

Previous studies have indicated that production of ethanol is favored under bacterial non-growth conditions (Ahmed et al., 2006). The reduction of acetic acid to ethanol with the formation of acetaldehyde as an intermediate has been proposed conforming with the Wood-Ljungdahl pathway (Phillips et al., 1994). These reduction/oxidation reactions also oxidize NAD(P)H to $NAD(P)^+$. Different reducing agents such as benzyl viologen, methyl viologen and neutral red have been used to regenerate NAD(P)H to increase ethanol production and reduce acetate concentration during syngas fermentation (Ahmed et al., 2006; Klasson et al., 1992).

Gaddy et al. (2007) recently described a method of increasing the NAD(P)H in *C. ljungdahlii* cells by limiting key nutrients, namely calcium pantothenate, cobalt chloride and vitamin B_{12}. Calcium pantothenate is a precursor of acetyl-CoA and by limiting its concentration, the rate of acetyl-CoA formation will be reduced relative to the CO oxidation rate in the Wood-Ljungdahl pathway. This will result in the build-up of reduced ferredoxin, resulting in regeneration of NAD(P)H. Cobalt is involved during the transfer of the methyl group of methyl tetrahydrofolate to the cobalt center of the corrinoid iron sulfur protein (C/FeSP) (Ragsdale and Pierce, 2008). Vitamin B_{12} plays an important role during acetogenesis as an integral component of the cobalamin-dependent methyl transferase synthase (MeTr) (Das et al., 2007; Lu et al., 1993). It is assumed that by reducing the tetrahydrofolate cycle rate, the ratio of NAD(P)H to NADH can be increased with subsequent increase in ethanol production (Figure 1).

A two-stage reactor comprising of a growth reactor and a product reactor has been proposed to meet the requirements of cell growth and ethanol production during syngas bacterial fermentation (Gaddy et al., 2007; Jain et al., 1993). Multi-stage fermentors were shown to have a higher ethanol productivity compared to single stage bioreactors in *Saccharomyces cerevisiae* fermentation (Chen, 1990; Laluce et al., 2002). Multi-stage fermentor systems with cell recycling were observed to be advantageous compared to cell immobilization techniques due to improved diffusion and total recycling of microorganisms creating a homogenous fermentation broth (Tashiro et al., 2005). Use of hollow fiber recycle membranes for cell recycling has been shown to offer several advantages due to high filtration area and relatively high flux due to cross filtration, but are associated with disadvantages such as low strength compared to ceramic or steel filters and inability for steam sterilization (Damiano et al., 1985).

The broad objective for the present study was to test the hypothesis of improving ethanol production during syngas fermentation by limiting key nutrients of the Wood-Ljungdahl

pathway. Specific objectives for the study were to test the effects of: a) limiting calcium pantothenate, vitamin B_{12} and $CoCl_2$ on ethanol production during syngas fermentation, and b) To design a continuous syngas fermentation system testing the significant interactions between the limiting nutrients.

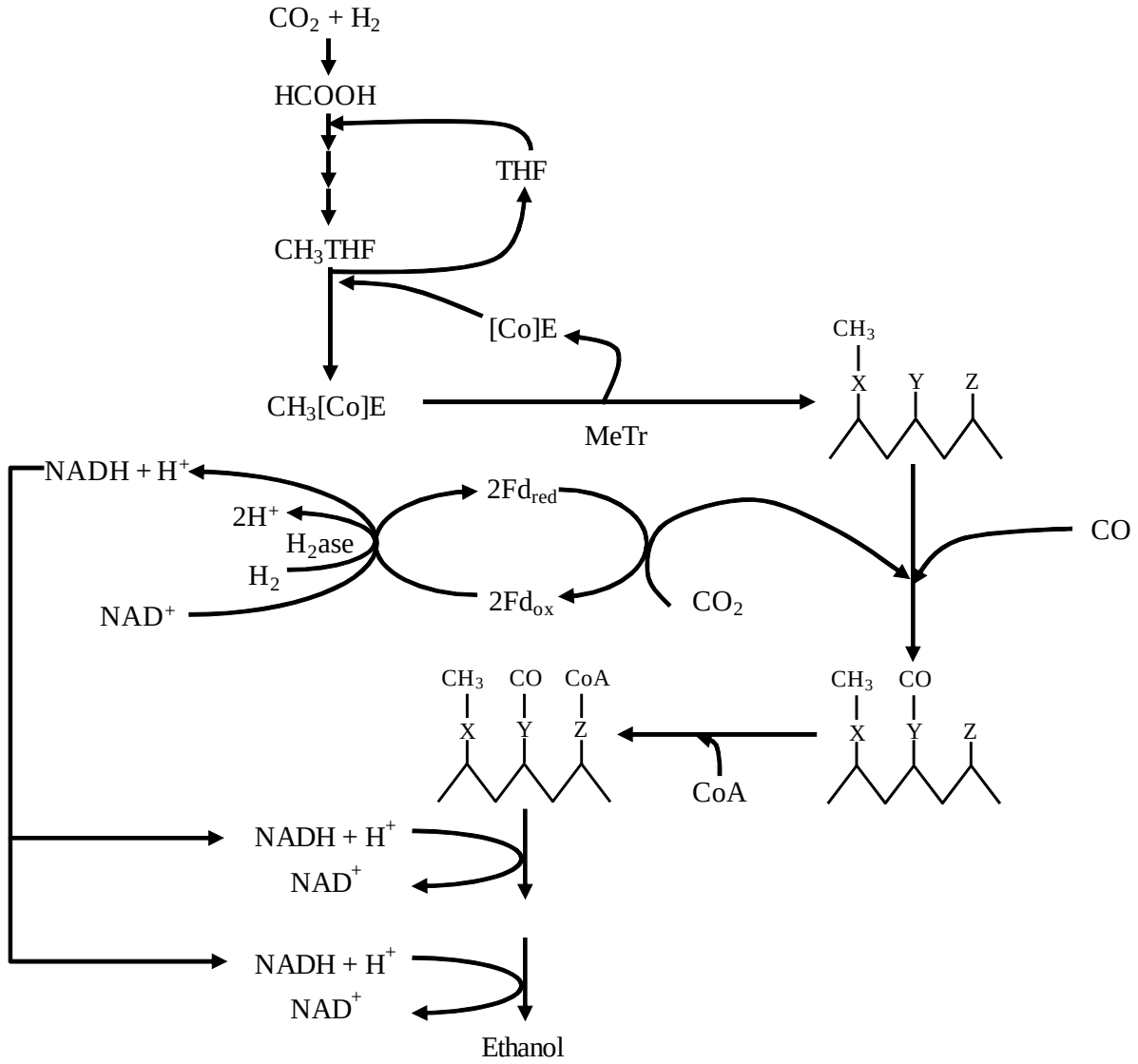

Figure 4.1. Modified Wood-Ljungdahl Pathway showing the mechanism of growth with CO or CO and H_2 as the carbon and energy source. THF- tetrahydrofolate, [Co]E-corrinoid enzyme, X Y Z- CO dehydrogenase with its three binding site, H2ase- hydrogenase, CoA-coenzyme A, MeTr- methyl transferase synthase. Fd_{ox} and Fd_{red} are the oxidized and reduced forms of ferredoxin. (Adapted and modified from Pezacka and Wood (1984); Wood (1991)).

Materials and Methods

Microbial catalyst and culture medium

Clostridium strain P11^T (ATCC PTA-7826) was originally obtained from Dr. Ralph Tanner, University of Oklahoma. The bacterial strain is currently maintained in our laboratory, and was subcultured and used as the starting inoculum at the rate of 10% of the working volume. All subculture fermentations were conducted in 250-mL serum bottles each with a 100 mL final working volume. The inoculum was prepared by a procedure termed as 'passaging' wherein the inoculum was sub-cultured sequentially with an incubation period of 48 h. The third passage was used as the inoculum for all treatment fermentations. The bacterium was maintained under strictly anaerobic conditions in a standard medium containing following components (per L): 30 mL mineral stock solution, 10 mL trace metal stock solution, 10 mL vitamin stock solution, 10 g corn steep liquor (CSL), 10 g morpholinoethanesulfonic acid (MES), 10 mL of 4% cysteine sulfide solution, and 0.1 mL 1% resazurin indicator (Tanner, 2002). A detailed composition of the stock solutions has been previously described (Kundiyana et al., 2010). CSL was centrifuged at 20,000 g for 10 min in a micro-centrifuge to remove the solids and the supernatant added to the fermentation medium. Removal of solids was found to be necessary to minimize the variability in subculture preparation because of the varying solids concentration in CSL, and to avoid solid precipitation after sterilization which was found to be interfering with cell biomass optical density estimation. The subculture bottles were purged with commercial syngas (Composition (volume %): 5% H_2, 15% CO_2, 20% CO, and 60% N_2) every 24 h and incubated at 37 °C with a constant agitation of 150 rpm on an orbital shaker.

Analytical Procedures

Samples were collected every 48 h for determining the time course for bacterial cell biomass, substrate, and product concentrations depending on the fermentation protocol. The cell biomass was determined by measuring sample absorbance at a wavelength of 660 nm (A_{660nm}) in 1-cm pathlength cuvettes using a UV-visible spectrophotometer (Cary 50 Bio, Varian, USA). Ethanol and acetic acid were separated by HPLC with an Aminex-HPX-87H ion exchange column (Biorad, Hercules, CA, USA). The eluent was 0.01N H_2SO_4 pumped at 0.6 mL min^{-1} and column temperature was 60 °C (Sluiter et al. 2005). Separated components were detected by a refractive index detector (1100 Series, Agilent, Santa Clara, CA, USA). Peaks were identified by comparison with known standards (Sigma-Aldrich, St. Louis, MO, USA).

Gas samples were collected every 24 h from the reactor headspace and analyzed for syngas component utilization. The total run time for sample analysis was 20 min. A ramped temperature profile was followed in the oven for resolving the overlapped peaks (32 °C for 12 min, then ramped to 236 °C at the rate of 30 °C min^{-1}). The separated components were detected using a thermal conductivity detector (TCD) maintained at an inlet and outlet temperatures of 200 °C and 230 °C respectively. Oxidation-reduction potential (ORP) of the fermentation broth was measured using a Ag/AgCl reference ORP electrode (InPro 3200/SG, Mettler Toledo, USA) attached to a transmitter (M400, Mettler Toledo, USA) and the data acquired using pDAQ 56 (Iotech Corporation, USA). Redox potential (E_h) was calculated vs. standard hydrogen electrode (SHE) using equation 1.

$$E_h = E_c + 222.4 \text{ mV} \quad [1]$$

where, E_c was the measured redox value and +222.4 mV is the standard electrode potential of Ag/AgCl electrode (Kakiuchi et al., 2007).

Analysis of variance of data was performed using the Mixed Procedure of SAS Release 9.1 (SAS, Cary, NC, USA) with a compound variance covariance structure and repeated measure design. Level of significance was tested at $\alpha = 0.05$.

Serum Bottle Fermentation

A two-level, three-factor design was used to determine the limiting effects of calcium pantothenate, vitamin B_{12} and $CoCl_2$ (Table 4.1). 'High' level concentration for the limiting nutrients was chosen based on the individual concentrations in the *Clostridium* strain P11 standard medium (Kundiyana et al., 2010). 'Low' level concentrations were chosen based on the concentrations previously indicated in similar nutrition limitation studies except for vitamin B_{12}, which was 2.5 times lower than the 'High' level. The nutrients were tested using a 2^3 factorial statistical design.

