EFFECT OF THE REDUCING AGENT DITHIOTHREITOL ON ETHANOL AND ACETIC ACID PRODUCTION BY *CLOSTRIDIUM* STRAIN P11

By

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PRODUCTION BY *CLOSTRIDIUM* STRAIN P11

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CHAPTER I

INTRODUCTION

Liquid fuels are the lifeline of transportation industry. It is a well-known that there is fast depletion of non-renewable sources of energy and an increasing demand for liquid fuels. It is projected that there would be a 1.7 % increase in global energy demand per year, making it almost 15.3 billion tons of oil equivalent by 2030 (Bilgen et al. 2004). Global warming due to the release of green house gases (GHG) like CO_2 can have dire consequences on our planet and an alarming 72% of GHG emissions comes from the transportation sector (Greene and Schafer 2003).

The use of renewable sources of energy can reduce our dependence on fossil fuels, which are limited in supply. The only sustainable energy sources for liquid fuels are the biofuels (Huber et al. 2006). Biofuels, especially bioethanol, is already used in the transportation industry as a fuel additive. Bioethanol is produced commercially from corn and other starch rich feedstocks. In Brazil, biofuels are produced from sugar cane. There is an ongoing research on the production of bioethanol from other sugar crops such as sweet sorghum and from cellulosic feedstocks such as energy dedicated crops, agricultural and forestry residues, and waste materials. The main advantages of using bioethanol in the transportation industry are:

- Using crops to produce ethanol helps in recycling the carbon emitted from liquid fuels and in combating global warming.
- Methyl *tert*-butyl ether (MTBE) was conventionally used as a fuel oxygenate. However, the use of MTBE is banned in 16 states in the U.S. as it has been identified as a ground water contaminant (EIA 2003). Moreover, oxygen content in ethanol (35% by weight) is nearly twice as much as in MTBE, making ethanol a better fuel oxygenate (RFA 2004).
- The need for fossil fuels in the transportation sector would be cut by approximately 44%, if gasoline was replaced by 95% ethanol blended fuel (E95) (Yacobucci 2008), reducing the dependence on other nations for oil.
- As of 2007, nearly 364,000 vehicles in the U.S. can use E85 blend (85% ethanol, 15% gasoline) as fuel (Figure 1.1), implying the increasing demand for fuel grade ethanol in the transportation sector (EIA 2008a).

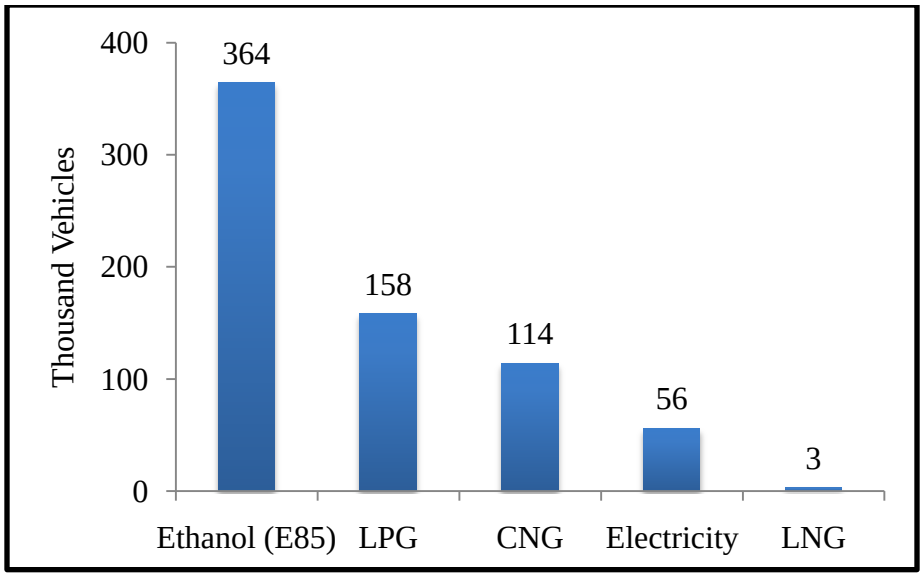

Figure 1.1 Number of alternate fuel based vehicles in the U.S. as of 2007. LPG – Liquefied Petroleum Gas, CNG – Compressed Natural Gas, LNG – Liquefied Natural Gas. Adapted from (EIA 2008a).

The energy equivalent of the total amount of fuel grade ethanol produced in U.S. was 0.784 Quadrillion Btu, whereas the consumption was 0.816 Quadrillion Btu for the year 2008 (EIA 2008b). Also, The Energy Independence and Security Act of 2007 has mandated that by the year 2022, the U. S. must produce 36 billion gallons of biofuels per year (EPA 2009), implying the importance of innovation in the field of biofuels production.

The three main feedstocks used in ethanol production are:

- Feedstocks that contain sugars like sugarcane.
- Feedstocks that contain starch like corn.

- Lignocellulosic feedstock like switchgrass, agricultural and forest residues. However, there are no commercial plants in operation from lignocellulosic feedstock.

Corn is the primary feedstock for ethanol production in the United States. Conventionally, ethanol is produced from corn by hydrolyzing starch. But there will not be enough corn to meet the rising demand for ethanol (Baker and Zahniser 2006a). The U.S. economy is affected by the increasing demand for corn and it is speculated that in the years to come "agflation" can worsen (Luchansky and Monks 2009). Agflation refers to the inflation in commodity prices due to the agricultural sector.

The production of ethanol from lignocellulosic feedstock can be done by two routes:

- Hydrolysis–Fermentation
- Gasification–Fermentation

In hydrolysis–fermentation, the lignocellulosic feedstock is pretreated to break down the outer lignin layer, thereby exposing cellulose and hemicelluloses for hydrolysis and subsequent fermentation of released sugars to ethanol (Mielenz 2001). Although ethanol yields are high in hydrolysis-fermentation, high costs of pretreatment and low ethanol titer are still obstacles for this technology.

In gasification–fermentation, the feedstock is pyrolyzed to produce synthesis gas (syngas), which is then converted to ethanol, either by a chemical method using Fischer–Tropsch process or using microbial catalysts such as autotrophic microorganisms (Datar et al. 2004). The main disadvantage of Fischer–Tropsch process is that it is an expensive

technology due to its operating conditions. Syngas fermentation produces alcohols and acids using acetogens such as , *Clostridium ljungdahlii* (Klasson et al. 1992), *Clostridium carboxidivorans* (Rajagopalan et al. 2002) and *Clostridium* strain P11 (Huhnke et al. 2008). These microbes produce alcohols like ethanol and butanol and acids like acetic acid and butyric acid through the Wood – Ljungdahl pathway, also called the acetyl–CoA pathway (Wood et al. 1986).

Advantages of syngas fermentation include the potential for a wider range of metabolites that can be produced using a specific acetogen and mild process conditions like low temperature and pressure. Syngas fermentation is also feedstock independent through the gasification process and can utilize lignin in biomass for the production of useful metabolites. This reduces the burden of waste disposal. However, syngas fermentation suffers from drawbacks like low ethanol productivity, low syngas solubility and sensitivity of microbial catalysts to environmental conditions such as pH, availability of nutrients and reducing equivalents.

Reducing agents act as artificial electron carriers that are oxidized by donating electrons in a redox reaction. The donated electrons can be used by acetogens to produce ethanol from acetyl–CoA. The addition of reducing agents to the fermentation medium has shown to enhance ethanol production during syngas fermentation. Some of the reducing agents that have increased ethanol production are neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (Ahmed 2006) and methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride) (Panneerselvam 2009; Rao et al. 1987). Although ethanol production increased by 60% and 22% with the addition of methyl viologen and neutral red, respectively, the maximum ethanol concentration attained were

only 1.3 and 0.6 g/L, respectively (Panneerselvam 2009).

There is a need to increase the final ethanol titer in the fermentation medium. Dithiothreitol (DTT) is a strong reducing agent and has the potential to enhance ethanol production by acting as an artificial electron carrier. Its application in syngas fermentation has not been studied.

The objective of this study was to determine the effect of various concentrations of DTT on the growth and product formation by *Clostridium* strain P11 during syngas fermentation using simulated and actual biomass-based syngas (producer gas) in two different media, namely yeast extract and corn steep liquor**.**

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Economic and environmental concerns, along with a decrease in petroleum imports and increasing greenhouse gas (GHG) emissions, has made innovation in the field of biofuels a national priority (Dale 2003). In the U.S., 97% of transportation fuel comes from crude oil (MacLean et al. 2004).

Advantages of bioethanol over fossil fuels are:

- Ethanol is a better additive and fuel oxygenate compared to MTBE (methyl *tert*-butyl ether), which is a ground water contaminant (Nadim et al. 2001). Mixing oxygenates with fuels reduces CO emissions (Nadim et al. 2001).
- Paves the way for the creation of new jobs, especially in the agricultural sector.
- Would help reduce GHG emissions. It has been estimated that in the short run there will be a 20% reduction in the GHG emissions (equivalent to 14 million metric tons of CO_2 for 2008 and 130 million metric tons of CO_2 for 2022) due to the use of ethanol as transportation fuel (NCGA(b) 2009).
- Reduces the import of foreign oil, making the U.S. more energy independent and secure.

- E10 (10% ethanol and 90% gasoline) and E85 (85% ethanol and 15% gasoline) makes bioethanol a more attractive option. Ethanol as a fuel in the transportation industry increased by nearly 700% in the last 10 years (RFA 2009) and the production of ethanol increased by 34% in 2007 compared to 2006 and by 38% in 2008 compared to 2007 (RFA 2009). Ethanol production in the U.S. increased from 9.2 billion gallons in 2008 to 10.6 billion gallons by the end of 2009, indicating a 42% increase in production (RFA 2010).

2.2 Bioethanol Production

The raw materials used in the production of biofuels are classified into three types (Balat and Balat 2009):

- Feedstocks containing sugars such as sugarcane, sweet sorghum and sugar beet.
- Feedstocks containing starch such as corn and sorghum.
- Feedstocks containing lignocelluloses such as grasses, forest and agricultural residues.

The ethanol industry in the United States is largely dependent on corn as the primary feedstock. However, there is not enough corn in the United States to meet the increasing demand for production of fuel grade ethanol (Baker and Zahniser 2006b). Discussion on the production of ethanol from corn and sugar-producing crops are beyond the scope of this study.

2.2.1 Lignocellulosic Ethanol

Lignocellulosic feedstocks comprise of wood, municipal waste, forest wastes, agricultural residues and grasses. The lignocellulosic feedstock constitutes are cellulose (40-60%), hemicellulose (20-40%) and lignin (10-25%) (Hamelinck et al. 2005). Lignin and hemicellulose forms the outer cell wall of the biomass and cellulose form the central material (Hamelinck et al. 2005). Hemicellulose and cellulose can be saccharified by acid and enzymatic hydrolysis to form simple sugars, whereas the lignin is not degradable. Hence, lignin is a residue after the hydrolysis process (Hamelinck et al. 2005). The main advantage of enzymatic hydrolysis is the mild treatment condition. The released sugars are then fermented using microorganisms to produce ethanol. These microbes consume the simple sugars like glucose derived from complex cellulose and hemicelluloses to produce ethanol and other useful byproducts. Ethanol is recovered from the fermentation broth by distillation.

Figure 2.1 shows the process flowchart of a typical bioethanol production process (RFA 2005). First, the biomass is reduced in size by grinding or chipping. In the next step, the biomass is treated with dilute sulfuric acid to facilitate the hydrolysis of hemicelluloses to simple sugars like xylose, arabinose, mannose and galactose (RFA 2005). After acid pretreatment, cellulase enzymes are used to breakdown cellulose to glucose (RFA 2005). This is followed by glucose fermentation by microorganisms to produce ethanol and carbon dioxide (RFA 2005). The main pentose sugar formed as a result of hemicellulose hydrolysis is xylose, which can also be femented to ethanol (RFA 2005). The final step in ethanol production is ethanol recovery from the fermentation broth, usually through distillation. In some cases, the lignin leftover in the hydrolysis

process is used for electricity production (RFA 2005).

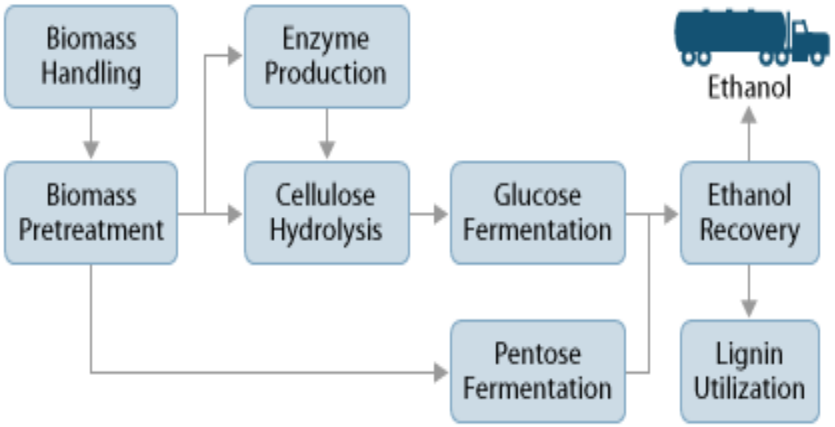

Figure 2.1 Flow chart for ethanol production from cellulosic biomass through the biochemical platform (RFA 2005).