Table 4.1. Concentrations of the three limiting nutrients

Variables	Levels	
	High (1)	**Low (0)**
Calcium pantothenate (A)	5.0×10^{-5} g L^{-1}	5.0×10^{-6} g L^{-1}
Vitamin B_{12} (B)	5.0×10^{-5} g L^{-1}	2.0×10^{-5} g L^{-1}
Cobalt chloride (C)	2×10^{-3} g L^{-1}	2.0×10^{-5} g L^{-1}

Table 4.2 shows the experimental design for each of the treatment bottles and their corresponding levels of individual nutrients. Factorial design experiments are advantageous in identifying critical factors compared to the traditional method of manipulating a single variable per trial since factorial design can identify main and interaction effect between tested variables. Factorial design and RSM have been used previously in optimizing culture conditions, optimizing process parameters such as pH, temperature, and aeration rate, and the feeding rate (Kalil et al., 2000).

Table 4.2. Experimental plan showing the treatment and the levels of tested independent variables. See Table 4.1 for the reference to individual levels. (n=2).

Treatment	Calcium Pantothenate	Vitamin B_{12}	$CoCl_2$
P	1	0	0
B	0	1	0
C	0	0	1
PB	1	1	0
PC	1	0	1
BC	0	1	1
PBC	1	1	1
CONT	0	0	0

Treatment bottles were prepared in 250 mL serum bottles with 100 mL working volume and inoculated with 10% *Clostridium* strain P11 inoculum from passage 3. The bottles were pressurized with a customized syngas mixture of 30% CO_2 , 40% CO, 30% H_2 to 239 kPa. Each treatment was conducted in duplicate and incubated at 37 °C for 21 d on an orbital shaker at 150 rpm. Headspace gas was exchanged every 24 h by flushing the treatments for 3 min.

Fermentor Studies

Results from serum bottle studies were replicated in series of continuous stirred tank fermentors. Two different reactor configurations were considered in 3-L fermentors with a maximum 2 L working volume. The first configuration included a single fermentor (Bioflo 110, New Brunswick, NJ USA) operated in a continuous mode with cell recycling using a 0.2 µ hollow fiber membrane (Model KRB12-3PS, GE HealthCare, USA) (Figure 4.2). Retentate returned from the recycle membrane contained cells, while cell-free permeate was collected downstream.

The fermentor was operated in a semi-continuous mode (syngas with a composition of H_2- 30%, CO_2- 30%; CO- 40% sparged in continuous mode and fermentation in batch mode) during the growth stage typically for 3 d. At the end of 3rd d, the operation was switched to

continuous mode and fresh fermentation media was added to the fermentor, composition of which was determined by experimental treatment.

The second reactor configuration involved design of two stirred-tank fermentors of equal volume in series with partial cell recycle (Figure 4.2). In this reactor scheme, the first reactor functioned as a 'Growth Reactor' (GR) and the second reactor as a 'Product Reactor'(PR). The GR was operated in semi-continuous mode (without cell recycle) for the first 3 d and then operated on a continuous mode (with cell recycle) until Day 10. On Day 7, 1.8 L of the media was flushed from the GR and the volume made with fresh limiting media. On Day 10, cell recycle was stopped on the GR, and PR was brought online with a working volume of 1 L. The PR was added with the inoculum from GR to a working volume of 2 L. Working volume in the GR was made up to 2 L by feeding in fresh media. Both fermentors were operated in semi-continuous mode for the next 3 d. At the end of Day 3, PR was operated in continuous mode with a constant media plus cells fed from the GR. The volume in the GR was continuously made up with fresh limiting media. Both the fermentors operated in a steady state in the continuous mode. The gas strategy in the second fermentor configuration differed from the first fermentor configuration as it flowed in the counter current direction to the feed media (i.e. syngas was first purged into the PR and the exhaust then sparged through the GR). Our previous studies have shown that during the solventogenic phase, increased consumption of H_2 and CO_2 was observed (Kundiyana et al., 2010). Based on this observation, an assumption was made that the increased availability of reducing equivalents may increase ethanol production.

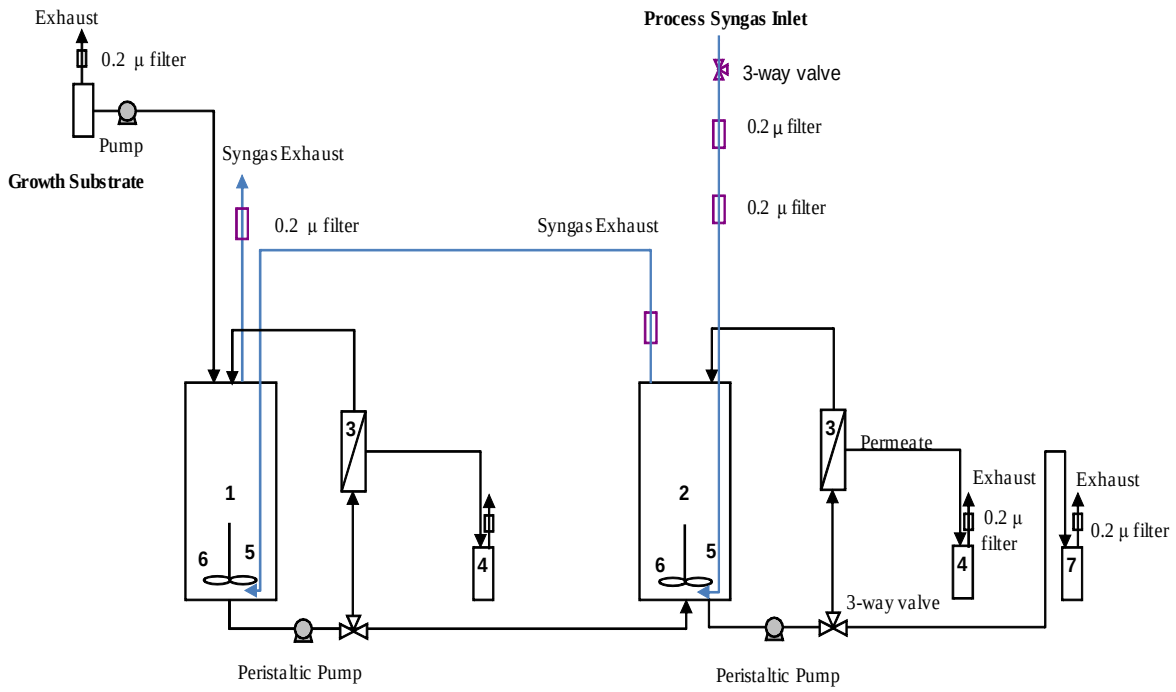

Figure 4.2. Schematic diagram of the continuous stirred tank reactor set-up to test the effect of the three limiting nutrients in single stage and two-stage reactor design. Single stage reactor was used for the first three treatments and the two stage reactor was used to test the nutrient limitation treatment. (1- Growth reactor; 2- Product reactor; 3- 0.2 µ hollow fiber membrane; 4- Permeate collection vessel; 5- Syngas sparging through a micro-sparger; 6- agitator with rushton impellers; 7; Bleed collection)

Syngas was bubbled through a single orifice sparger attached with a micro-sparger with a mean pore size of 2 µ (Mott Corporation, USA) at the rate of 100 cc min^{-1}. Positive impact of micro-spargers on gas mass transfer limitations has been described previously (Birch and Ahmed, 1996; Kundiyana et al., 2010). The fermentor was constantly stirred at 100 rpm using an agitator assembly with two Rushton impellers.

Specific growth rate (µ, equation 2) was calculated using the measured cell densities during the initial growth phase. The specific growth rate (µ) was subsequently used to determine the dilution rate (D) for each fermentor operation. Using the substrate feed-rate, and the known values transfer tube diameter (0.0048 m) and the pump capacity (60 Hz, 14.4 rpm) of 23.5 mL

min^{-1}, the peristaltic feed pump setting on the fermentor was calculated. Permeate from the recycle module was collected in a separate reservoir.

$$\mu = \frac{\ln x_2 - lnx_1}{t_2 - t_1} \quad [2]$$

$$D = \frac{F}{V} \quad [3]$$

At steady state,

$$\mu = D \quad [4]$$

where,

F- flowrate of the feed media (L h^{-1})

V- working volume of the reactor, L

x_t- Cell density at time t

Results and Discussion

Productivity profile during fermentation can be altered by optimizing the chemical and physical environment of the process to which the fermenting organism is exposed. In this study, the effect of some of the key limiting nutrients involved in the Wood-Ljungdahl pathway have been tested in an effort to improve the syngas conversion efficiencies. In addition, a unique two stage continuous fermentor design was used to test the significant effects from the serum bottle fermentation in fermentor studies.

Serum Bottle Fermentation

A 2^3 factorial incomplete block design was designed to test the effect of the three limiting nutrients, calcium pantothenate, vitamin B_{12} and $CoCl_2$. Figures 4.3 and 4.4 show ethanol and acetic acid productivity for the eight treatments. Acetic acid production began after Day 1 and

production of ethanol began after Day 3 for most treatments. The overall effect of the treatments ($F_{7,8}$, $p < 0.05$) and the interaction of treatment with time ($F_{77,88}$, $p < 0.0001$) were observed to be significant for both acetic acid and ethanol production. The high concentration of calcium pantothenate resulted in greater concentration of acetic acid and ethanol after 9 d. However, the two-way interaction between vitamin B_{12} and $CoCl_2$ was significant for ethanol production ($F_{1,88}$, $p = 0.0002$). In general, both acetic acid and ethanol production ceased after 9 d.

Treatments BC, PC, and control produced the highest ethanol concentrations after 9 d with maximum ethanol produced in the treatment with three limiting nutrients (2.15 g L^{-1}) at the end of 21 d. Treatments with the higher level of calcium pantothenate and vitamin B_{12} produced less ethanol than treatments with lower level of these nutrients over the course of the experiment. At the end of 9 d when ethanol production ceased, calcium pantothenate was observed to have a significant effect on ethanol production ($p<0.05$). The two-way interaction is evidenced by the fact that the mean ethanol production for treatments containing high levels of both vitamin B_{12} and $CoCl_2$ or low levels of both vitamin B_{12} and $CoCl_2$ were 1.67 and 1.84 g L^{-1}, respectively. However, mean ethanol production was less than 0.81 g L^{-1} for treatments where either vitamin B_{12} or $CoCl_2$ was at a high level and the other was at a low level.

All treatments produced more than 3 g L^{-1} acetic acid after nine days with treatments vitamin B_{12} and $CoCl_2$ limitation producing the greatest concentrations of acetic acid after 9 d. Maximum acetic acid concentration of 5.19 g L^{-1} was produced in the treatment with $CoCl_2$ limitation at the end of 21 d. The three way interaction between calcium pantothenate, $CoCl_2$ and vitamin B_{12} was observed to be significant at all sampling times ($F_{11,88}$, $p < 0.0001$). In general, acetic acid production began to decrease after Day 5, which corresponds to when the cells began to produce more ethanol.