Advantages of bioethanol from lignocellulosic materials through the biochemical platform are:

- Food security is not threatened when lignocellulosic materials are used for fuels production (Kim and Dale 2004).
- High ethanol yields are obtained through the biochemical platform.

Disadvantages of bioethanol from lignocellulosic materials through biochemical platform are:

- Expensive pretreatment step and formation of inhibitory compounds such as hydroxymethylfurfural (HMF).
- Carbon within the lignin is never used for ethanol formation.

2.3 Gasification–Fermentation

Gasification-fermentation is a two-step process, comprising of gasification of

cellulosic feedstock and fermentation of generated synthesis gas (syngas). Although there is no current commercial production of ethanol using gasification-fermentation technology, there are much research and innovations made in this field. The production of ethanol from lignocellulosic feedstocks though gasification-fermentation would overcome many of the disadvantages of direct fermentation of cellulosic biomass.

Lignocellulosic biomass is converted into syngas in a thermal process called gasification (Maschio et al. 1994), in which the biomass undergoes partial oxidation at temperatures above about 800°C to form syngas. Syngas is a mixture of carbon monoxide (CO), carbon dioxide (CO_2), hydrogen (H_2), nitrogen (N_2) and some hydrocarbons (Phillips et al. 1994). The thermochemical reactions that produce syngas components include partial oxidation, complete oxidation, and water-gas shift reaction as shown in Table 2.1 (McKendry 2002). The composition of syngas depends on the composition of the feedstock and gasification conditions (Klasson et al. 1992).

Syngas can be converted to ethanol either by chemical catalysts (Fischer–Tropsch process) or microbial catalysts. The discussion on Fischer–Tropsch process is beyond the scope of this study.

Table 2.1 Thermochemical reactions during the production of synthesis gas. Adapted from (McKendry 2002).

Reaction	Equation
Partial oxidation	$C + 1/2O_2 \leftrightarrow CO$
Complete oxidation	$C + O_2 \leftrightarrow CO_2$
Carbon-water reaction	$C + H_2O \leftrightarrow CO + H_2$
Water–gas shift	$CO + H_2O \leftrightarrow CO_2 + H_2$
Methane formation	$CO + 3H_2 \leftrightarrow CH_4 + H_2O$

Syngas can be fermented by acetogens to ethanol and acetic acid (Najafpour and Younesi 2006). Some acetogens are capable of fermenting synthesis gas into butanol and butyric acid in addition to ethanol and acetic acid. The fermentation is strictly anaerobic in nature. The overall reactions involved in syngas fermentation to produce acetate and ethanol are shown below (Klasson et al. 1992):

$$6CO + 3H_2O \rightarrow CH_3CH_2OH + 4CO_2 \quad (2.1)$$

$$2CO_2 + 6H_2 \rightarrow CH_3CH_2OH + 3H_2O \quad (2.2)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (2.3)$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (2.4)$$

A process flow diagram for the production of ethanol employing gasification-fermentation technology is shown in Figure 2.2. The process consists of several steps. The biomass is first gasified to produce syngas, which then flows through a scrubber to remove ash and tar materials that could inhibit the microbial catalysts. Then, the syngas enters the fermentor, where it is utilized by the microbes to produce metabolites like ethanol, acetic acid and butanol. Ethanol is then recovered by distillation and dehydration to produce fuel grade ethanol.

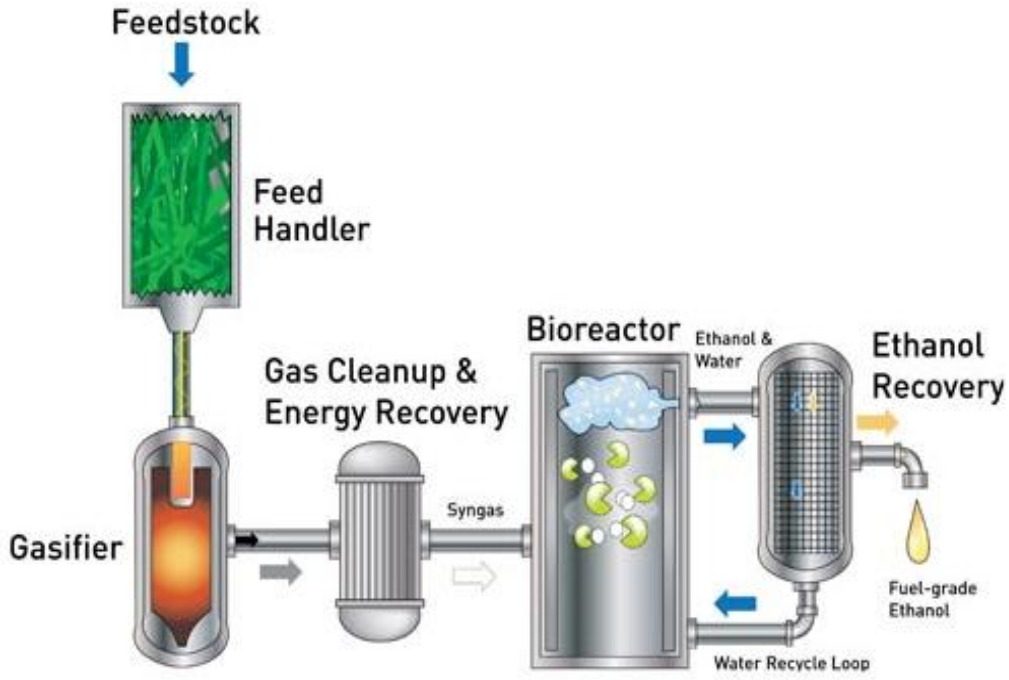

Figure 2.2 Process flow diagram for the production of ethanol employing gasification-fermentation technology (Coskata 2009).

Advantages of gasification−fermentation are:

- Feedstock flexibility – a wide range of raw materials like biomass, municipal waste can be used.
- Low operating pressures and temperatures and high end product specificity (Grethlein and Jain 1992; Klasson et al. 1992).
- Lignin (which is not used in direct fermentation) is converted to CO, H_2 and CO_2, which are eventually utilized for ethanol production (Reed et al. 1980).
- No pretreatment of biomass or hydrolytic enzymes are required.

Disadvantages of gasification−fermentation are:

- Production of tars and other impurities in syngas such as nitric oxide (NO_x) and ammonia can inhibit the microbial catalysts (Ahmed 2006).

- Sensitivity of microbial catalysts to environmental conditions such as pH, O_2 concentration and redox potential.
- Gas−liquid mass transfer limitations due to low solubility of H_2 and CO in the fermentation media (Worden et al. 1997).
- Low productivity in bioreactors, primarily due to low cell density and inhibition of microbes by the products and reactants (Worden et al. 1997).

2.4 Acetogens

Acetogens are the class of autotrophic microbes that utilize CO_2 and CO to produce ethanol and acetic acid (Zeikus et al. 1985). They are also chemoorganotrophs that can consume organic substrates to produce metabolites. Acetogens produce acetic acid as a primary product (Ljungdhal 1986). There are many microorganisms that produce acetic acid and ethanol through the Wood–Ljungdahl pathway. Details on some of the industrially important microbes that utilize syngas components are given below.

Peptostreptococcus productus, a gram–positive, mesophilic bacteria can produce acetate by utilizing either CO or CO_2 in the presence of H_2 (Lorowitz and Bryant 1984). *Clostridium thermoaceticum* is an anaerobic thermophilic bacterium that can grow optimally at pH 7 – 8 and between 55 and 60°C to produce acetic acid (Sugaya et al. 1986). *Clostridium ljungdahlii*, a rod shaped gram–positive acetogen, is the first known microbe to utilize syngas to produce ethanol and acetic acid (Klasson et al. 1992). The acetic acid production was favored at pH 5 – 7 and ethanol production was favored at pH 4 – 4.5 (Klasson et al. 1992). This bacterium produces acetic acid during the growth phase and ethanol during stationary phase (Klasson et al. 1992).The highest ethanol and acetic acid concentrations obtained with cell recycle system in a continuous stirred-tank

reactor (CSTR) employing *C. ljungdahlii* at the end of 560 h of operation were 48 g/L and 3 g/L, respectively (Klasson et al. 1993). The ratios of product concentrations (ethanol to acetate) were between 1.2 g/g and 16 g/g (Klasson et al. 1993). *Clostridium autoethanogenum* was isolated from rabbit feces, which can utilize carbon sources like CO, CO_2 and organic carbon sources such as xylose, pyruvate and fructose to produce ethanol and acetic acid (Abrini et al. 1994).

Clostridium carboxidivorans P7, an anaerobic spore forming, gram positive acetogen, was isolated from the sediments of an agricultural lagoon at Oklahoma State University (Liou et al. 2005). This microbe was able to grow on CO, CO_2 and H_2 to produce ethanol, acetate, butyrate and butanol. The strain P7 produced 4.4 g/L ethanol, 0.7 g/L acetate and 1.7 g/L butanol (Liou et al. 2005). The product profile of the strain P7 was evaluated in a bubble column reactor and the results showed that the amount of ethanol, acetic acid and butanol produced after 10 days of fermentation were 1.6 g/L, 0.3 g/L and 0.6 g/L, respectively (Rajagopalan et al. 2002). In another study conducted with the same microorganism in a 3 L bioreactor (chemostat mode), the acetic acid and ethanol yields were found to be 8 g/g cell and 3 g/g cell, respectively, after 21 days of fermentation (Ahmed et al. 2006).

Clostridium strain P11 is a gram positive acetogen, capable of utilizing syngas to produce ethanol and acetate (Huhnke et al. 2008). Strain P11 was able to produce 0.5 g/L ethanol and 4.5 g/L acetic acid after 300 h of fermentation in batch studies without the addition of reducing agents (Panneerselvam 2009). However, the same study showed that the maximum ethanol concentration obtained with the addition of methyl viologen and neutral red were 1.3 and 0.6 g/L, respectively. In another study, the maximum amount of

products produced by strain P11 were 25.3 g/L ethanol (after 1416 h of fermentation), 9.3 g/L of 2-propanol (after 576 h), 4.8 g/L acetic acid (after 576 h) and 0.5 g/L of 1-butanol (after 312 h) in a 100 L bioreactor (Kundiyana et al. 2010). It was also found that ethanol production was maximum when the concentrations of Cu^{2+}, Ni^{2+}, Zn^{2+}, SeO_4^- and WO_4^- in the fermentation media were 0, 8.5, 35, 7 and 5 µM, respectively (Saxena 2008). Acetogens produce metabolites like ethanol and acetic acid by utilizing CO, CO_2 and H_2 through the acetyl–CoA or the Wood–Ljungdahl pathway (Wood et al. 1986).

2.5 Acetyl–CoA Pathway or Wood–Ljungdahl Pathway

The acetyl–CoA pathway was discovered in *C. thermoaceticum* (Wood et al. 1986). The acetyl–CoA pathway was named the Wood Ljungdahl pathway to honor the discoverers, Harland Wood and Lars Ljungdahl (Ahmed 2006). Before the discovery of acetyl–CoA pathway, only two pathways were believed to be involved in the utilization of CO_2 for autotrophic growth of microbes, i.e. the Calvin cycle and reductive tricarboxylic acid cycle (Wood et al. 1986). The acetyl–CoA formed in the acetyl–CoA pathway serves as the precursor for the production of carbohydrates, amino acids, nucleotides and lipids (Ljungdhal 1986).

One of the main purposes of the acetyl–CoA pathway is to conserve energy by forming acetate through assimilation of carbons (Drake 1994). The ability for certain microbes to utilize CO is made possible through the conversion of CO to CO_2 in the acetyl–CoA pathway (Ragsdale 2004). In this pathway, CO acts both as an electron donor and a carbon source (Ragsdale 2004). The CO_2/CO redox potential is -558 mV at pH 7 and the potential of CO in acting as an electron donor is significantly higher than that of nicotinamide adenine dinucleotide (NADH) (Ragsdale 2004). The reduction of CO_2 to

acetyl–CoA occurs through two branches, namely the carbonyl and methyl branch. In the methyl branch, CO_2 is reduced to a methyl corrinoid protein and in the carbonyl branch, CO_2 is reduced to a carbonyl group by carbon monoxide dehydrogenase (CODH) as shown in Figure 2.3 (Wood et al. 1986).

2.5.1 Methyl Branch

The methyl branch leads to the formation of methyl corrinoid protein. If CO is available as a carbon source, it is first converted to CO_2 by carbon monoxide dehydrogenase (CODH) (Ljungdhal 1986). Formate dehydrogenase (FDH) catalyzes the reversible reduction of carbon dioxide to formate (Ljungdhal 1986). Even though ferredoxin is an electron donor in most microbes, in case of *Clostridium thermoaceticum* nicotine adenine dinucleotide phosphate (NADPH) acts as electron donor in the conversion of CO_2 to formate (Ljungdhal 1986) as shown in Eqn. 2.5.

$$CO_2 + NADPH \rightarrow HCOO^- + NADP^+ \quad (2.5)$$

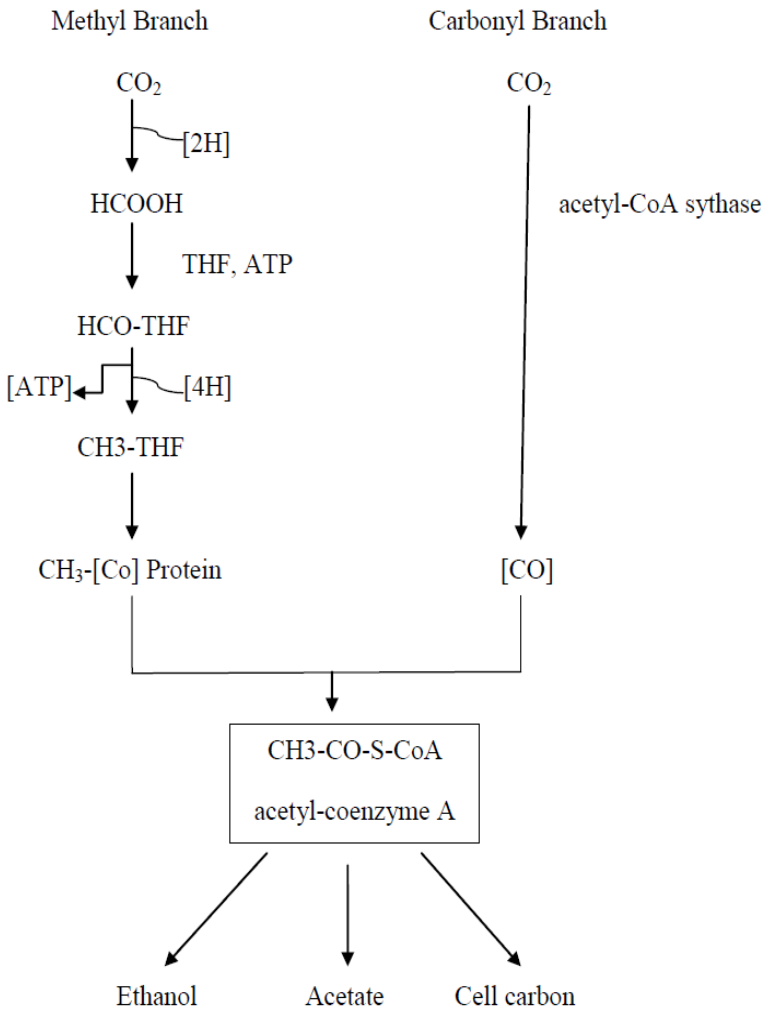

Figure 2.3 Simplified representation of the acetyl–CoA pathway in acetogens. Adapted from (Drake 1994).

The reduction of formate to a methyl group is facilitated through a four step conversion process, involving four different enzymes namely formyl–H_4 folate synthetase, methenyl–H_4 folate cyclohydrolase, methylene–H_4 folate dehydrogenase and methylene–H_4 folate reductase (Ljungdhal 1986). The production of formyl–H_4 folate from formate and tetrahydrofolate (H_4 folate) is catalyzed by formyl–H_4 folate synthetase as shown in Eqn. 2.6 (Ljungdhal 1986).

$$HCOOH + H_4folate + ATP \rightarrow HCO - H_4folate + ADP + P_i \quad (2.6)$$

Formyl–H_4 folate is then dehydrated to methenyl–H_4 folate, catalyzed by methenyl–H_4 folate cyclohydrolase as shown in Eqn. 2.7 (Ljungdhal 1986).

$$HCO - H_4folate + H^+ \rightarrow CH - H_4folate^+ + H_2O \quad (2.7)$$

Methylene –H_4 folate dehydrogenase catalyzes the reduction of methenyl–H_4 folate to methylene–H_4 folate. One NADPH molecule is oxidized for this reduction reaction as shown in Eqn. 2.8 (Ljungdhal 1986).

$$CH - H_4folate^+ + NADPH \rightarrow CH_2 - H_4folate + NADP^+ \quad (2.8)$$

The reduction of methylene–H_4 folate to methyl–H_4 folate is catalyzed by methylene–H_4 folate reductase as shown in Eqn. 2.9 (Ljungdhal 1986).

$$CH_2 - H_4folate^+ + ferredoxin_{red} \rightarrow CH_3 - H_4folate^+ + ferredoxin_{ox} \quad (2.9)$$

The methyl group in the methyl–H_4 folate is transferred to a corrinoid protein, catalyzed by methyl transferase as shown in Eqn. 2.10 (Ljungdhal 1986).

$$CH_3 - H_4folate^+ + E - [Co] \rightarrow H_4folate + E - [Co] - CH_3 \quad (2.10)$$

2.5.2 Carbonyl Branch

The end product of the carbonyl branch is a carbonyl group, which later merges with a methyl group to form acetyl–CoA, which is the precursor for the formation of ethanol and acetic acid. Acetyl–CoA synthase or CODH catalyzes the formation of the

carbonyl group [CO] from CO_2 through a reduction reaction (Diekert and Wohlfarth 1994) as shown in Eqn. 2.11 (Ljungdhal 1986).

$$CO_2 + 2H^+ + 2e^- \leftrightarrow [CO] + H_2O \quad (2.11)$$

The methyl group merges with the carbonyl group to form acetyl–CODH moiety, which then condenses with free coenzyme to form acetyl–CoA, catalyzed by the acetyl–CoA synthase or CODH as shown in Eqn. 2.12 (Ljungdhal 1986).

$$E–[CO]–CH_3 + [CO] \rightarrow E–[Co] + \text{Acetyl CoA} \quad (2.12)$$

2.5.3 Metabolism of Acetyl–CoA

Acetyl–CoA is consumed for the formation of acetate, adenosine triphosphate (ATP), ethanol and cell mass. Microbes favor the formation of acetate from acetyl–CoA as it involves the formation of ATP, which is vital for their growth. Figure 2.4 shows the schematic for the production of acetate, ethanol, butanol and butyrate from acetyl–CoA in *Clostridium acetobutylicum* (Vasconcelos et al. 1994).

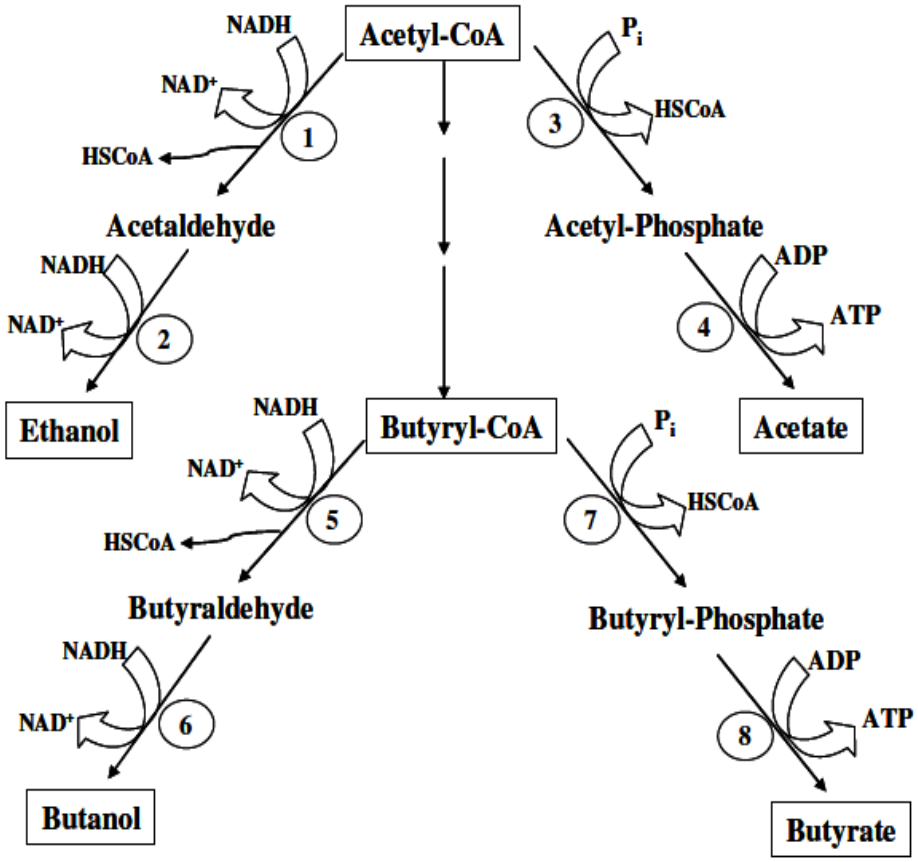

Figure 2.4 Metabolism of acetyl–CoA *in Clostridium acetobutylicum.* Adapted from (Vasconcelos et al. 1994). 1–acetaldehyde dehydrogenase, 2–alcohol dehydrogenase, 3–phosphotransacetylase, 4–acetate kinase, 5–butyraldedyde dehydrogenase, 6–butanol dehydrogenase, 7–phosphotransbutyrylase, 8–butyrate kinase.

2.5.3.1 Formation of Acetate from Acetyl−CoA

The production of acetate from acetyl−CoA is called acidogenesis, which involves two enzymes, namely phosphotransacetylase and acetate kinase. Acetyl–CoA is transformed into acetyl-phosphate by the addition of phosphate and removal of CoA

group, catalyzed by phosphotransacetylase as shown in the Eqn. 2.13 (Rao and Mutharasan 1989).

$$\text{Acetyl}-\text{CoA} + P_i \rightarrow \text{Acetyl}-\text{phosphate} + \text{CoA} \quad (2.13)$$

Acetyl-phosphate is then converted to acetate, accompanied by the phosphorylation of adenosine diphosphate (ADP) to ATP. This reaction is catalyzed by the enzyme acetate kinase as shown in Eqn. 2.14 (Rao and Mutharasan 1989).

$$\text{Acetyl}-\text{phosphate} + \text{ADP} \rightarrow \text{Acetate} + \text{ATP} \quad (2.14)$$

2.5.3.2 Formation of Ethanol from Acetyl – CoA

The production of alcohols from acetyl−CoA is referred to as solventogenesis and formation of ethanol from acetyl−CoA involves two enzymes, namely acetaldehyde dehydrogenase and alcohol dehydrogenase (Rao and Mutharasan 1989). The first step involves the conversion of acetyl−CoA to acetaldehyde, accompanied by the oxidation of NADH and removal of the CoA group. This step is catalyzed by acetaldehyde dehydrogenase as shown in the equation 2.15 (Rao and Mutharasan 1989).

$$\text{Acetyl}-\text{CoA} + \text{NADH} + H^+ \rightarrow \text{acetaldehyde} + \text{NAD}^+ + \text{CoA}-\text{SH} \quad (2.15)$$

In the final step, acetaldehyde formed in the previous reaction is converted to ethanol. This is a reduction reaction, accompanied by the oxidation of NADH to NAD^+, catalyzed by alcohol dehydrogenase as shown in Eqn. 2.16 (Rao and Mutharasan 1989).

$$\text{Acetaldehyde} + \text{NADH} + H^+ \rightarrow \text{Ethanol} + \text{NAD}^+ \quad (2.16)$$

During the stationary phase of cell's life cycle, NADH formation is observed and ethanol serves as the terminal electron acceptor. Hence, acetic acid production is favored during growth phase and ethanol production is favored during the stationary phase (Klasson et al. 1992). The formation of ethanol is dependent on the level of NADH within the cell and butanol production depends on the level of both NADH and NADPH (Rao and Mutharasan 1989). *Clostridium acetobutylicum* releases hydrogen to get rid of excess reducing equivalents (Gray and Gest 1965; Rao and Mutharasan 1989). Electrons are released in the form of molecular hydrogen through the oxidation of ferrodoxin (FDH_2) by hydrogenase enzyme during both acidogenic and solventogenic phases (Rao and Mutharasan 1989).

There are many factors that can affect the production of alcohols and acids, such as pH, NADH, reducing agents and nutrient availability in the fermentation medium (Adler and Crow 1987; Gottschal and Morris 1981; Rao and Mutharasan 1989; Rao et al. 1987). In a study conducted with *C. acetobutylicum*, it was concluded that the drop in pH from 7.0 to 5.0 was necessary for initiating solventogenesis (Gottschal and Morris 1981).

The amount of solvent produced was also found to be related to the number of spores formed by the cell culture and the cell morphology of *C. acetobutylicum* (Adler and Crow 1987). Nutrient content of fermentation media also plays a vital role in production of acids and alcohol. In a study with *C. acetobutylicum*, limiting the iron content in fermentation media resulted in an increase of butanol yield from glucose from 20% to 30% (Junelles et al. 1988).