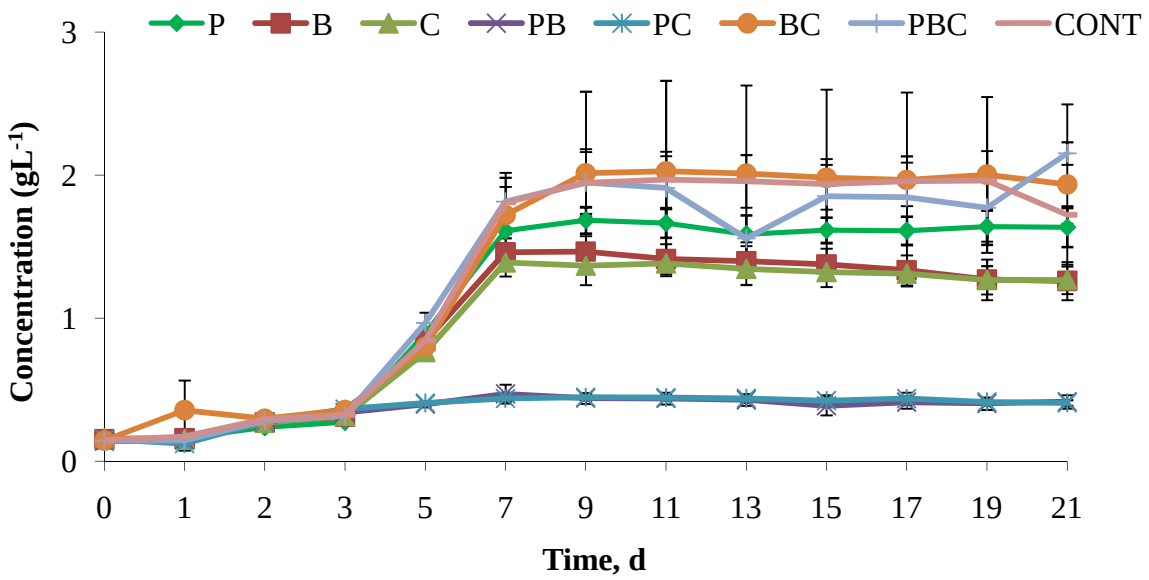

Figure 4.3. Ethanol production profile for the serum bottle study testing the three limiting nutrients in a 2^3 factorial design. (n = 2) (P: calcium pantothenate limitation; B: vitamin B_{12} limitation; C: $CoCl_2$ limitation; Cont: Standard *Clostridium* strain P11 medium)

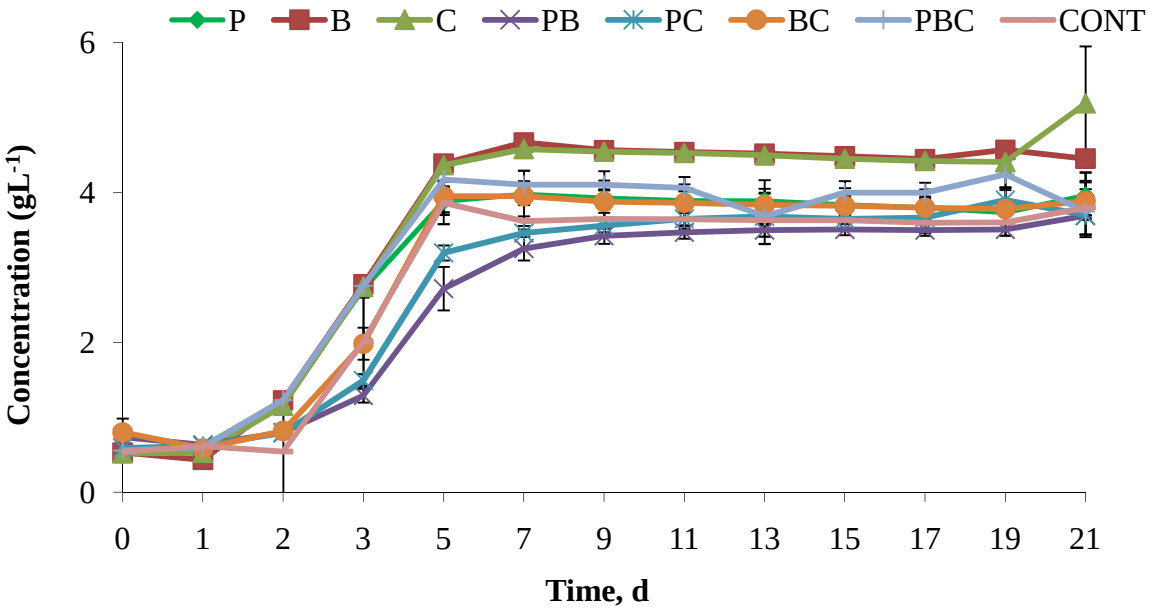

Figure 4.4. Acetic acid production profile for the serum bottle study testing the three limiting nutrients in a 2^3 factorial design. (n= 2) (P: calcium pantothenate limitation; B: vitamin B_{12} limitation; C: $CoCl_2$ limitation; Cont: Standard *Clostridium* strain P11 medium).

Fermentor Studies

Results from the serum bottle fermentations showed that two-way interaction between vitamin B_{12} and $CoCl_2$ was significant for ethanol production, and the three-way interaction between calcium pantothenate, vitamin B_{12} and $CoCl_2$ was significant for acetic acid product. These results were further translated into single stage and two stage fermentor studies. In the single stage fermentor design the three treatments tested were: Control (Trt1): Treatment with standard *Clostridium* strain P11 fermentation medium; Trt2: Treatment testing the limiting concentration of vitamin B_{12} and $CoCl_2$; Trt3: Treatment testing the limiting concentration of calcium pantothenate, vitamin B_{12} and $CoCl_2$. The single stage fermentor study indicated that the product yield in Trt 3 was higher compared to the other treatment which was subsequently tested in the two-stage series reactor design. Figures 4.5 and 4.6 show the ethanol and acetic acid yields for the reactor studies. In general, it was observed that the product yields were higher when the cell biomass concentration in the corresponding fermentor was higher. When Control, Trt2 and Trt3 (all treatments were operated as single stage with different nutrient treatment) are compared, it can be seen that Trt3 was associated with higher ethanol and acetic acid yields. When the reactor designs were compared with similar nutrient limitation, i.e. Trt3 with single stage and Trt 4 with two stage, the ethanol and acetic acid concentrations were higher in Trt4 (two stage reactor with the same media as Trt3). The control treatment (standard *Clostridium* strain P11 fermentation medium) was associated with a longer lag time and a concomitant decrease in acetic acid production, a trend commonly observed in similar syngas fermentation studies using this medium (Ahmed et al., 2006; Kundiyana et al., 2010; Kundiyana et al., 2010). The higher product yields associated with treatments with one or more of limiting nutrients, indicates that the tested nutrients are part of the complex nutrients playing a key role in the activity of enzymes involved in modulating the Wood-Ljungdahl pathway. The nutrients might

have resulted in the build-up of NAD(P)H before the ethanol production stopped (Gaddy et al., 2007). Furthermore, the utilization of cell recycling might have contributed to the increased product yields since cell-recycling has been shown to offer homogeneity of the fermentation medium, allow total recycling of microbial cells and overcome diffusional limitations (Ferras et al., 1986). When cell recycling was coupled to a two stage fermentor design (Trt4), product concentrations were higher compared to a single stage fermentor design. Similar two-stage fermentor designs in which, the strategy of operating the first reactor as a growth reactor and the second reactor as product reactor, and by using cell bleed with high cell densities from the growth reactor to be reused as inoculum in the second reactor has been shown to be associated with higher ethanol production rates (Ben Chaabane et al., 2006; Nishiwaki and Dunn, 1999; Nishiwaki and Dunn, 2005). The authors also indicate that it is necessary to achieve high cell mass concentration to attain higher productivities (Nishiwaki and Dunn, 2005).

However, one of the reasons for low ethanol yield in this run was due to low cell biomass concentration of *Clostridium* strain P11, a factor which was found necessary for higher ethanol concentration in previous study with *C. ljungdahlii* (Gaddy et al., 2007). When the observed biomass data was fit to the logistic equation, it was observed that the carrying capacity coefficient (k_c) for the four treatments 1, 2, 3 and 4 were 0.21, 0.19, 0.12, 0.21 (GR) and 0.01 (PR), respectively (Shuler and Kargi, 2002). The apparent decrease in cell biomass concentration in response to nutrient limitation might have resulted in the cells switching to solvent production to reduce the demand for ATP (Hill et al., 1993).

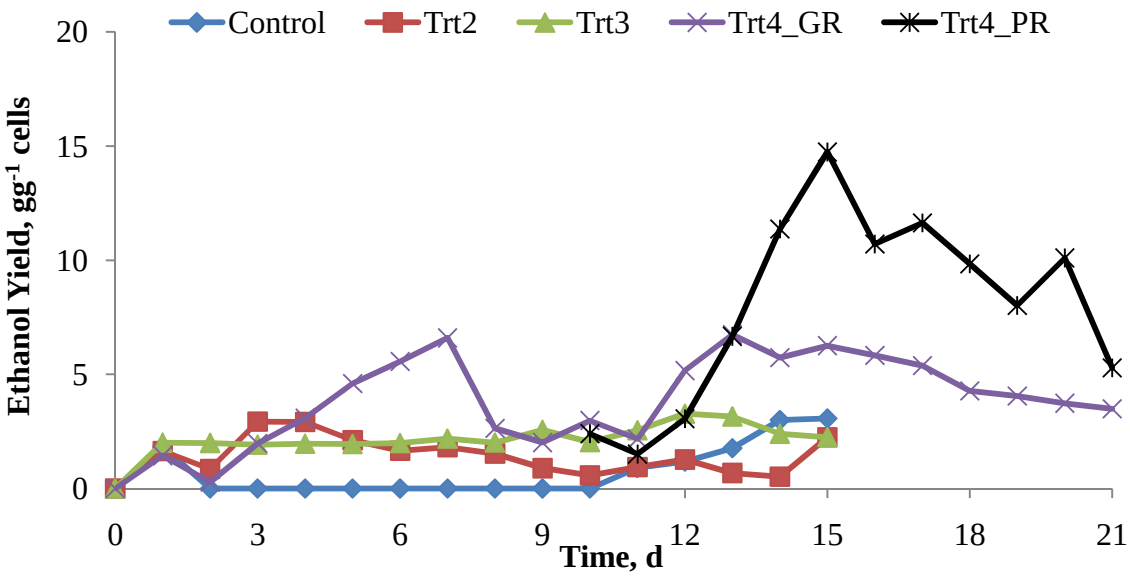

Figure 4.5. Ethanol yield for the reactor studies testing the different interactions between the three limiting nutrients. (Control: Standard *Clostridium* strain P11 medium, Trt2: Treatment with limiting vitamin B_{12} and $CoCl_2$, Trt3: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation, Trt4_GR: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation in the Growth Reactor, Trt4_PR: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation in the Product Reactor). Note: 0 d on PR corresponds with the 10 d of the two-stage fermentor run.

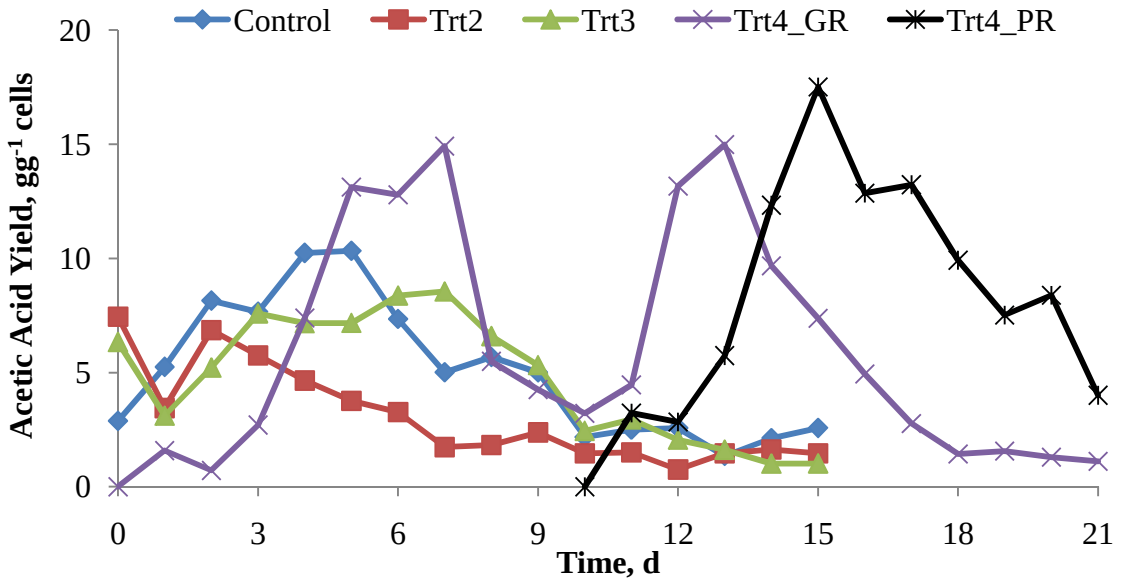

Figure 4.6. Acetic acid yield for the reactor studies testing the different interactions between the three limiting nutrients. (Control: Standard *Clostridium* strain P11 medium, Trt2: Treatment with limiting vitamin B_{12} and $CoCl_2$, Trt 3: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation, Trt4_GR: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation in the Growth Reactor, Trt4_PR: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation in the Product Reactor). Note: 0 d on PR corresponds with the 10 d of the two-stage fermentor run.

One possible strategy of achieving higher cell biomass concentration is by allowing the cells to grow for a longer time and concentrating the cells by flushing out cell free media, as was done with Treatment 4. This resulted in cell mass concentration comparatively higher than Trt 1 (Control), 2 and 3, and compared to previous syngas fermentation studies with *Clostridium carboxidivorans* P7 (Ahmed et al., 2006). However, in Treatment 4, it was observed that the product yields decreased with the progression of fermentation, possibly due to strain degeneration coupled with nutrient limitation. Similar observations were made during acetone-butanol-ethanol (ABE) fermentation with *C. acetobutylicum* P262, where the authors observed strain degeneration associated with high cell densities leading to reduced capacity to produce solvents (Ennis and Maddox, 1989). This may indicate the need to conduct future serum bottle experiments studying the interaction between limiting nutrients and cell mass concentration.

The redox and pH profile in Treatment 4 (limiting three nutrients) fermentor study is shown in Figure 4.7. The pH profile shows similar trend for acetic acid production and the switch to solventogenesis in the Treatments 1, 2 and 3, with single-stage fermentor design. In treatment 4 with a two-stage fermentor design, the operation strategy (explained previously in Materials and Methods) was different than other treatments, resulting in a different pH profile. Redox value was measured only in the product reactor (PR, Figure 1). With the onset of fermentation and acetic acid production, the redox value decreased to -235 mV and then started increasing when the fermentation switched to solvent production. At the end of Day 4, the redox value peaked and stabilized, corresponding to the cessation in ethanol formation. The initial redox value profile observed in this study was similar to the trend observed in previous syngas fermentation studies, but a positive redox value during later stages indicates a different trend (Ahmed et al., 2006; Kundiyana et al., 2010). Redox potential has been found to be an important process parameter that can be regulated to shift microbial metabolism. Lamed and Zeikus (1980)

observed that the presence of H_2 altered electron flow to lactate or ethanol formation in fermentation with strains of *Clostridium thermocellum* growing on cellobiose. The cessation of ethanol formation at the end of the Day 4 may have been due to the unavailability of reduced electron acceptors, lack of regeneration in reducing equivalents and/or the reduced activity of key Wood-Ljungdahl pathway enzymes such as formate dehydrogenase and carbon monoxide dehydrogenase that are linked to reduced electron acceptors (Ljungdhal, 1986; Pierce et al., 2008).

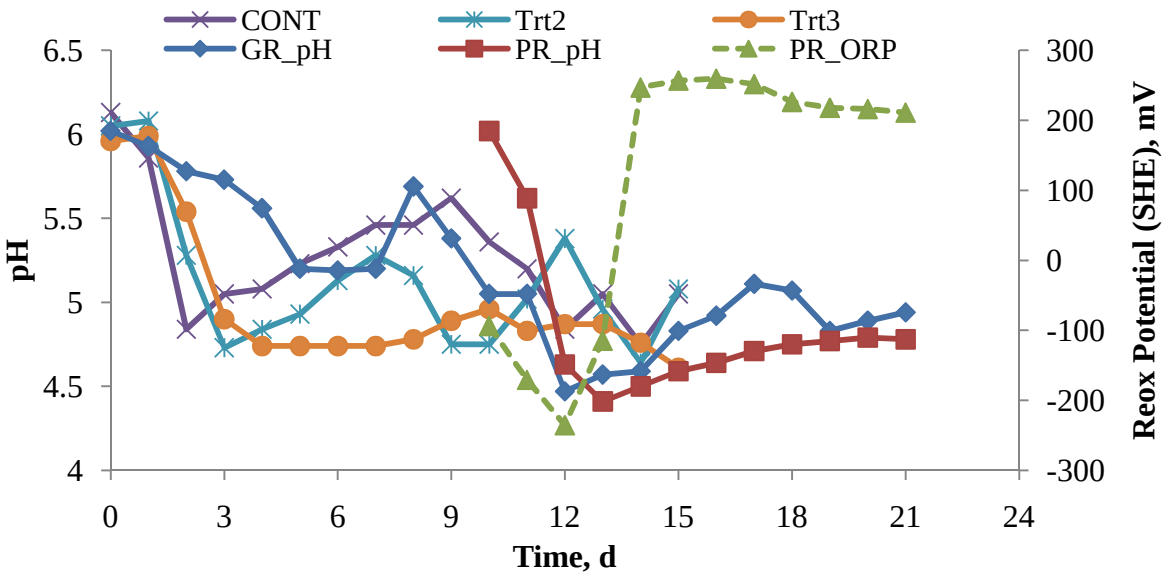

Figure 4.7. pH profile observed during the four treatments conducted in fermentors (Control: Standard *Clostridium* strain P11 medium, Trt2: Treatment with limiting vitamin B_{12} and $CoCl_2$, Trt3: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation, GR_pH: Trt4: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation in the Growth Reactor, PR_pH: Treatment with limiting vitamin B_{12}, $CoCl_2$ and calcium pantothenate limitation in the Product Reactor, PR_ORP: Redox potential measured in product reactor during Trt4). Note: 0 d on PR corresponds with the 10 d of the two-stage fermentor run.

Conclusion

The present study indicates that nutrient limitation coupled with a two stage fermentor design offers operational strategies for improved syngas productivity and higher syngas

conversion efficiencies. The study shows a significant interaction effect between the limiting concentration of calcium pantothenate, vitamin B_{12} and $CoCl_2$ in the *Clostridium* strain P11 fermentation medium. The effect of tested nutrients plays a role in modulating the reduction cycle of the Wood-Ljungdahl pathway in relation to the oxidation cycle, causing an increased availability of reducing equivalents. However, presence of CO (40%) in the feed syngas imparted an inhibitory effect on hydrogenases resulting in the fermentation medium becoming oxidized. This resulted in the depletion of available reducing equivalents, with a corresponding leveling of solvent production in the second stage reactor. Future work should focus on correlating nutrient limitation with partial pressure of individual syngas components, and understand the overall effect on the activity of key enzymes such as alcohol dehydrogenases, hydrogenases and CODH's involved in the Wood-Ljungdahl pathway.

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CHAPTER V: Syngas Fermentation in a 100-L Pilot Scale Fermentor: Design and Process Considerations

"The following chapter has been published in the *Journal of Bioscience and Bioengineering* and appears in this thesis with the journal's permission."

"Kundiyana, D.K., Huhnke, R.L., Wilkins, M.R., 2010. Syngas fermentation in a 100-L pilot scale fermentor: Design and process considerations. *Journal of Bioscience and Bioengineering*, 109, 492-498."

Abstract

Fermentation of syngas offers several advantages compared to chemical catalysts such as higher specificity of biocatalysts, lower energy costs, and higher carbon efficiency. Scale-up of syngas fermentation from a bench scale to a pilot scale fermentor is a critical step leading to commercialization. The primary objective of this research was to install and commission a pilot scale fermentor, and subsequently scale-up the Clostridium strain P11 fermentation from a 5-L fermentor to a pilot scale 100-L fermentor. Initial preparation and fermentations were conducted in strictly anaerobic conditions. The fermentation system was maintained in a batch mode with continuous syngas supply. The effect of anaerobic fermentation in a pilot scale fermentor was evaluated. In addition, the impact of improving the syngas mass transfer coefficient on the utilization and product formation was studied. Results indicate a six fold improvement in ethanol concentration compared to serum bottle fermentation, and formation of other compounds such as isopropyl alcohol, acetic acid and butanol, which are of commercial importance.

Keywords*: Syngas; Ethanol; Anaerobic; Clostridium; Biomass*

Introduction

Ethanol is typically produced either by direct fermentation of fermentable sugars in sugar cane or sweet sorghum, or by enzymatic or chemical hydrolysis of starch, cellulose, and hemicellulose to sugars that are then fermented by microbes to produce ethanol. One of the major disadvantages with the utilization of straw, wood and other cellulosic biomass is the presence of a large proportion of non degradable components such as lignin. Gasification of cellulosic biomass can be one approach of overcoming this hurdle in the utilization of biomass. Gasification produces synthesis gas (syngas) in which CO and H_2 are the essential components for subsequent ethanol production. Fermentation of syngas offers several advantages such as higher specificity of biocatalysts, lower energy costs, greater resistance to catalyst poisoning and the requirement for a fixed CO:H_2 ratio (Bredwell et al., 1999; Klasson et al., 1992).

Since biological fermentation of syngas is irreversible in nature, it ensures complete conversion and prevents the formation of thermodynamic equilibrium. Production of acetate by utilizing CO, CO_2 and H_2 can be summarized by following reactions (Ljungdahl, 1986).

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \qquad (\Delta G° = -37.8 \text{ KJ/g mol } CH_3COOH) \quad [1]$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \qquad (\Delta G° = -18.6 \text{ KJ/g mol } CH_3COOH) \quad [2]$$

Stoichiometry for ethanol production is not available since an autotrophic ethanol producing microorganism has not been identified. Hence based on the reactions [1] and [2] above, following stoichiometry for ethanol production has been proposed (Vega et al., 1989).

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \qquad (\Delta G° = -59.9 \text{ KJ/g mol } C_2H_5OH) \quad [3]$$

$$2CO_2 + 6H_2 \rightarrow C_2H_5OH + 3H_2O \qquad (\Delta G° = -23.2 \text{ KJ/g mol } C_2H_5OH) \quad [4]$$

Organisms able to reduce CO_2 to acetate via the acetyl-CoA or the Wood-Ljungdahl pathway are termed acetogens. This class of bacteria are anaerobic and produce acetate as their major fermentation end product (Wood et al., 1982). Acetogens play an important role in scavenging the carbon dioxide and converting it to acetate. Acetogens are strictly anaerobic bacteria utilizing C_1 compounds such as H_2-CO_2, CO or formate (Diekert and Wohlfarth, 1994).