An increase in cell concentration and decrease in lag phase was measured in syngas fermentation with P11 when glucose was used as the substrate (Panneerselvam 2009).

The effect of reducing agents on fermentation is discussed in the next section.

2.6 Reducing Agents

Redox potential is the ability of a solution or chemical to undergo oxidation or reduction reaction (IFIS 2009). The reduction or oxidation of hydrogen depends on the redox potential of the chemical substance that interacts with it. Hydrogen would be either oxidized or reduced based on the redox potential of the target chemical compound (Frankman 2009). Redox potential is measured in units of volts (V).

Monitoring the redox potential of fermentation broth helps in better understanding the fermentation process. Commercially available redox probes are used in monitoring the redox potential during the course of fermentation. The advantages of monitoring the redox potential level in fermentation are (Yang et al. 2007):

- Redox potential of the fermentation media is closely related to the metabolic activity inside the cell. Hence, controlling the redox potential appropriately would help increase or decrease formation of a product. For example, maximum ethanol production in fermentation by *Saccharomyces cerevisiae* was observed at a redox potential of -150 mV Standard Hydrogen Electrode (SHE).
- To ensure that the microbes grow in an environment that has the optimum redox potential for growth.
- In cases of anaerobic fermentation, the redox potential can help in measuring trace concentrations of dissolved oxygen (<1 ppm), which is difficult to measure with conventionally available DO sensors.

- Change in the redox potential of fermentation broth can indicate the change in nutrient concentration.

The following table shows different reducing agents used in fermentation processes (Frankman 2009). Some of the most notable ones are methyl viologen, neutral red and cysteine as they have already been determined to have an influence on ethanol production by strain P11.

Table 2.2 Various reducing agents used in fermentation processes.

Chemical	Microorganism	Concentration	Reference
Titanium (III)-citrate	*Methanobacterium thermoautotrophicum*	60 mM	(Jee et al. 1987)
Potassium ferricyanide	*Methanobacterium thermoautotrophicum*	0.1M	(Jee et al. 1987)
Hydrogen sulfide	*Methanobacterium thermoautotrophicum*	10 – 3,200 ppm	(Jee et al. 1987)
Sodium thioglycolate	*Thermoanaerobacter ethanolicus* *Clostridium acetobutylicum*	0.025 – 0.5 g/L	(Rao et al. 1987)
Cysteine	*Thermoanaerobacter ethanolicus* *Clostridium acetobutylicum*	0.025 – 0.5 g/L	(Rao et al. 1987)
Ascorbic acid	*Thermoanaerobacter ethanolicus* *Clostridium acetobutylicum*	0.025 – 0.5 g/L	(Rao et al. 1987)
Sodium sulfide	*Thermoanaerobacter ethanolicus* *Clostridium acetobutylicum*	0.025 – 0.5 g/L	(Rao et al. 1987)
Methyl viologen	*Thermoanaerobacter ethanolicus* *Clostridium acetobutylicum*	0.01 – 0.1 g/L	(Rao et al. 1987)
Sodium sulfide hydrate	*Clostridium thermosuccinogenes*	35 g/L	(Sridhar and Eiteman 2001)
Neutral red	*Clostridium carboxidivorans*	0.1 – 1 mM	(Ahmed 2006)
Neutral red	*Clostridium carboxidivorans*	0.1 – 1 mM	(Ahmed 2006)
Methyl viologen	*Clostridium* strain P11	0.1 mM	(Panneerselvam 2009)
Neutral red	*Clostridium* strain P11	0.1 mM	(Panneerselvam 2009)
Benzyl viologen	*Clostridium* strain P11	0.1 – 0.5 mM	(Panneerselvam 2009)

Reducing agents help increase alcohol production in fermentation processes by the following methods:

- Reducing agents can act as artificial electron carriers and alter the $NADH/NAD^+$ ratio. In *Clostridium* species, the shift towards solventogenesis phase is facilitated by altering the carbon flow with the addition of a reducing agent (Hipolito et al. 2008). The pathways involved in production of alcohols require high NADH levels (Rao et al. 1987). Therefore, alcohol production can be increased by increasing the NADH levels within the cells (Rao et al. 1987).
- Reducing the redox potential of the fermentation media (Jones and Pickard 1980). It was reported that negative redox potentials in the fermentation broth initiates solventogenesis in syngas fermentation with *Clostridium* strain P11 (Frankman 2009).
- Increasing the enzymatic activity of aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) (Girbal et al. 1995a). ALDH and ADH are the two enzymes involved in the production of aldehyde from acetyl−CoA and alcohol from aldehyde, respectively.

Reducing agents have been used in many fermentation studies that involve ethanol production. The addition of methyl viologen to the fermentation medium increased ethanol production from 1.41 g/L to 2.92 g/L in *Thermoanaerobacter ethanolicus,* whereas the lactate production decreased from 13.7 g/L to 12.0 g/L (Lamed and Zeikus 1980; Rao et al. 1987). Methyl viologen most significantly influenced the flow of electrons among other reducing agents studied such as sodium thioglycolate, ascorbic acid, cysteine and sodium sulfide (Rao et al. 1987).

It is also reported that the addition of 1 mM neutral red in a continuous culture of *Clostridium acetobutylicum* improved ethanol yield by threefold and decreased the total acetic acid yield by twofold (Girbal et al. 1995b). Neutral red increased alcohol yield by favoring the NADH related pathways (Girbal et al. 1995b). A decrease in yield of ethanol, hydrogen, lactate and an increased acetate production was observed in *Thermoanaerobium brockii* when electron acceptors were added to the fermentation media (Lamed and Zeikus 1980). There are very few studies exploring the application of reducing agents in syngas fermentation.

The effect of neutral red on ethanol and acetic acid production by *C. carboxidivorans* P7 was studied in both batch and semi-batch fermentations (Ahmed 2006). After 38 h of fermentation, neutral red was added to the medium. The concentrations of neutral red were 0.1, 0.4 and 1 mM. For batch studies, the results showed that ethanol productivity increased as the concentration of neutral red increased. For 0, 0.1, 0.4 and 1 mM neutral red, amounts of ethanol per cell mass were found to be 0.39 g/g cells, 0.67 g/g cells, 0.96 g/g cells and 1.92 g/g cells, respectively, after 4.5 days of fermentation; whereas, amount of acetic acid per cell mass were 11.8 g/g cells, 10.4 g/g cells, 7.2 g/g cells and 4.9 g/g cells, respectively (Ahmed 2006). This indicates that acetic acid production decreased with increasing concentrations of neutral red. It was also reported that the addition of neutral red had no effect on cell growth (Ahmed 2006).

In semi-batch studies, addition of 0.1 mM neutral red resulted in an ethanol yield of 6.6 g ethanol/g cell, whereas the medium with no neutral red produced 3 g/g after 230 h of fermentation (Ahmed 2006). The medium with 0.1 mM neutral red produced 6.7 g acetic acid/g cell, whereas the control medium produced 7 g/g after 230 h of fermentation

(Ahmed 2006).

The addition of 0.2 mM neutral red to the fermentation media at 67 h resulted in an ethanol yield of 4.9 g ethanol /g cell after 192 h of fermentation, whereas the control treatment only produced 1.8 g/g. In contrast, the control treatment produced more acetic acid (14 g acetic acid/g cell) than the treatment with 0.2 mM neutral red (9.8 g /g) after 192 h of fermentation (Ahmed 2006).

The effect of three different reducing agents, namely neutral red, methyl viologen and benzyl viologen on ethanol production by *Clostridium* strain P11 was studied in 250 ml serum bottles containing yeast extract based fermentation medium (Panneerselvam 2009). The reducing agents were added after 91 h of fermentation and the tests were followed for 300 h. The maximum ethanol concentration obtained with the addition of methyl viologen and neutral red were 1.3 and 0.6 g/L, respectively after 300 h of fermentation. Ethanol yields (g ethanol per g cells) were 7.5 g/g and 2.8 g/g with the addition of 0.1 mM methyl viologen and 0.1 mM neutral red, respectively (Panneerselvam 2009). In contrast, the control treatments had an ethanol yield of 2.4 g/g, indicating that addition of 0.1 mM methyl viologen enhanced ethanol yield by over threefold (Panneerselvam 2009). Acetic acid production decreased with the addition of methyl viologen (Panneerselvam 2009).

2.6.1 Dithiothreitol

Dithiothreitol (DTT), also called as Cleland's reagent, is a water soluble reducing agent with a redox potential of -332 mV at pH 7 and -366 mV at pH 8.1 (Cleland 1964). The molecular formula of DTT is $C_4H_{10}O_2S_2$ and its molecular weight is 154.25 g/mol (Cleland 1964). DTT has many applications which include:

- Reduction of redox potential. Addition of DTT significantly reduced the redox potentail of the fermentation broth involving 4-Decanolide production by *Sporidiobolus johnsonii* and *S. ruinenii* (Wang et al. 2000).
- Electron donor. DTT was used as an electron donor to increase the efficiency of hydrogen production by bacterial hydrogenase system (Krasnovsky et al. 1980).
- Enzyme protector. DTT can protect many enzymes from denaturation due to its ability to form disulfide bonds with enzyme active sites that are otherwise susceptible to damage by oxygen (Asada et al. 1981).

The addition of DTT reversed the oxidation of thiol groups in membrane vesicles that play a role in K^+ influx in *Escherichia coli,* thereby restoring cell growth and survival (Bagramyan et al. 2000). In yeast fermentation, addition of 2 mM of DTT protected the thiol groups (SH) of some of the key enzymes like the hexokinase, glyceraldehyde 3-phosphate dehydrogenase and adenosine kinase involved in ATP formation, thereby increasing the duration of ATP formation from 42 h to 100 h (Asada et al. 1981). DTT (1 mM) was also used to prevent the oxidative deactivation of NADH oxidase in *Lactobacillus brevis* (Hummel and Riebel 2003). In another study, DTT was used to increase the activity of endoproteolytic enzymes in malt brewing by preventing the oxidation of proteolytic enzymes (Jones and Budde. 2003). DTT also prevents the oxidation of NADH, NAD(P)H, reduced ferrodoxin by diamide (N, N, N', N'-tetramethylazoformamide) in *Clostridium* species. Diamide oxidizes the intracellular glutathione, thereby affecting the cell growth and protein synthesis (O'Brien et al. 1970).

Based on many studies that showed DTT has positive effects on alcohol production and enzymes involved in fermentation processes and the fact that no reports

were found in the literature on the use of DTT in syngas fermentation, the effect of this reducing agent on ethanol production from syngas using *Clostridium* strain P11 was investigated in the present study. It was hypothesized that DTT can increase ethanol production by acting as an electron donor to regenerate NADH from NAD^+. NADH is directly involved in the ethanol production pathway.

The anticipated advantages of the use of DTT in syngas fermentation include:

- DTT is a strong reducing agent and donates 2 electrons per molecule to help regenerate NADH from NAD^+.
- DTT is a less expensive reducing agent compared to neutral red and methyl viologen on a molar basis (Figure 2.5). The cost of DTT is $491/mol, whereas neutral red and methyl viologen costs are $5540/mol and $8711/mol, respectively.

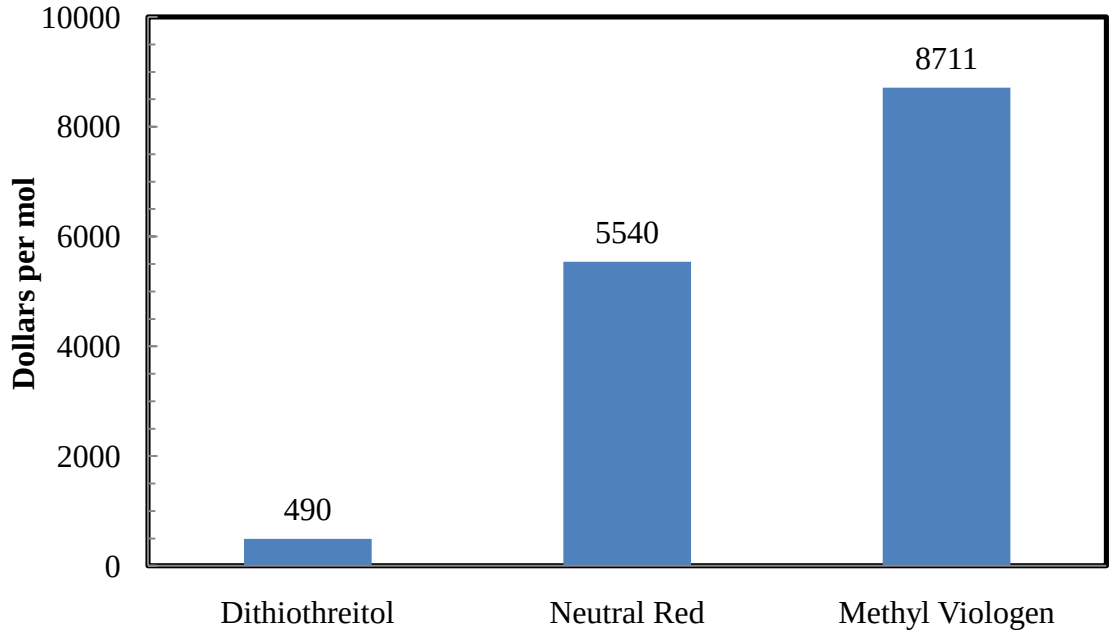

Figure 2.5 Cost of various reducing agents. Based on cost as of 11/03/2009 of dithiothreitol from Gold Biotechnology and neutral red and methyl viologen from Sigma Aldrich.