Production of ethanol by acetogens follows an analogous path similar to the Wood-Ljungdahl pathway for acetate production by acetogens such as *Clostridium acetobutylicum* (Jones and Woods, 1986). Oxidation of H_2 to 2[H^+] or of CO with H_2O to CO_2 and 2[H^+] produces the reducing equivalents for the conversion of CO_2 and CO to acetate or alcohols (Henstra et al., 2007). Ethanol production is a non-growth related product formed during anaerobic syngas fermentation. From an economic perspective, several acetogenic organisms have been shown to produce ethanol. Prominent among these are *Clostridium ljungdahlii* and *Clostridium carboxidivorans*, which produced up to 460 mM and 175 mM of ethanol, respectively (Gaddy et al., 2007; Liou et al., 2005). Recently *Clostridium* strain P11 was reported to produce 200 mM of ethanol using CO, and is currently the organism of choice at our research facility for syngas to ethanol conversion (Saxena and Tanner, 2006).

Commercialization of fermentation involves the scale-up of laboratory fermentation to pilot scale and industrial scale fermentors. With an increase in the fermentor volume, a larger deviation in the ideal behavior can be observed. A large 'non-ideal' fermentor differs from its 'ideal' laboratory counterparts in terms of macroscopic homogeneity of the fermentation broth, and influx of disturbances (Patnaik, 2002). Larger fermentors are also characterized by severe and uncertain changes in the extracellular environment. This is important because a constant interaction exists between the cells and the environment in terms of inward transport of the nutrients and outward transport of the products.

With an increase in fermentor size, mass transfer becomes a critical issue. Mass transfer can be increased by considering several design strategies, such as increasing the gas flow-rate per liquid volume (Ungerman and Heindel, 2007) or use of micro-spargers to produce micro-bubbles (Birch and Ahmed, 1996). Mean bubble diameter and gas hold-up affects both the interfacial area and mass transfer coefficient, which in turn are affected by the fluid dynamics existing within a fermentor (Heijnen and Van't Riet, 1984).

Corn steep liquor (CSL) offers a cheaper nutrient alternative compared to traditionally used yeast extract, thereby, improving the overall process economics. However, one of the main disadvantages of using CSL is its variable composition primarily due to the type of corn and the conditions employed for starch processing (Liggett and Koffler, 1948). Previously, CSL has been successfully incorporated in the industrial production of penicillin. CSL contains a group of active biological compounds favoring the metabolism of bacteria, yeast and mold (Johnson, 1946). In a fermentation medium with *Zymomonas mobilis*, CSL provided a complete source of nutrients and other growth factors such as trace elements and vitamins (Lawford and Rousseau, 1996). In a previous study it was observed that clarified CSL was 1.33 times more effective compared to whole CSL on a volume basis (Lawford and Rousseau, 1997).

Objectives of the present study were to validate the fermentation of *Clostridium* strain P11 in a pilot scale fermentor, validate anaerobic fermentation procedure in a pilot scale fermentor and to assess the feasibility of fermentor run and the system utility designed over an extended fermentation period.

Materials and Methods

Fermentor Description

A pilot scale fermentor, Biostat D-75 (Sartorius BBI, USA) conforming to the ASME Section VIII, Division I code for an Unfired Pressure Vessel, was installed and commissioned to study syngas fermentation. The fermentor was installed in line with the existing fluidized bed and down draft gasifiers existing in our research facility (Figure 5.1).

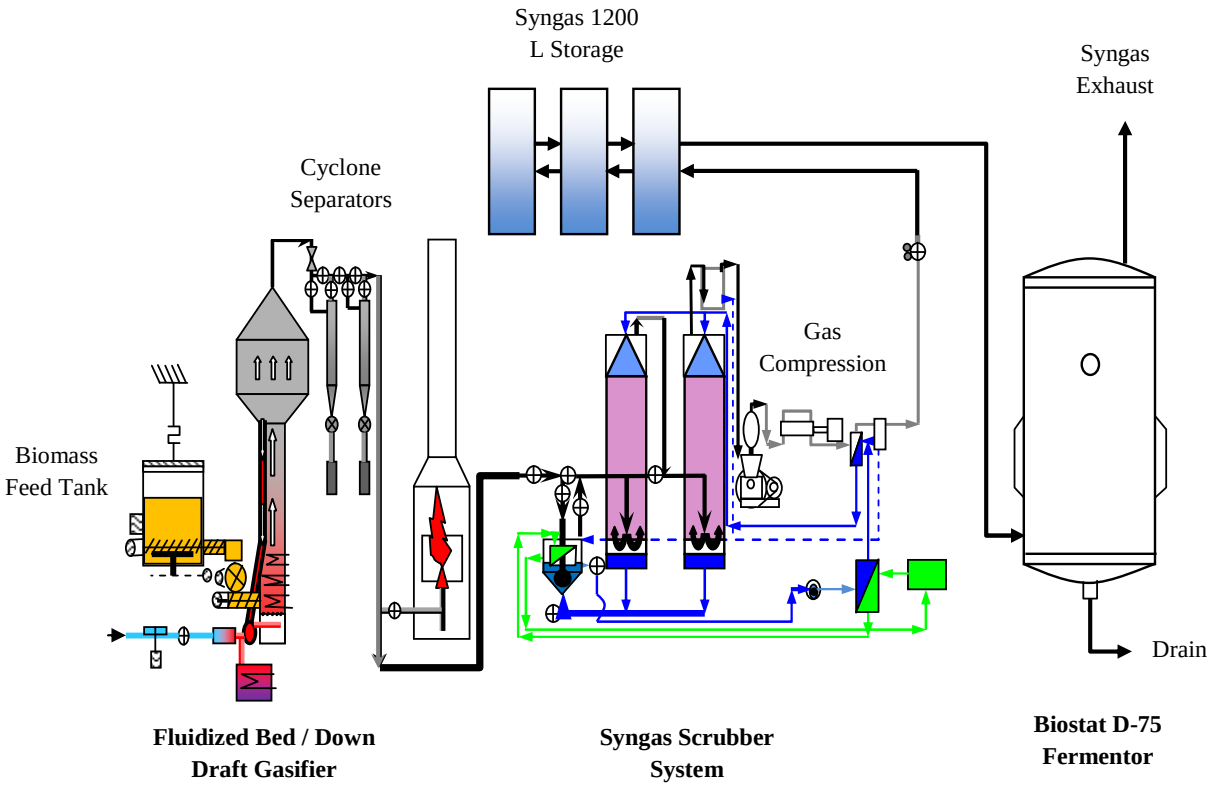

Figure 5.1. Schematic representation of the Oklahoma State University Syngas-Fermentation pilot scale process.

Biostat D-75 is a stirred tank reactor (STR) which was operated in a semi continuous mode i.e. syngas is purged continuously through the fermentation medium while the medium is not replaced. Vessel surface in contact with the liquid media is constructed of 316L stainless steel (SS). The jacketed vessel consists of a closed loop circulation for steam and water for a precise temperature control via a PID (proportional-integral-derivative) control loop. The vessel

is equipped with a pH probe, temperature probe, dissolved oxygen (DO) probe, foam sensor, two septum ports with 19-mm adapters for external media addition and needle inoculation and a single orifice sparger (SOS). The fermentor is equipped with four removable standard baffles, and a top driven agitator shaft mounted with three standard six blade Rushton impellers. The instrumentation and control of the fermentor includes a digital microprocessor controlled system, with an operator interface terminal (OIT) that ensures a direct digital control of the process parameters: temperature, speed, gas flow rate, pH, foam control and control loops for external media component addition. Measurement and control firmware includes Multi Fermentor Control System (MFCS) Data Acquisition (DA) system for real time data logging. Table 5.1 shows the detailed dimensions for the Biostat D-75.

Table 5.1. Dimensions of the Biostat D-75 fermentor used for syngas fermentation studies

Parameters	Reference Symbol	Biostat D-75
Total volume	V_t	0.1 m^3
Working volume (maximum)	V_w	0.075 m^3
Working volume (minimum)	V_w	0.03 m^3
Vessel diameter	D_t	0.406 m
Vessel height	H_t	0.914 m
Impeller diameter	D_I	0.152 m
D_I/D_t Ratio		0.38
Agitator speed range (1119 Js^{-1})		60-600 rpm
Tip Speed		4.796 ms^{-1}
Max. working pressure (176.7°C, full vacuum)		3.48 bar

Utility Sizing

The Biostat D-75 fermentor requires several utility requirements for optimum, and trouble free operation (Figure 5.2). The utility supply for the fermentor should also meet minimum quality requirements to ensure high quality processing and maintain a sterile fermentation environment. The different utilities required for the Biostat D-75 include clean and dry compressed air, utility steam, clean steam, softened water, and de-ionized water.

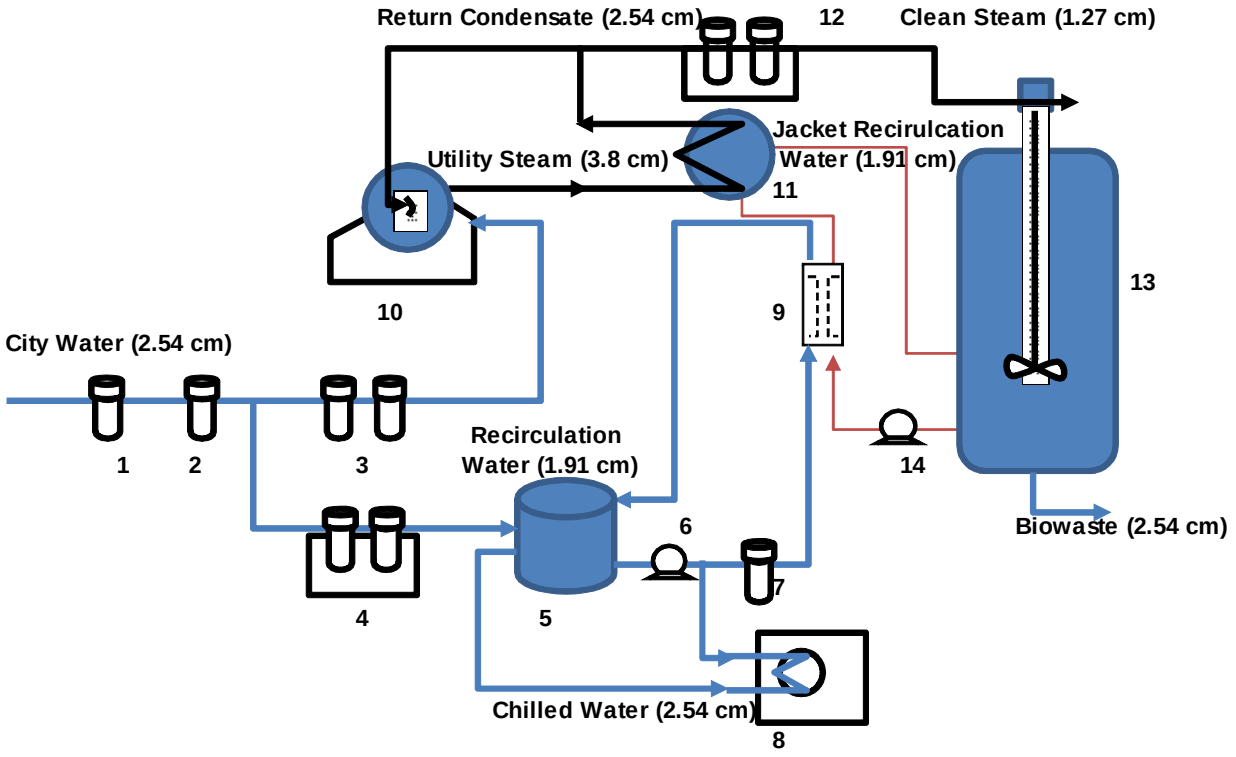

Figure 5.2. Schematic representation of the utility design for the Biostat D-75 fermentor. Piping dimensions are shown in parentheses. The steam and water utilities used in the fermentor are shown. Temperature inside the fermentor is maintained by jacket circulation water. 1- Water prefilter; 2- Activated carbon bed; 3- Twin water softener bed; 4- Anion-Cation Exchange Bed; 5- 1900 L water storage tank; 6- circulation pump; 7- 0.2 micron stainless steel filter; 8- Water chiller; 9- Plate heat exchanger; 10- Vertical boiler; 11- Tubular heat exchanger; 12- Clean steam filtration station; 13- Biostat D-75 fermentor; 14- Water circulation pump.

The utility requirements were sized to accommodate two additional 100-L fermentors with an additional 15% contingency. The utility system is flexible and can also handle the requirements of a much larger fermentor, for instance, a 1000-L fermentor. Table 5.2 shows the minimum flow capacity (pressure and flowrate) of the utilities installed on the fermentor required to ensure trouble free operation of the fermentor. Plant steam and cooling water is required for maintaining the fermentor jacket temperature, which in turn maintains the desired fermentor temperature during sterilization and fermentation incubation. Clean steam is required for maintaining the differential pressure between the agitator seal housing and the fermentor vessel. Compressed process air is required for the operation of pneumatic valves on the fermentor.

Table 5.2. Utility requirements for Biostat D-75

Utility	**Capacity**
Cooling Water (60 psig)	3.15×10^{-4} $m^3 s^{-1}$
Plant utility steam (45 psig)	8.44×10^{-3} kg s^{-1}
Clean steam (30 psig)	2.52×10^{-3} kg s^{-1}
Process and Instrumentation air (80 psig)	2.08×10^{-3} $m^3 s^{-1}$
Electrical	120-208/3/60 15 amps

A vertical high pressure tubeless natural gas fired boiler (Model HE-15 Lattner, Federal Corporation, USA) was installed to meet the steam requirement for the fermentor operation. The boiler package conforms to the American Society of Mechanical Engineers Controls and Safety Devices (ASME CSD-1) requirements for the state of Oklahoma code for pressure vessels, delivering 147 kW at 1135 kPa with an input of 200 kW. The main steam header was branched to a clean steam filtering station mounted with two sintered 316L SS filters (Spirax Sarco, USA)

with a steam filter efficiency of 2 micron or less. The unit conformed to the 3-A accepted practice number 609-01 for the production of culinary steam meeting the clean steam requirement for the fermentor. A condensate receiver (Model FHC-112, Armstrong, USA) was installed to continuously return the steam condensate back to the boiler feed tank. A modular water treatment system (US Filter-Siemens, USA) consisting of pre-filters, activated carbon, and ion-exchange resin based twin water softener was installed for pre-treating water to the required quality standards for fermentor and boiler operation. An anion-cation based exchange bed unit was installed to deliver de-ionized quality water (18 MΩ resistivity), required for the media preparation and fermentor jacket circulation. The jacket circulation water was continuously fed through a chiller (Model # 8301, Unitrol, USA) with a nominal rating of 35 kW.

A 0.00873 m^3s^{-1} capacity rotary screw compressor, Model Ingersoll Rand UP6-5-TAS (Ingersoll Rand, USA), was installed to deliver high quality (dust- and oil-free) compressed air for the operation of pneumatic valves on the fermentor. The compressor was pre-equipped with integrated dryer and prefilters. Two additional filters were installed in series to remove oil aerosols and water up to 0.01 micron, and dry particles removal down to 1 micron.

Several modifications were sought on the fermentor to improve the performance during syngas fermentation. Mixing plays an important role in the distribution of gas and the overall mass transfer coefficient. One approach considered in the present research was the installation of a 5.1 cm SS micro-sparger (Mott Corporation, USA) for increased gas mass transfer in the fermentation medium. The positive effect of a micro-sparger in improving the mass transfer coefficient by generating very fine gas bubbles for effective gas dispersion has been indicated earlier. The bubble size generated from a micro-sparger is a function of the pore size, gas exit velocity, liquid viscosity, and face velocity across the sparger pores. The bubble size was not evaluated either by us or by the manufacturer purely due to the lack of equipment to test the

bubble size. However, visual examination indicated that the bubbles generated were much smaller compared to the SOS and uniform in size, larger in number, and uniformly distributed throughout the fermentation medium. Table 5.3 shows the dimension of the micro-sparger installed on the Biostat D-75 fermentor. The syngas feed inlet line was modified to install a new bypass line to prevent the dosing of plant steam into the fermentation medium during the fermentor sterilization.

Table 5.3. Engineering design for micro-sparger installed at the end of SOS in the Biostat D-75 fermentor.

Design Parameters	Engineering Data
Sparger outer diameter	3/8”
Porous Length	2”
Mean Pore Size	2 micron
Impingement Length	2”
Media Grade	2
End Fitting	½” MNPT to 1/8” FNPT reducer bushing
Material	316L SS porous/ 316 SS hardware

A stainless steel exhaust gas condenser was fabricated in-house to minimize the evaporation of fermentation medium and to prevent the loss of fermentation gas products in the exhaust gas stream (Figure 5.3). The main fermentation products during ethanol fermentation are ethanol, 2-propanol and 1-butanol. Since these products are volatile at the fermentation temperature of 37 °C, it is necessary to capture volatilized products for accurate analysis of the fermentation products. Water from the 1900 L water tank was used as the cooling medium in a cross flow direction to maximize the fermentation product condensation in the condenser.

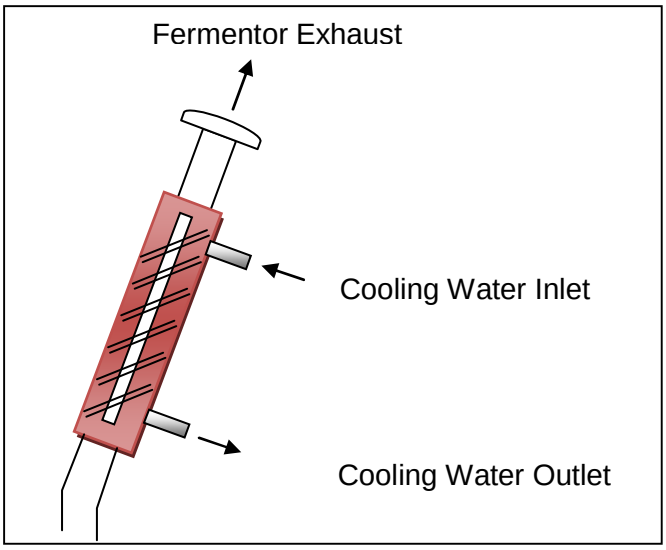

Figure 5.3. Schematic of the exhaust gas condenser built in-house for Biostat D-75

Fermentation

Clostridium strain P11 fermentation medium was maintained in the laboratory in serum bottles by a periodic subculturing regime. Protocol for the inoculum preparation for the fermentor involves repeated inoculum transfer also termed as 'passaging'. *Bottled Gas* refers to the mixed gas purchased commercially having a customized composition similar to gas produced by gasifying switchgrass at the OSU Biomass Gasification Facility. *Producer Gas* refers to syngas produced by gasifying switchgrass at our facility. The composition of the bottled gas was 5% H_2, 15% CO_2, 20% CO, and 60% N_2. Composition of the producer gas varied between the different gasification batches, feedstock, and process conditions. Differences in gas composition were observed in the quality of gas generated from the fluidized bed and downdraft gasifiers. The gas composition from the fluidized bed gasifier was comparable to the bottled syngas (H_2-5%, CO- 20%, CO_2-15%). On the other hand, the gas composition from the downdraft gasifier ranged from about 7-12% H_2, 12-18% CO, and 10-17% CO_2. Sampling and instrument error of approximately 0.1% existed during the gas analysis. O_2 was also observed in the producer gas from both gasifiers ranging between 0.04% - 2.6%.

Clostridium strain P11^{T} originally was obtained from Dr. Ralph Tanner, University of Oklahoma. All subculture fermentations were conducted in 250 mL serum bottles each with a 100 mL final working volume. *Clostridium* strain P11 subculture currently maintained in the laboratory was used as the starting inoculum at the rate of 10% of the working volume. The bacterium was maintained under strictly anaerobic conditions in a semi-defined medium containing following components (per L): 30 mL mineral stock solution, 10 mL trace metal solution, 10 mL vitamin solution, 10 g corn steep liquor (CSL), 10 g morpholinoethanesulfonic acid (MES), 10 mL of 4% cysteine sulfide solution, and 0.1 mL 1% resazurin indicator (Tanner, 2002). Detailed composition of the stock solutions has been described previously (Huhnke et al., 2008). CSL was previously centrifuged at 13000 revolutions per minute (rpm) for 10 min a micro-centrifuge to remove the solids. Removal of solids was found to be necessary to minimize the variability in subculture preparation because of the varying solids concentration in CSL. Removing the solids also helps in cell optical density measurement in a UV spectrophotometer. Bottled syngas was used for all inoculum preparation. The serum bottles were purged with bottled gas every 24 h. All fermentation serum bottles were incubated at 37 °C with a constant agitation of 150 rpm on an orbital shaker.

Passage 1 was prepared by inoculating the previously maintained *Clostridium* strain P11 inoculum and fermenting the serum bottles for 36 h. In a similar procedure, Passage 2 and Passage 3 were prepared which were then incubated for 72 and 60 h, respectively. Passage 3 was prepared in five serum bottles giving a total inoculum volume of 500 mL required for the preparation of the final inoculum. The final inoculum was prepared in a 7.5 L Bioflo 110 (New Brunswick Scientific Corporation, USA) STR operated in a semi-continuous fermentation mode.

Preparation of the final inoculum in a 7.5 L STR (NBSC, USA) is an elaborate process. The total working volume in the reactor was 5 L. The only difference between the media

composition in serum bottles and the 5L fermentor was the reduced addition of MES (@ 0.5% vv^{-1}), addition of CSL with solids, and addition of cysteine sulfide after sterilization in the fermentor. These media components were prepared in Pyrex glass bottles and added externally into the fermentation medium. All media components were weighed and added to the reactor, and the volume was made to 4.5 L by adding deionized water (DI). Dissolved oxygen (DO) and pH probe calibration procedures are explained in the section below. Experience has shown that it is a good practice to check the condition of gaskets and O-rings on the head-plate, and for any leaks from the fermentor by purging compressed nitrogen gas before sterilization. Immediately after sterilization, N_2 was continuously purged through the fermentor. The medium components were autoclaved and cooled to ambient temperature before adding 1% (vv^{-1}) cysteine sulfide. N_2 is continuously purged for another 3 h and later switched to syngas for 3 h. Passage 3 inoculum is then added at the rate of 10% (vv^{-1}) under sterile anaerobic conditions.

Biostat D-75 Fermentor Operation

The fermentor operation requires a thorough manual cleaning followed by sterilization-in-place (SIP) before the start of a fermentation batch. Experience working with pilot scale fermentors has shown that it is a good practice to test the SIP procedure prior to attempting fermentation. This procedure ensures that the fermentor is performing normally, and any leaks or other maintenance requests are rectified before the start of the actual fermentation run. During this preliminary SIP, the modified bypass line was opened to the fermentor rather than to the drain.