However, disadvantages of using DTT include:

- Oxidation of DTT is an irreversible process. Hence, there would be no regeneration of reduced form of DTT (Krasnovsky et al. 1980).
- Downstream processing and waste disposal could be an issue if large quantities of DTT were to be used in the fermentation process.

The effect of various concentrations of DTT on the growth and product formation by *Clostridium* strain P11 during syngas fermentation in yeast extract and corn steep liquor media was investigated in the present study.

CHAPTER III

MATERIALS AND METHODS

3.1 Microbial Catalyst

Clostridium strain P11 provided by Dr. Ralph S. Tanner, University of Oklahoma, was used as the microbial strain P11 is gram positive and rod shaped and has the ability to ferment syngas (CO, CO_2 and H_2), as well as some sugars, to produce acids and alcohols. The optimum pH and temperature for growth of strain P11 are 6.1 and 37°C, respectively. Subcultures of strain P11 were maintained at room temperature with feeding of syngas once in every 15 days.

3.2 Culture Medium

The microbial culture was grown under strict anoxic conditions in a defined medium that was previously optimized (Saxena 2008). The medium consisted of the following components (per liter) as shown in Table 3.1: 1 g yeast extract (Difco laboratories, Detroit, MI) or 10 g corn steep liquor (Sigma Aldrich, St Louis, MO) depending on the type of fermentation medium, 10 g morpholinoethanesulfonic acid (MES), 1 ml resazurin (0.1%), 30 ml minerals stock solution, 10 ml vitamins stock solution, 10 ml trace metals stock solution and 10 ml of 4% cysteine sulfide solution.

Minerals stock solution

The minerals stock solution consisted of the following components (per liter): 100 g ammonium chloride, 4 g calcium chloride, 20 g magnesium sulfate, 10 g potassium chloride and 10 g potassium phosphate monobasic.

Trace metal stock solution

The trace metal stock solution consisted of the following components (per liter): 2 g nitrilotriacetic acid, 1 g manganese sulphate, 0.8 g ferrous ammonium sulphate, 0.2 g cobalt chloride, 1 g zinc sulphate, 0.2 g nickel chloride, 0.02 g sodium molybdate, 0.1 g sodium selenate and 0.2 g sodium tungstate.

Vitamins stock solution

The vitamins stock solution consisted of the following components (per Liter): 0.005 g p-(4)-aminobenzoic acid, 0.002 g d-biotin, 0.005 g pantothenic acid (calcium salt), 0.002 g folic acid, 0.01 g MESNA, 0.005 g nicotinic acid, 0.01 g pyridoxine, 0.005 g riboflavin, 0.005 g thiamine, 0.005 g thioctic acid and 0.005 g vitamin B-12.

Table 3.1 Media compositions used in this study.

Components	Yeast Extract Media (per liter)	Corn Steep Liquor Media (per liter)
Yeast extract	1 g	-
CSL	-	10 g
Minerals	30 ml	30 ml
Vitamins	10 ml	10 ml
Trace metals	10 ml	10 ml
MES buffer	10 g	10 g
Resazurin (0.1%)	1 ml	1 ml
Cysteine sulfide (4%)	10 ml	10 ml

3.3 Simulated Synthesis Gas

Commercial syngas composed of 5% H_2, 15% CO_2, 20% CO and 60% N_2 by volume (Airgas, Inc) was used in this study. The above gas composition is similar to the composition of syngas obtained by gasifying switchgrass (Rajagopalan et al. 2002).

3.4 Biomass Synthesis Gas (Producer Gas)

The biomass-based producer gas (obtained by gasifying switchgrass in a down draft gasifier at Oklahoma State University) was used in this experiment. The producer gas used in the 1.0 g/L yeast extract media experiment consisted of 8.24% H_2, 14.21% CO_2, 15.38% CO, 56.9% N_2 and 5.21% methane by volume. The producer gas used in the 10 g/L corn steep liquor media experiment consisted of 8.76% H_2, 14.36%, CO_2, 13.67% CO, 60% N_2, 3.19% methane and 0.41% acetone by volume. Previous studies have shown that the producer gas also contains 1.4% ethylene, 0.35% ethane, 0.1% acetylene and 150 ppm nitric oxide (Ahmed 2006).

3.5 Preparation of DTT stock solution

Dithiothreitol (DTT), purchased from Gold Biotechnology Inc, St Louis, MO (catalog # DTT 20; CAS# 27565-41-9) was used for the experiments. Dithiothreitol is added into the fermentation media in liquid form and is prepared fresh just before its addition into the fermentation media (within 1-2 hours before adding the DTT into treatment bottles). The steps involved in the preparation of DTT stock solution are described below.

1. The DTT stock solution was prepared by dissolving 12.5 g of DTT powder in Erlenmeyer flask that contained about 50 ml of freshly prepared fermentation medium. The DTT stock solution was prepared in the same fermentation medium (yeast extract or corn steep liquor media) as in Table 3.1 in order to have consistent composition of mineral, trace metals and vitamins in all the treatments with and without DTT.
2. The stock DTT solution was then transferred into a 100 ml volumetric flask and filled to 100 ml with the fermentation medium. The DTT stock solution was then carefully transferred to a clean 250 ml serum bottle. The stock solution was sparged with N_2 for about 15 minutes to remove any dissolved O_2 that could otherwise oxidize DTT and reduce its efficiency as a reducing agent.
3. The serum bottle containing stock DTT solution was then sealed with a gas impermeable butyl rubber stopper and an aluminum cap. This stock solution was used to prepare the treatments with various concentrations of DTT.

3.6 Batch Studies

Batch experiments were done in 250 ml serum bottles (Wheaton, NJ) with 100 ml of fermentation medium (Table 3.1), which was prepared and sparged with N_2. The medium was then dispensed into serum bottles inside a glove box under strict anoxic conditions. This was followed by addition of 1 ml of 4% cysteine sulfide solution and the bottles were then sterilized in an autoclave at 121°C for 20 minutes (Primus Sterilizer Co. Inc, Omaha, NE). After the bottles were cooled to room temperature, DTT stock solution was added filter sterilized according to Table 3.2 to each treatment using a syringe equipped with 0.2 µm nylon filters (VWR, Arlington Heights, IL). Four concentrations of DTT, 2.5, 5.0, 7.5 and 10.0 g/L and a control (no DTT), were used in this study.

Table 3.2 Amount of fermentation media and dithiothreitol (DTT) in various treatments.

DTT (g/L)	Initial volume of fermentation medium (ml)	Volume of DTT stock solution added (ml)
0.0	100	-
2.5	98	2.0
5.0	96	4.0
7.5	94	6.0
10.0	92	8.0

The serum bottles were then fed with syngas at 239 kPa (absolute) and inoculated with 5% (v/v) of strain P11 culture. The experiment was conducted twice to test the

statistical significance of the effect of DTT on ethanol production from the obtained results. Each time, the test was run in duplicate for each DTT concentration and control.

Bottles were placed on an orbital rotary shaker (Innova 2100, New Brunswick Scientific Edison NJ) at 150 rpm and incubated at 37ºC in a temperature-controlled room. The fermentation was followed for a period of 360 h (15 days). Samples were collected from the serum bottles every 24 h. Headspace gas was replaced with fresh syngas at 239 kPa (absolute) each day after the sample was collected from the serum bottle. Cell concentration, pH, acetic acid, ethanol and butanol concentrations were measured.

3.7 Analytical Procedures

3.7.1 Cell Mass Measurement

The optical density (OD) of the samples was measured at 660 nm using a UV–Vis spectrophotometer (Varian Inc., Palo Alto, CA). Samples with OD values above 0.4 were diluted so that the OD was within the linear range of the calibration curve between cell mass and OD. The equation used to determine the cell mass from O.D value is given below (Panneerselvam 2009).

$$\text{Dry Cell Weight (g/L)} = 0.396 \times \text{OD} - 0.0521 \quad (3.1)$$

3.7.2 Acetic Acid and Butanol Analysis for Samples from Experiment with Simulated Syngas

Acetic acid in the samples was analyzed using Agilent GC 6890 gas chromatography (Agilent Technologies, Wilmington, DE) setup fitted with PoraPak QS 80/100 column, which was connected to a flame ionization detector. The GC was operated at an isothermal temperature of 210ºC with helium as the carrier gas, whose

flow rate was set at 25 ml/min. Chromatograms were analyzed using Agilent CHEMSTATION® data analysis software.

3.7.3 Acetic Acid, Acetone and Isopropanol Analysis for Samples from Experiment with Producer Gas

Acetic acid, acetone and isopropanol in the samples were analyzed using Agilent GC 6890 gas chromatography (Agilent Technologies, Wilmington, DE) setup fitted with a Supelco 25461 capillary column, which was connected to a flame ionization detector. The GC was operated with a ramping temperature profile that reached a maximum of 235ºC with hydrogen as the carrier gas, whose flow rate was set at 25 ml/min. Chromatograms were analyzed using CHEMSTATION® data analysis software.

3.7.4 Ethanol Analysis

Ethanol concentration was measured using an YSI 2700 Biochemistry analyzer (YSI Life Sciences, Yellow Springs, OH). The instrument uses an enzymatic method to detect ethanol concentration in the sample.

3.7.5 Statistical Analysis

An analysis of variance was calculated using SAS (Release 9.2, Cary, NC). Ethanol concentration was the dependent variable and dithiothreitol concentration was the independent variable. A Dunnett's test (Dunnett, 1955) was used to compare each DTT concentration to the control (without DTT). A 95% confidence level was used to determine if obtained results with each DTT concentration were different from the control.

CHAPTER IV

RESULTS AND DISCUSSION

The effect of the reducing agent dithiothreitol (DTT) on ethanol and acetic acid production was evaluated in two different fermentation media, 1.0 g/L yeast extract (YE) and 10 g/L corn steep liquor (CSL) and using simulated syngas and actual biomass producer gas.

4.1 Simulated Syngas

4.1.1 Yeast Extract Medium and Dithiothreitol

4.1.1.1 Fermentation Pattern

The fermentation pattern of strain P11 in the control media (without DTT) in 1 g/L YE medium is shown in Figure 4.1. The maximum cell mass obtained in the YE medium was 0.35 g/L after 192 h of fermentation, however, the cell mass decreased to 0.15 g/L after 360 h. The maximum amount of acetic acid produced in YE medium was 4 g/L after 216 h of fermentation. A decrease in acetic acid concentration was also observed in the YE medium. The final acetic acid concentration was 3.45 g/L after 360 h of fermentation.

The consumption of acetic acid was 14% in the YE medium. The maximum amounts of ethanol produced after 360 h were 0.50 g/L in YE medium. Also, acetic acid consumption by strain P11 occurred at the same time as ethanol production, indicating that acetic acid was reduced to form ethanol.

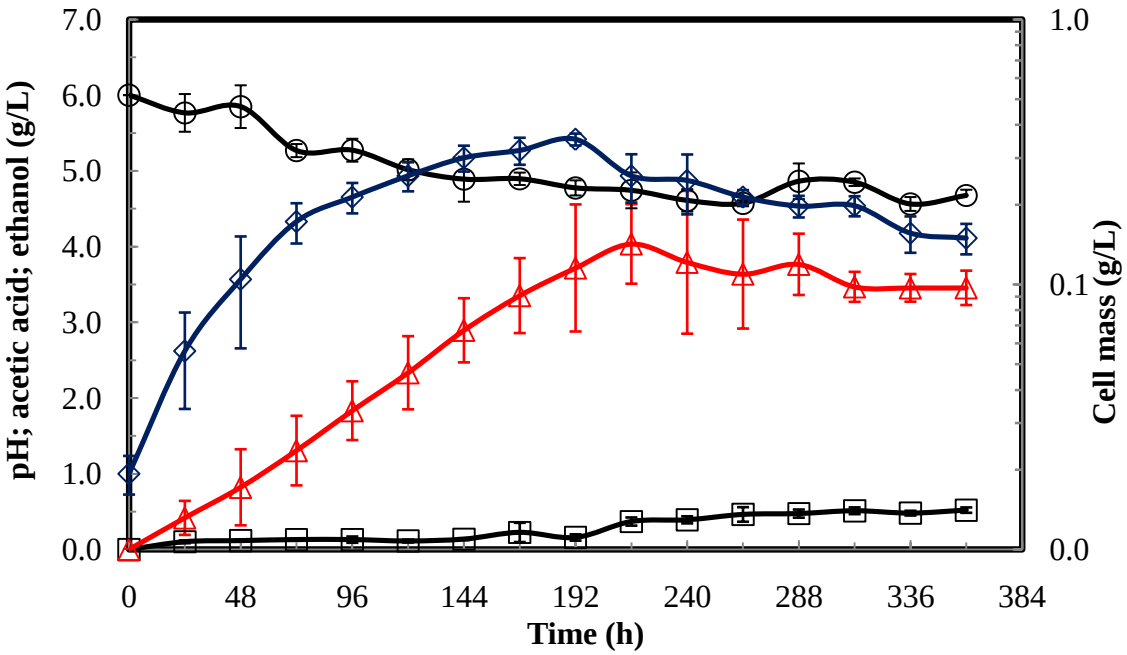

Figure 4.1 Kinetics of growth of *Clostridium* strain P11 and ethanol and acetic acid production in 1.0 g/L yeast extract medium with simulated syngas and without dithiothreitol: (◇) cell mass; (□) ethanol; (Δ) acetic acid; (O) pH.