For accurate process measurement, it is necessary that the attached probes are properly maintained and calibrated according to the manufacturer's recommendation. Easyferm Plus VP 120 pH probe (Hamilton, USA) was calibrated with respect to standard solutions before SIP.

From a good manufacturing practice (GMP) perspective, it is necessary to ascertain the condition of the DO probe membrane, and replace the electrolyte solution. Calibration of the DO probe is usually done during or after SIP, and is calibrated against two reference points 'zero' and 'span'. During sterilization, the DO probe was zero calibrated, but the 100% span was not calibrated due to the limitation existing in the calibrating procedure of a DO probe in a larger anaerobic fermentor. For the 100% span calibration of the DO probe that is performed after SIP, high purity compressed air has to be purged through the fermentation medium. This step poses significant process limitation under anaerobic fermentation conditions because purging of compressed air needs to be followed by purging with nitrogen gas to make the medium anoxic. This step proves uneconomical and impractical under pilot scale and commercial production environments. Our research group is in the process of finding a solution for the optimal functionality of the DO probe.

A total fermentation working volume of 70 L was prepared in a manner similar to the media preparation in a 7.5-L fermentor. Following the pre-SIP, fermentation media components were weighed and added to the fermentor. Detailed *Clostridium* strain P11 media composition has been described earlier. The fermentor with fermentation medium, was sterilized with the bypass line opened to the drain.

Following sterilization N_2 and syngas was purged continuously through the fermentor as explained in the previous section. Cysteine sulfide (1% v v^{-1}) was dosed anaerobically using a peristaltic pump, followed by the addition of *Clostridium* strain P11 inoculum (10% v v^{-1}). During the fermentation growth phase, bottled syngas was purged through the fermentation medium. After seven days, gas source was switched to switchgrass producer gas. This minimizes interference or inhibition of *Clostridium* strain P11 cell growth from tar and other

compounds during the growth phase of the cells and reduces the variability due to producer gas composition. Temperature, pH, agitation rate, and gas flow rate were registered real time using Biostat D-75 MFCS software.

Samples were collected to determine the time course for bacterial cell biomass, substrate, and product concentrations (ethanol acetic acid, isopropyl alcohol and 1-butanol). The cell biomass was determined by measuring sample absorbance at a wavelength of 660 nm (A_{660}) in 1cm pathlength cuvettes using a UV-visible spectrophotometer (Cary 50 Bio, Varian, USA). Product concentrations were separated by HPLC with an Aminex-HPX-87H ion exchange column (Biorad, Hercules, CA, USA). The eluent was 0.01N H_2SO_4 pumped at 0.6 mL min^{-1} and column temperature was 60 °C (Sluiter et al., 2005). Separated components were detected by a refractive index detector (1100 Series, Agilent, Santa Clara, CA, USA). Peaks were identified by comparing with known standards (Sigma-Aldrich, St. Louis, MO, USA). Ethanol production was calculated by subtracting initial concentrations from concentrations at other time points. The inlet and outlet gas samples were analyzed using a gas chromatograph (6890 Agilent Co., USA) equipped with a Hayesep-DB column and a thermal conductivity detector with argon as the carrier gas. A ramped temperature profile included 40 °C for 6 min after which the temperature was increased to 140 °C for 20 °C at an increment of 100 °C min^{-1}.

Results and Discussion

A fermentor is central to the economical production of useful biological products like ethanol, acetic acid, 2-propanol and 1-butanol. An ideally designed fermentor will provide a controlled environment for optimal growth and product formation. Of the different fermentor designs currently available in the market today, STR either in a batch or in continuous mode is the most desired and widely used fermentor system for industrial applications.

Fermentor Design and Operation

The present study did not evaluate the effect of increasing gas flow rate at constant impeller speed due to limited availability of the constant supply of producer gas. In this study, we have successfully incorporated the use of a micro-sparger to improve the mass transfer rates without considering changes in the impeller configuration or the gas flow rates. Micro-spargers have been shown to produce micro-bubbles that improved the overall mass transfer coefficient in fermentation systems (Birch and Ahmed, 1996).

The impeller blade and baffle design used in Biostat D-75 as well as other vessel geometries used in the present study corresponds to a standard reactor configuration (Vega et al., 1989). A dual six-blade impeller along with four symmetrically located baffles was found to reduce vortex formation, thereby improving the mass transfer performance.

The fermentor was run for a period of 59 d without any major downtime in the utility requirement. Unexpected failure was observed in the operation of the air compressor (49 d) leading to the unavailability of compressed air to the fermentor. This resulted in fermentor temperature to drop to 30.2 °C after a failure time of 6 h. On the resumption of compressed air supply, the bacterial cells resumed metabolism, as evident by the change in product profile and cell densities. No failure was observed in case of steam boiler, and as per the manufacturer's recommendation, it was necessary to blow-down and monitor the feed water quality on a daily basis. This practice also qualified per se GMP requirement. The performance of utility set-up on the fermentor validates the sizing and the functionality of the designed system to meet the utility requirement of the fermentor, in order to keep the fermentation medium viable and sterile.

The process parameters on the fermentor included syngas/producer gas flow-rate (0.9 standard liters per minute, SLPM), agitation (150 rpm), and temperature (37 °C). Syngas and

producer gas was purged continuously through the fermentation medium, which was different from the subculture bottle fermentation where the gas exchange was done every 24 h. Agitation in the fermentor was also different compared to serum bottles and it is assumed that with Ruston impellers, the gas mass transfer rates will be improved when compared to mass transfer rates in serum bottles.

Fermentor Product Profile

The main products analyzed from the Biostat D-75 fermentation were ethanol, acetic acid, 2-propanol, and 1-butanol (Table 5.4). The product profile can be divided into two main phases considering ethanol productivity as the criteria: Phase 1 extends from Day 0 to Day 24 (acetogenic phase) and Phase 2 from Day 24 to Day 59 (solventogenic phase) (Figure 5.4). Results for 1-butanol are not discussed because of low productivity.

In Phase 1, ethanol concentration increased from 0.17 g L^{-1} on Day 1 to 4.46 g L^{-1} with a productivity of 0.18 g $L^{-1}d^{-1}$ at the end of 24 d. The productivity for acetic acid was 0.15 g $L^{-1}d^{-1}$, with the concentration increasing from 1.24 g L^{-1} on Day 1 to 4.82 g L^{-1} at the end of 24 d. Comparatively, 2-propanol showed a higher productivity of 0.39 g $L^{-1}d^{-1}$ with concentration increasing from 0 g L^{-1} on Day 1 to 9.25 g L^{-1} at the end of 24 d.

Phase 2 corresponded with a linear increase in ethanol production while the acetic acid and 2-propanol concentration was constant. The ethanol concentration increased to 24.57 g L^{-1} with a productivity of 0.87 g $L^{-1}d^{-1}$ at the end of 59 d. The productivity for ethanol was comparable to productivity observed with *C. carboxidivorans* in a 7.5 L fermentor (Ahmed et al., 2006).

Table 5.4. Product concentration recorded during the *Clostridium* strainP11 fermentation run in Biostat D-75.

Product	Time Period (day)	Concentration (g L^{-1})
Ethanol	59	25.26
2-propanol	24	9.25
Acetic acid	24	4.82
n-butanol	13	0.47
Total Cell biomass concentration	59	1.13

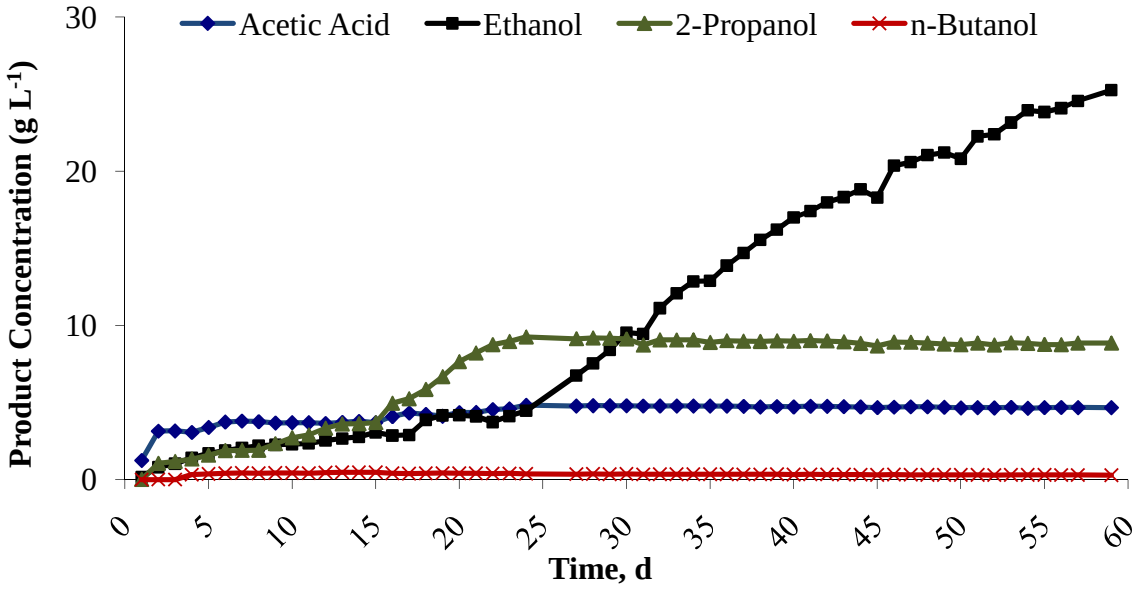

Figure 5.4. Product profile for Biostat D-75 fermentor during the time course of fermentation.

Production of acids in the first stage is necessary for the production of solvents in the second stage (Worden et al., 1991). From a bioenergetics point of view, production of acid is necessary for the generation of ATP and no consumption of electrons, whereas, alcohol production is associated with consumption of electrons and no production of ATP. 2-propanol concentration also decreased in Phase 2 reducing from 9.25 g L^{-1} at the end of 24th d to 8.86 g L^{-1} on 59th d with a corresponding productivity of -0.02 g $L^{-1}d^{-1}$. Reason for decrease in 2-propanol

concentration is unknown. 2-propanol production has not been previously reported during a syngas fermentation. In related work in our lab, both *Clostridium* strain P11 and *C. carboxidivorans* were cultured in glucose media containing acetone (unpublished data). *Clostridium* strain P11 reduced acetone to 2-propanol, but *C. carboxidivorans* did not. This indicates that *Clostridium* strain P11 has the necessary enzyme system to reduce acetone to 2-propanol.

The ethanol concentration in the fermentation run is higher compared to previously reported concentrations during syngas fermentation with *Clostridium carboxidivorans* and *Clostridium* strain P11 as the microbial agent (Saxena and Tanner, 2006). Formation of 2-propanol has not been shown in previous studies while the production of acetic acid was comparable to other studies (Ahmed et al., 2006; Datar et al., 2004). Probably the higher productivity in the present run could be due to pilot scale fermentor, gas sparging strategy, process modifications done on the fermentor, and comparatively longer fermentor incubation time compared to previous studies.