4.1.1.2 Cell Growth

Growth profiles of *Clostridium* strain P11 in YE media containing various concentrations of DTT were similar until 168 h (Figure 4.2). Cells were in the exponential phase in the first 144 h, after which, cells entered the deceleration phase followed by the stationary phase. However, cell concentration declined after 192 h in the medium without DTT. A maximum cell mass concentration of 0.37 g/L was observed after 192 h in the medium with a DTT concentration of 2.5 g/L. The difference in cell

mass concentration in the first 168 h for all DTT concentrations tested was insignificant. However, a clear difference in cell mass concentration was measured between the medium without DTT and media with DTT after 192 h. The specific growth rate for strain P11 decreased with the increase in DTT concentration (Table 4.1).

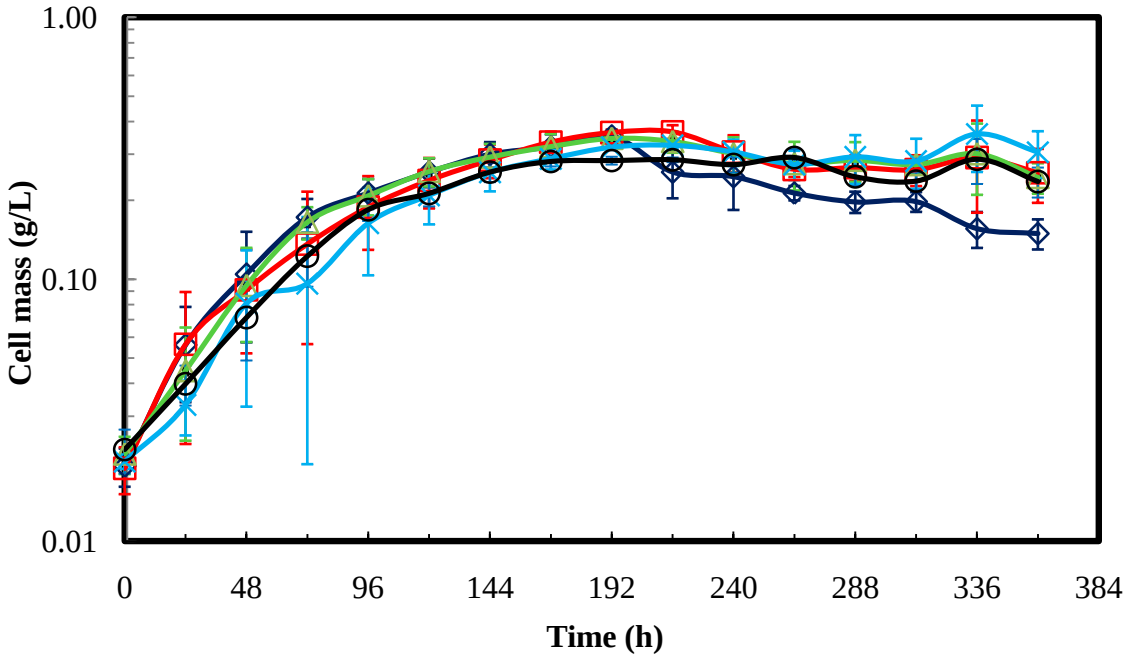

Figure 4.2 Kinetics of cell mass production in 1.0 g/L yeast extract media with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

Table 4.1 Specific growth rates and ethanol yields in 1.0 g/L yeast extract media with simulated syngas and various dithiothreitol (DTT) concentrations using *Clostridium* strain P11.

DTT (g/L)	Initial cells (mg/L)	Specific growth rate, μ (h^{-1})	Ethanol yields[a] (g/g)
0.0	19.3	0.035	3.48
2.5	18.9	0.033	2.98
5.0	21.5	0.028	3.60
7.5	20.1	0.022	7.60
10.0	22.4	0.022	11.42

[a] Values are calculated after 360 h in g ethanol/g cell mass

4.1.1.3 pH and Pressure Profiles

The pH of the fermentation medium with all tested DTT concentrations decreased similarly with time (Figure 4.3). This was largely influenced by the production of acetic acid. The pH of the media decreased from 6 to 4.5 in the first 264 h. It then increased to 4.8 between 264 and 360 h, which was also the time at which ethanol production rate increased. This indicates that ethanol production occured during the stationary phase in the pH range of 4.5 to 4.8. The differences in pH profiles with all tested DTT concentrations were statistically insignificant ($p < 0.05$).

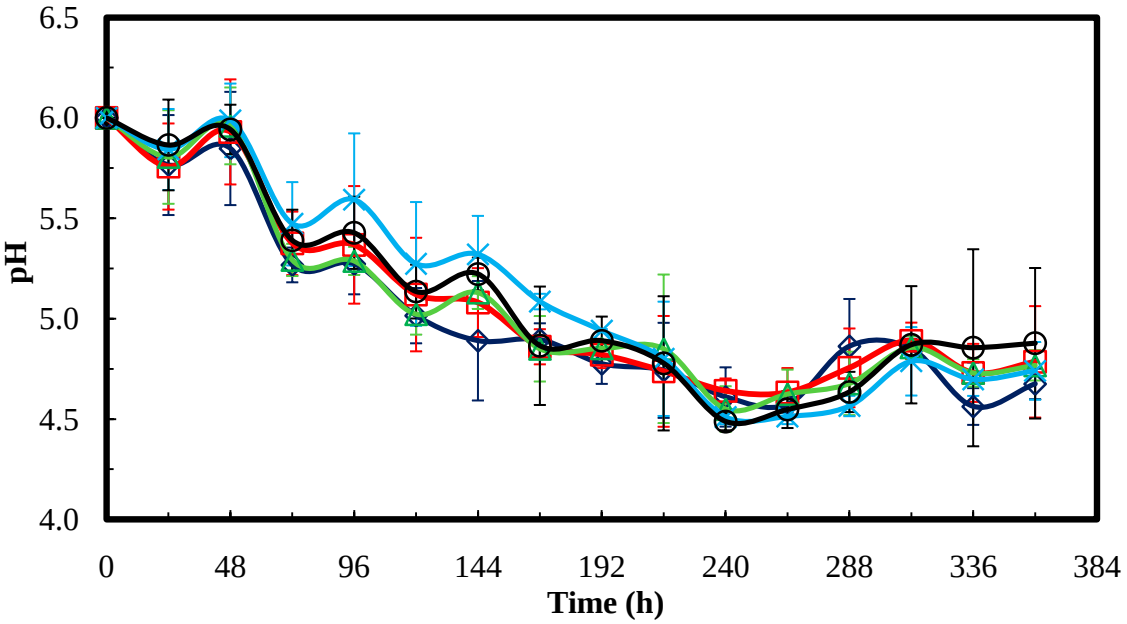

Figure 4.3 pH profile during syngas fermentation in 1.0 g/L yeast extract media with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

Figure 4.4 shows changes in head space pressure in the fermentation bottles after every 24 h. Initial pressure was set to 239 kPa (absolute) by purging the head space every 24 h after taking samples from all media to determine product and cell mass concentrations. More syngas was consumed in the first 192 h (most of the growth phase) compared to the rest of the fermentation period (stationary and death phases). The increase in syngas consumption from 24 to 96 h is due to the increase in cell mass concentration, which required more substrate for growth and product formation. Almost no gas consumption was measured after 264 h in the media with DTT concentrations below 5 g/L. However, syngas consumption was measured in the media with DTT above 7.5 g/L after 264 h. This indicates higher cell activity in the 7.5 g/L DTT medium (after 264 h) favored ethanol production, which will be explained in the next section.

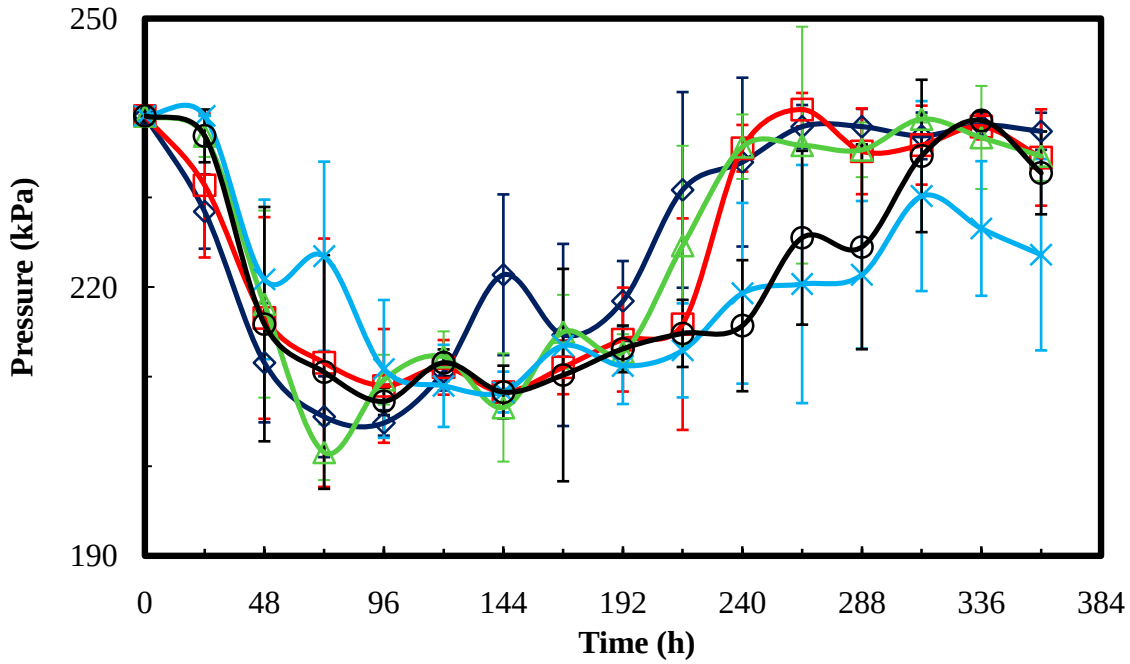

Figure 4.4 Pressure profile during syngas fermentation in 1.0 g/L yeast extract media with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

4.1.1.4 Product Profile

Acetic acid and ethanol were the main products found in all media. Butanol was also produced during the fermentation process in all media, however, the concentration of butanol was below 0.1 g/L, which is much less compared to the two main products (i.e., ethanol and acetic acid). Acetic acid is a primary metabolite and its production is associated with cell growth. Hence, there was production of acetic acid until 216 h in the media that contained 0, 7.5 and 10 g/L DTT (Figure 4.5). However, acetic acid production was only noticed in the first 192 h in media that contained 2.5 and 5.0 g/L DTT. There was larger variability in the amounts of acetic acid measured after 216 h in media with DTT concentrations above 5.0 g/L, which cannot be explained. However, there was a general decrease in acetic acid concentration in all media after 216 h. This

coincides with an apparent increase in pH in the media that was observed after 240 h (Figure 4.3). In addition, the decrease in acetic acid corresponded with an increase in ethanol production, suggesting that acetic acid was utilized by P11 for ethanol formation. A decrease in acetic acid combined with an increase in ethanol production was also observed in another syngas fermentation study using *Clostridium* strain P11 and reducing agents (Panneerselvam 2009).