Figure 5.5 shows the cell concentration with corresponding pH profile observed during the Biostat D-75 fermentation. The cell concentration increased to 0.44 g L^{-1} at the end of Day 2 with a corresponding pH of 5.62. From Day 3 to Day 7, the cell concentration essentially remained constant, however the pH dropped exponentially to 4.95. The gas source was changed to biomass producer gas at the end of Day 7, which caused further lag in the fermentation with no change in pH or cell concentration. This is probably because the bacterial cells were adapting to the producer gas environment. Producer gas components such as nitric oxide and acetylene has been shown to inhibit hydrogenase activity and result in a growth lag in acetogens (Tibelius and Knowles, 1984). The total cell density in the fermentation broth peaked at the end of 14 d

reaching a concentration of 0.84 g L^{-1} before plateauing until the end of the fermentation. This may also correspond with the switch in *Clostridium* strain P11 metabolism from acetogenic to solventogenic phase, in accordance with the non-growth associated production of solvents (Ahmed et al., 2006). The total cell concentration at the end of 59th d was 0.87 g L^{-1}. The cell densities observed in the present study were much higher compared to earlier studies with *Clostridium carboxidivorans* $P7^T$ (Ahmed et al., 2006; Datar et al., 2004).

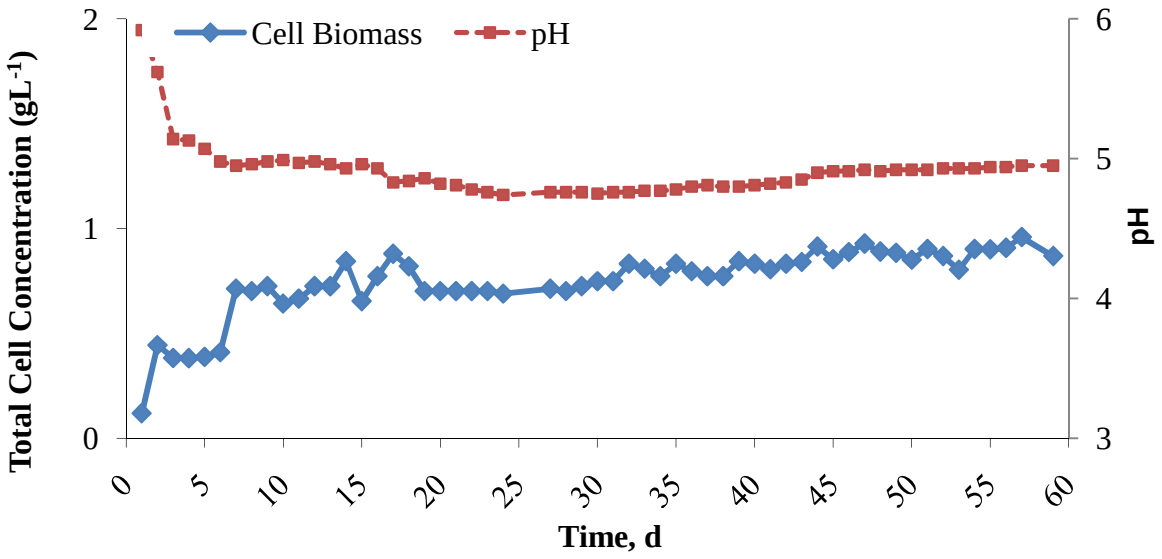

Figure 5.5. *Clostridium* strain P11 cell concentration and pH profile observed during Biostat D-75 fermentation run.

The pH continued to decrease to 4.74 observed at the end of 24 d before the fermentation switched to solvent production. Regulation of pH further alters the flow of electrons either to acetate or solvent production and it is necessary for the bacterial cells to produce acid to ensure subsequent solvent production (Worden et al., 1991). It is also expected that the drop in pH observed during the time course of fermentation, may also increase the mass transfer performance due to the presence of carbonic acid (H_2CO_3), produced by simultaneous chemical reaction and CO_2 mass transfer during syngas fermentation (Kapic et al., 2006). The authors also

observed that presence of carbonic acid resulted in the decrease of medium pH which had an instantaneous effect on the solubility of CO and a greater sensitivity to its mass transfer.

Large scale production of inoculum is one of the critical factors for maintaining a viable inoculum volume in large scale fermentations and for multi stage inoculum development. Once the process operating conditions have been optimized, the reaction rate can be improved by increasing the microbial cell density. A high *Clostridium* strain P11 cell concentration was observed in the fermentation medium, which was beneficial in efficiently converting the syngas components, and may have resulted in high productivity observed in the present run. A high cell concentration was shown to impact substrate utilization (Datar et al., 2004). In ethanol fermentation using yeast cells it was found that high cell densities was necessary to achieve high fermentation rates (BOSTID, 1983). It can also be deliberated that a high cell concentration, the inoculums will have a greater probability of survivability and adaptation in presence of inhibitors.

The ethanol productivity was much lower than that observed for fermentations of glucose and other monosaccharides to ethanol. Low cell densities and low mass transfer rates are the main reasons for low ethanol productivity during syngas fermentations. Recently, several patent applications have been filed detailing the use of hollow fiber membrane reactors for syngas fermentation (Tsai et al., 2009a, 2009b, 2009c)). In these reactors a biofilm forms on the side of the membrane in contact with the liquid media with syngas diffusing through the membrane and into the biofilm. Mass transfer rates are much greater in these reactors and the cells are immobilized in the reactor by the biofilm, thus, increasing cell density and preventing cell washout if continuous operation is used. Other novel strategies such as altering media composition, nutrient feed rate, operating conditions such as temperature, agitation and pH, and syngas composition and feed-rate, have also been mentioned in order to maintain a steady state

condition associated with ethanol productivities greater than 10 g L^{-1} and a stable culture growth (Gaddy et al. 2007).

Effect of Contaminants on the Fermentor Product Profile

The effect of biomass producer gas components, specifically nitric oxide and acetylene, has been shown to inhibit the hydrogenase activity and subsequent uptake of hydrogen. A study with nitric oxide and *Clostridium carboxidivorans* showed that at a nitric oxide concentration of 150 ppm, hydrogenase activity was consistently inhibited (Ahmed et al., 2006). In addition, the authors also observed that tar in biomass producer gas was responsible for cell dormancy rather than ash. This may be the reason for the lag observed in cell growth when the feed gas was switched from bottled gas to producer gas.

Acetogens are strict or obligate anaerobes. However, in the present study the producer gas generated from the gasifier was constantly contaminated with oxygen, reasons for which are presently being investigated. Oxygen concentration as measured by headspace gas analyzed on the gas chromatography ranged between 400 to 26000 ppm (data not shown). In spite of the O_2 presence in the gas feed, *Clostridium* strain P11 inoculum demonstrated growth and product formation. Ability of *Clostridium* strain P11 to grow and metabolize under micro-aerophilic conditions (5% O_2 concentration) is surprising and encouraging from a process scale-up perspective. Acetogens isolated from anoxic soils and termite guts were speculated to contain certain mechanisms to cope with O_2 in an in-situ environment. Acetogens such as *Moorella thermoacetica* and *Clostridium magnum* displayed peroxidase and NADH oxidase activity which enables it to grow in media supplemented with 21% O_2 (vv^{-1}) (Karnholz et al., 2002). It can be speculated that under prolonged exposure to O_2 in the producer gas, it is possible that some of

these oxygen consuming enzymes were over expressed enabling *Clostridium* strain P11 to grow and metabolize the syngas components to ethanol, 1-butanol.

Conclusion

In this study, the installation and operation of a pilot scale fermentor with improved productivity was successful compared to serum bottle fermentation and bench scale stirred tank reactors. The study also elaborates the sizing and design of utilities required for a trouble free operation of a fermentor. The fermentor was operated over an extended period of time. Included in this study was a staggered procedure for preparing final inoculum for the pilot scale fermentor. Future research should address the effect of different nutrients using a statistically designed procedure.

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APPENDIX

APPENDIX 1: Detailed composition of Proflo Cotton Seed Extract (Adapted from (Zabriskie et al., 1980)

AMINO ACIDS*			
	%		%
Lysine	4.26	Alanine	4.13
Histidine	2.89	Cystine	1.68
Arginine	12.12	Valine	4.51
Aspartic Acid	9.72	Methionine	1.63
Threonine	3.50	Isoleucine	3.25
Serine	4.82	Leucine	6.31
Glutamic Acid	22.95	Tryosine	1.15
Proline	4.15	Phenylaianine	5.66
Glycine	4.40	Tryptophan	2.87

VITAMIN CONTENT			
	$\mu g\ g^{-1}$		$\mu g\ g^{-1}$
Carotene	0.11	Pantothenic Acid	12.510
Total Tocoherols	14.00	Choline	33.480
Ascorbic Acid	25.11	Pyriodoxine	0.885
Thiamine	4.36	Biotin	0.784
Riboflavin	5.11	Folic Acid	1.500
Niacin	83.90	Inositol	0.364

MINERAL CONTENT		SOLUBLES*	
	ppm		%
Calcium (Ca)	2530	Total Solubles	32.560
Chloride (Cl)	632	Soluble Amino Nitrogen	1.650
Iron (Fe)	109	Soluble Phosphorous (P)	0.346
Magnesium (Mg)	7480	Soluble Iron (Fe)	0.00036
Phosphorous (P)	13100	Soluble Magnesium (Mg)	0.467
Potassium (K)	17300		
Sulphates (SO_4)	15600		

*Percent of Total Protein
*All values determined relative to one gram of Proflo autoclaved in 100 mL of water for 15 min at 120°C.

VITA

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Scope and Method of Study: Biomass gasification followed by fermentation of syngas to ethanol is a potential process to produce bioenergy. The process is currently being researched under laboratory- and pilot-scale in an effort to optimize the process conditions and make the process feasible for commercial production of ethanol and other biofuels such as butanol and propanol. The broad research objectives for the research were to improve ethanol yields during syngas fermentation and to design a economical fermentation process. The research included four statistically designed experimental studies in serum bottles, bench-scale and pilot-scale fermentors to screen alternate fermentation media components, to determine the effect of process parameters such as pH, temperature and buffer on syngas fermentation, to determine the effect of key limiting nutrients of the acetyl-CoA pathway in a continuous series reactor design, and to scale-up the syngas fermentation in a 100-L pilot scale fermentor.

Findings and Conclusions: The first experimental study identified cotton seed extract (CSE) as a feasible medium for *Clostridium* strain P11 fermentation. The study showed that CSE at 0.5 g L^{-1} can potentially replace all the standard *Clostridium* strain P11 fermentation media components while using a media buffer did not significantly improve the ethanol production when used in fermentation with CSE. Scale-up of the CSE fermentation in 2-L and 5-L stirred tank fermentors showed 25% increase in ethanol yield. The second experimental study showed that syngas fermentation at 32 °C without buffer was associated with higher ethanol concentration and reduced lag time in switching to solventogenesis. Conducting fermentation at 40 °C or by lowering incubation pH to 5.0 resulted in reduced cell growth and no production of ethanol or acetic acid. The third experiment studied the effect of three limiting nutrients, calcium pantothenate, vitamin B_{12} and $CoCl_2$ on syngas fermentation. Results indicated that it is possible to modulate the product formation by limiting key nutrients of acetyl-CoA pathway and using a continuous fermentation in two-stage fermentor design to improve ethanol yields. The last experimental study was conducted to commission a pilot scale fermentor, and subsequently scale-up the *Clostridium* strain P11 fermentation from a bench-scale to a pilot scale 100-L fermentor. Results indicated a six-fold improvement in ethanol concentration (25.3 g L^{-1} at the end of 59 d) compared to previous *Clostridium* strain P11 and *Clostridium carboxidivorans* fermentations plus the formation of other compounds such as isopropyl alcohol, acetic acid and butanol, which are of commercial importance.

ADVISER'S APPROVAL: Raymond L. Huhnke