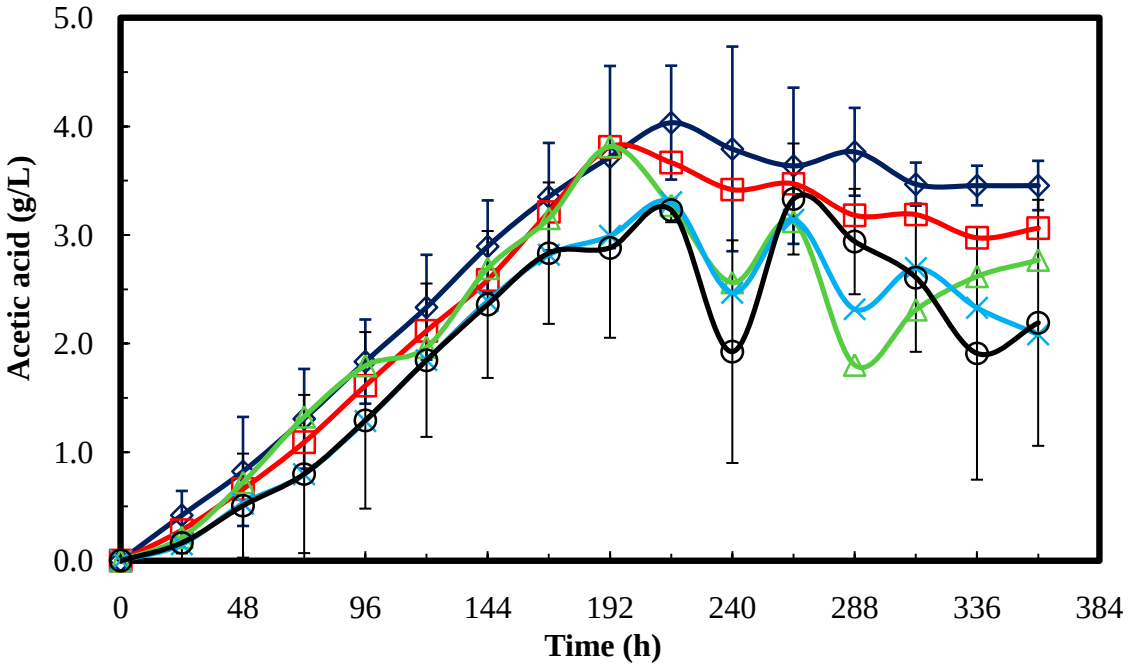

Figure 4.5 Kinetics of acetic acid production in 1.0 g/L yeast extract media with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

Ethanol production in media with and without DTT is shown in Figure 4.6. It can be seen that slight amounts of ethanol (< 0.2 g/L) were produced during the growth phase. However, more ethanol was produced during the stationary phase. The highest concentration of ethanol (2.7 g/L) was obtained in the medium with 10 g/L DTT after

360 h. This is over 500% more ethanol production than in the medium without DTT. In addition, 2.3 g/L of ethanol was produced in the medium with 7.5 g/L DTT. However, the cocentration of ethanol produced in the media with DTT concentrations of 5.0 g/L or less was 0.9 g/L.

Ethanol concentrations in fermentations containing 2.5 and 5.0 g/L DTT were not significantly different compared to the control ($p < 0.05$). This indicates that the concentration of DTT in the medium should be above 5.0 g/L to substantially enhance ethanol production using *Clostridium* strain P11. More variability in ethanol concentrations was measured in media with 7.5 and 10 g/L DTT compared to media with less DTT concentrations. There was no significant difference in ethanol production in media with 7.5 and 10 g/L DTT concentrations ($p < 0.05$). However, the amounts of ethanol produced between 288 and 360 h in 7.5 and 10 g/L DTT media were statistically significant than in the medium without DTT ($p < 0.05$).

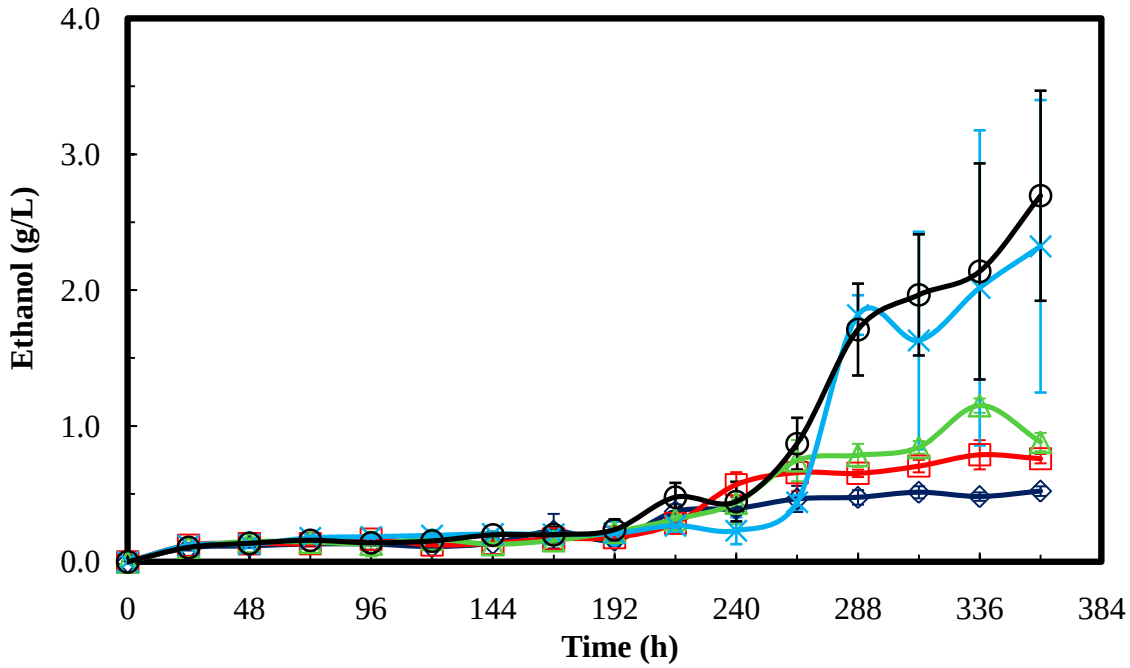

Figure 4.6 Kinetics of ethanol production in 1.0 g/L yeast extract media with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The addition of DTT did not have an effect on ethanol production during the growth phase (Figures 4.2 and 4.6). Its effect started after the cells entered the stationary phase when acetic acid production ceased (Figure 4.5). This indicates that there is no need to add DTT until the cells enter the stationary phase, which could result in reducing the amount of DTT needed to enhance ethanol production. It is hypothesized that DTT donated electrons, which were used to reduce more NAD^+ to NADH. The regenerated NADH probably might have contributed to the increased production of ethanol in the presence of DTT. The overall effect of DTT in enhancing solventogenesis is similar to other reducing agents used in previous studies (Ahmed 2006; Panneerselvam 2009; Peguin et al. 1994; Rao and Mutharasan 1986; Rao and Mutharasan 1988). The ethanol

yield in the presence of 10 g/L of DTT was 11.42 g ethanol/g cell mass (Table 4.1). However, it was only 3.48 g/g in the absence of DTT.

The ethanol yield increased as the concentration of DTT increased above 5.0 g/L. It was shown that the addition of 0.1 mM of neutral red in batch reactors during syngas fermentation with *Clostridium carboxidivorans* P7 increased the ethanol yield from 0.05 to 0.2 g ethanol/g cells (Ahmed 2006). Results from another study using *Clostridium* strain P11 during syngas fermentation with 0.1 mM methyl viologen showed a maximum ethanol production of 1.3 g/L and ethanol yield of 7.5 g/g after 300 h compared to 0.51 g/L of ethanol concentration and 2.2 g/g ethanol yield without the addition of methyl viologen (Panneerselvam 2009). In the same study, the addition of 0.1 mM neutral red to the fermentation medium slightly enhanced ethanol production and yield. More ethanol production and greater yield was observed in the present study; however, the concentration of DTT used in the present study to enhance ethanol production and yield is much higher than the concentrations of other reducing agents used earlier. The addition of DTT when cells enter stationary phase could decrease the amount of DTT required to affect ethanol production, which warrants further investigation. Results from an experiment with DTT, neutral red and methyl viologen confirmed that the use of 10 g/L DTT (64 mM) in the fermentation medium was twice as efficient in enhancing ethanol production compared to 0.1 mM methyl viologen and 0.1 mM neutral red (Table A.1).

4.1.2 Corn Steep Liquor Medium and Dithiothreitol

4.1.2.1 Fermentation Pattern

The fermentation pattern of strain P11in the control media (without DTT) in 10 g/L CSL medium is shown in Figure 4.7. The maximum cell mass was 0.43 g/L in CSL medium after 144 h. Cell death was less in CSL medium than in control of YE medium (Figure 4.1) and the cell mass in CSL medium after 360 h was 0.35 g/L. The maximum cell mass concentration in CSL medium was 23% more than the maximum cell mass concentration obtained with YE medium. However, the cell mass concentration after 360 h of fermentation in CSL medium was about 130% more than the cell mass concentration after 360 h in YE medium. This could be attributed to the rich nutrient content of CSL medium.

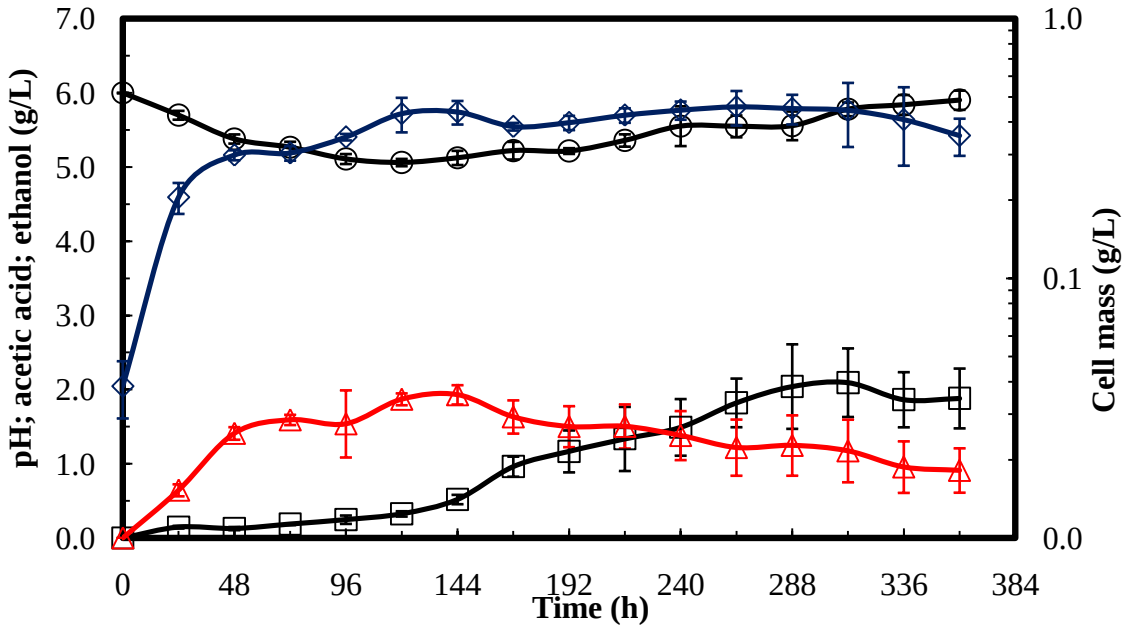

Figure 4.7 Kinetics of growth of *Clostridium* strain P11 and ethanol and acetic acid production in 10 g/L corn steep liquor medium with simulated syngas and without dithiothreitol: (◇) cell mass; (□) ethanol; (Δ) acetic acid; (O) pH.

The maximum amount of acetic acid produced in CSL medium was 1.93 g/L after 144 h of fermentation (Figure 4.7) and 4 g/L after 216 h in YE medium (Figure 4.1), which is nearly 107% more than the amount of acetic acid produced in CSL medium. A decrease in acetic acid concentration was also observed in both CSL and YE media. The final acetic acid concentration (after 360 h of fermentation) was 0.91 g/L in CSL medium and 3.45 g/L in YE medium. The consumption of acetic acid was 53% in the CSL medium, whereas, it was only 14% in the YE medium. The maximum amounts of ethanol produced after 360 h were 0.50 g/L in YE and 1.88 g/L in CSL media, respectively (Figures 4.1 and 4.7). The increase in ethanol concentration in CSL medium could be due to the higher cell mass concentration compared to YE medium. Also, acetic acid consumption by strain P11 occurred at the same time as ethanol production, indicating that acetic acid was reduced to form ethanol.

4.1.2.2 Cell Growth

Cells grown in CSL medium were in the exponential growth phase for the first 48 h (Figure 4.8). Cells in media with 0 g/L, 2.5 g/L and 5 g/L DTT remained in stationary phase from 96 h to 288 h, however, cells in treatments with 7.5 g/L and 10 g/L DTT remained in stationary phase from 48 h to 120 h, after which cell death was observed. It is clear that addition of higher concentrations of DTT (7.5 g/L or higher) reduced the final cell concentration. The maximum cell concentration obtained was 0.45 g/L in the control treatment after 264 h. After 120 h of fermentation, treatments with 7.5 g/L and 10 g/L DTT produced significantly less cell mass compared to the control ($p < 0.05$).

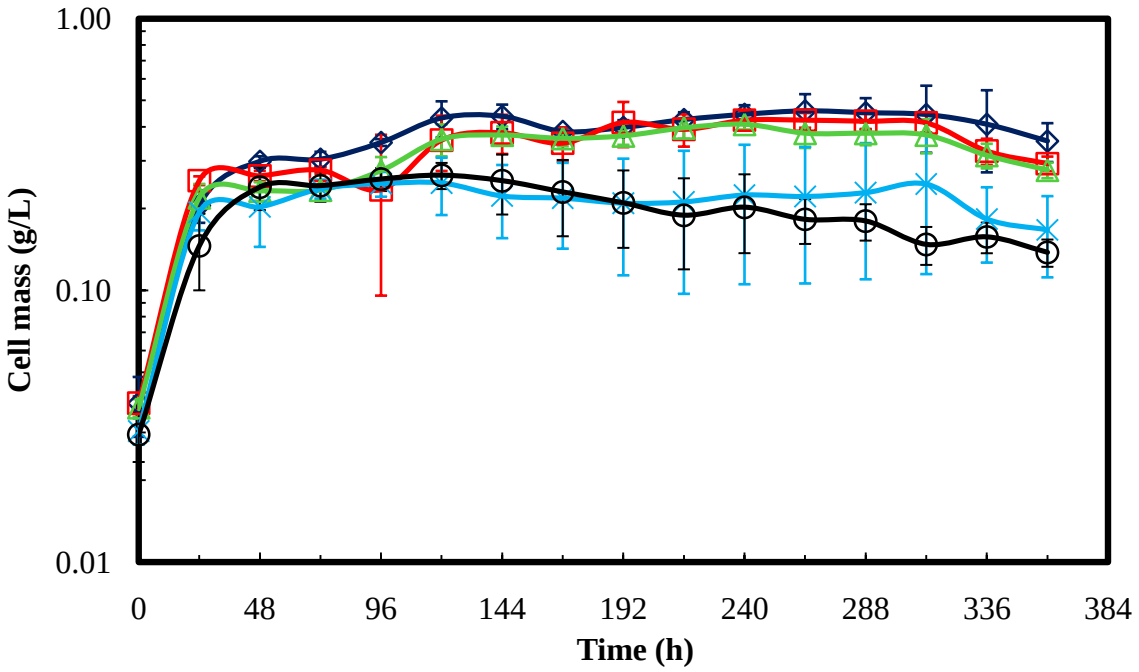

Figure 4.8 Kinetics of cell mass production in 10 g/L corn steep liquor media with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

4.1.2.3 pH and Pressure Profiles

The pH profile during the course of fermentation was similar for all DTT treatments from time 0 h to 48 h as shown in Figure 4.9. The pH dropped from an initial value of 6 to 5.4 in 48 h. However, the pH change was different for different treatments from 48 h to 360 h. In the control, the pH continued to decrease from 5.4 (at 48 h) to 5.0 (at 120 h). This is due to the increase in acetic acid concentration in the fermentation broth with time. After this, the pH increased to 6.0 (at 360 h). This is clearly due to consumption of acetic acid, which could have been used for ethanol production. In 10 g/L DTT media, the pH reached a value of 6 after 144 h of fermentation as almost all the

acetic acid in the broth was consumed by the bacteria. The pH profile for control and 2.5 and 5 g/L DTT were similar after 216 h.

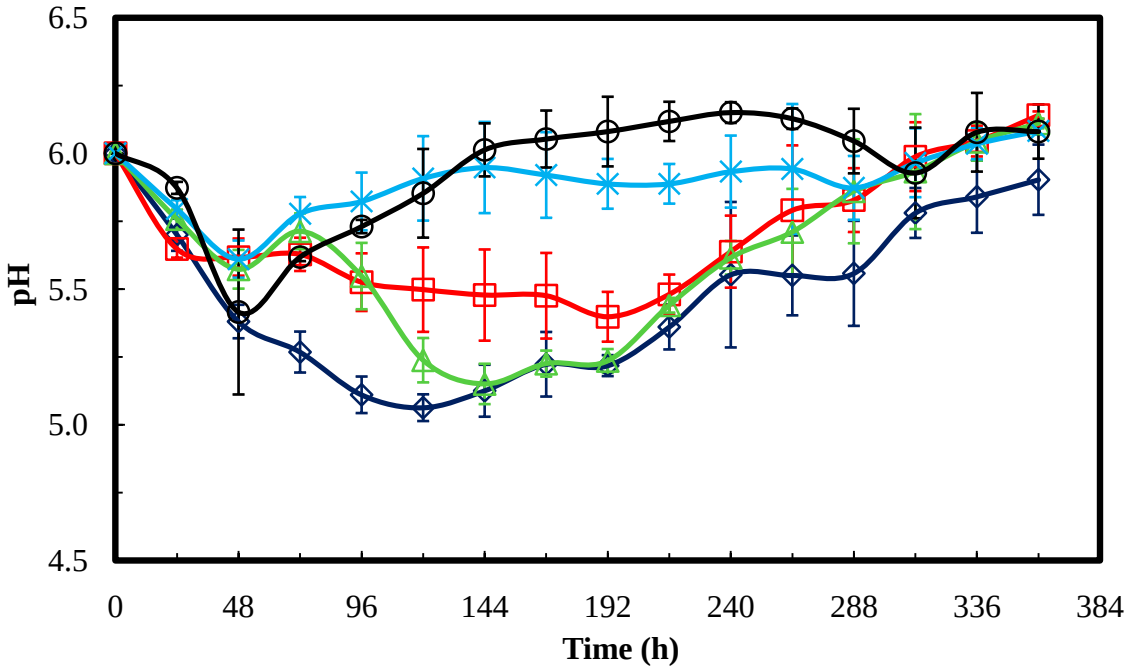

Figure 4.9 pH profile during syngas fermentation in 10 g/L corn steep liquor with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The change in headspace pressure in the serum bottles during the course of fermentation is shown in Figure 4.10. The initial pressure was set to 239 kPa (absolute) by filling the head space with fresh syngas every 24 h after taking the liquid samples. Consumption of syngas was observed until 288 h, after which there was no consumption of syngas. The treatment that contained 10 g/L DTT consumed the least amount of syngas and hence produced the least amount of cells (Figure 4.8). Since the gas consumption in 10 g/L DTT treatment ceased at around 192 h, the cell concentration also rapidly declined in that treatment.

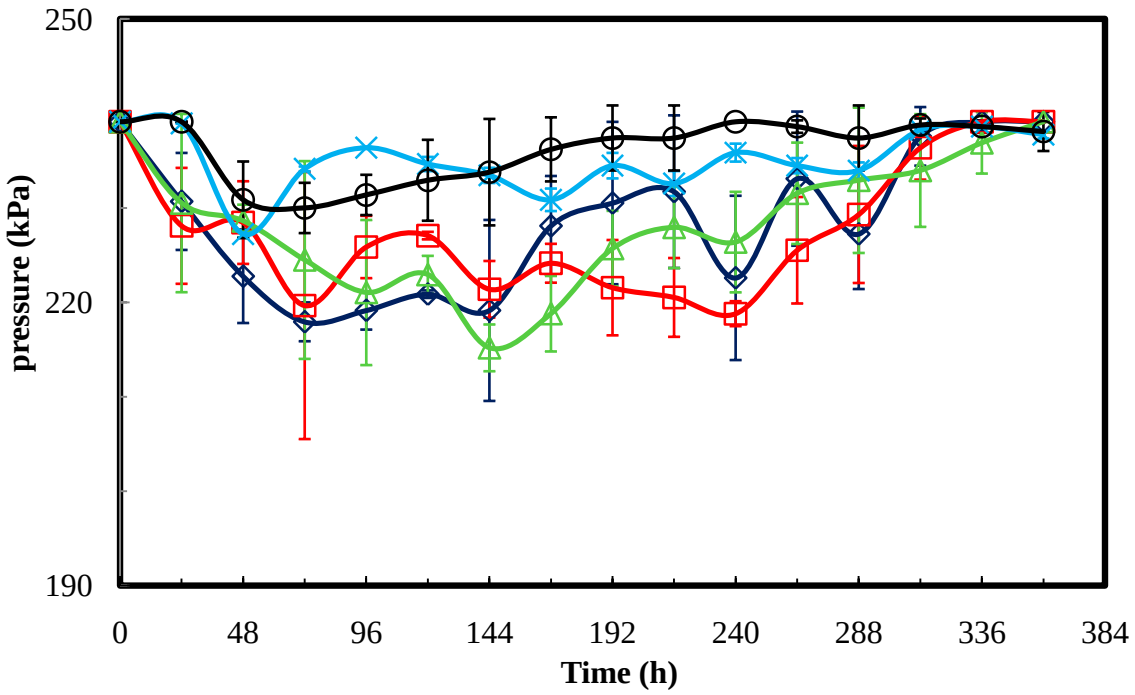

Figure 4.10 Pressure profile during syngas fermentation in 10 g/L corn steep liquor with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

4.1.2.4 Product Profile

The primary products of the fermentation are ethanol, acetic acid and butanol. As acetic acid is a growth related product, the production of acetic acid only was seen when strain P11 was in the growth phase (Figure 4.11). In the control treatment, the maximum acetic acid concentration was 2 g/L at 144 h, after which it decreased with time. The acetic acid concentration in the control at the end of 360 h of fermentation was 1 g/L, implying that the cells consumed about 50% of the acetic acid present in the fermentation broth (assuming there was no acetic acid production after 144 h).

In the case of treatments with DTT, the maximum acetic acid concentrations were less than the control and the percentages of acetic acid consumed by the strain P11 cells

were higher. The general trend observed was that acetic acid concentration decreased with increasing DTT concentration. In 10 g/L DTT medium, the maximum acetic acid concentration was 0.9 g/L at 72 h and the cells consumed almost all the acetic acid by 144 h. The presence of DTT in the fermentation broth seems to have stimulated the cells to consume more acetic acid. This is probably because DTT helps in regeneration of NADH from NAD^+, which in turn is directly involved in ethanol production. For production of ethanol, acetic acid could have been used as a substrate, so the consumption of acetic acid increased with increasing DTT concentrations. The acetic acid concentrations (after 48 h) in the DTT 7.5 g/L and DTT 10 g/L treatments were significantly less than the control treatments ($p < 0.05$).

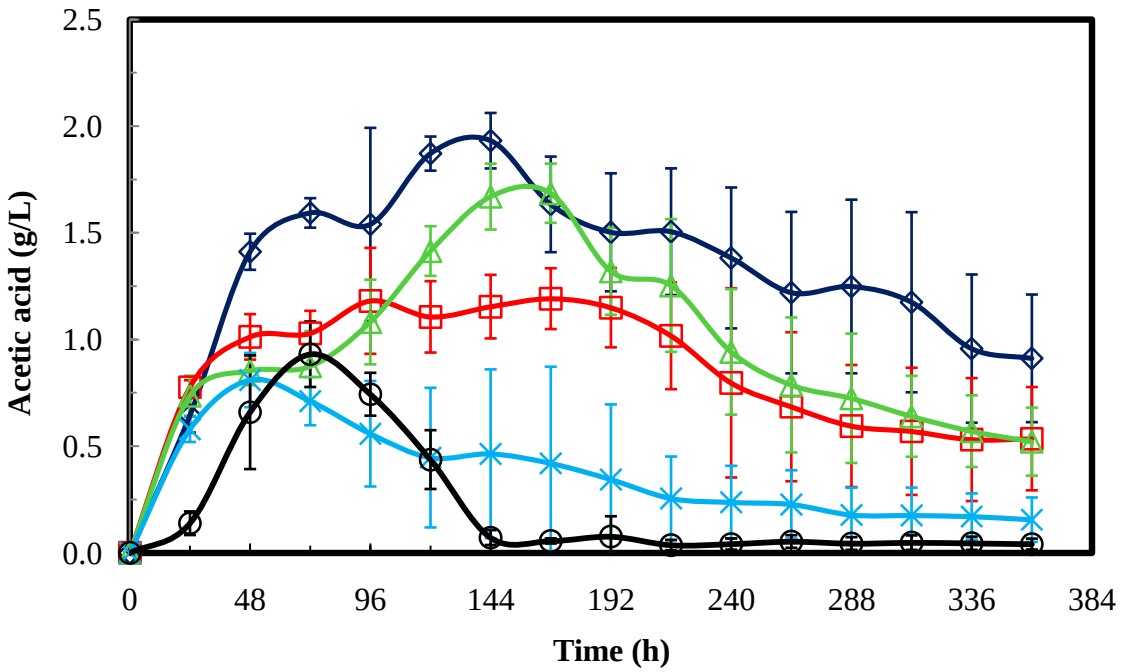

Figure 4.11 Kinetics of acetic acid production in 10 g/L corn steep liquor media with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The profile for ethanol production in different treatments is shown in Figure 4.12. At 96 h, the ethanol concentration was 0.96 g/L in 7.5 g/L DTT; whereas, ethanol concentration was only 0.25 g/L in the control medium. The ethanol concentration at 216 h was similar in all treatments (~ 1.3 g/L), after which the ethanol production began to vary among the treatments. The greatest ethanol concentration observed was 2.54 g/L in the 5 g/L DTT medium at 360 h, in contrast to 1.88 g/L ethanol in the control treatment at 360 h. The amount of ethanol produced in 5 g/L DTT treatment was 35% more than the control treatment. The concentration of ethanol at 360 h in 2.5 g/L, 5 g/L and 7.5 g/L DTT treatments were significantly higher than the control ($p < 0.05$).

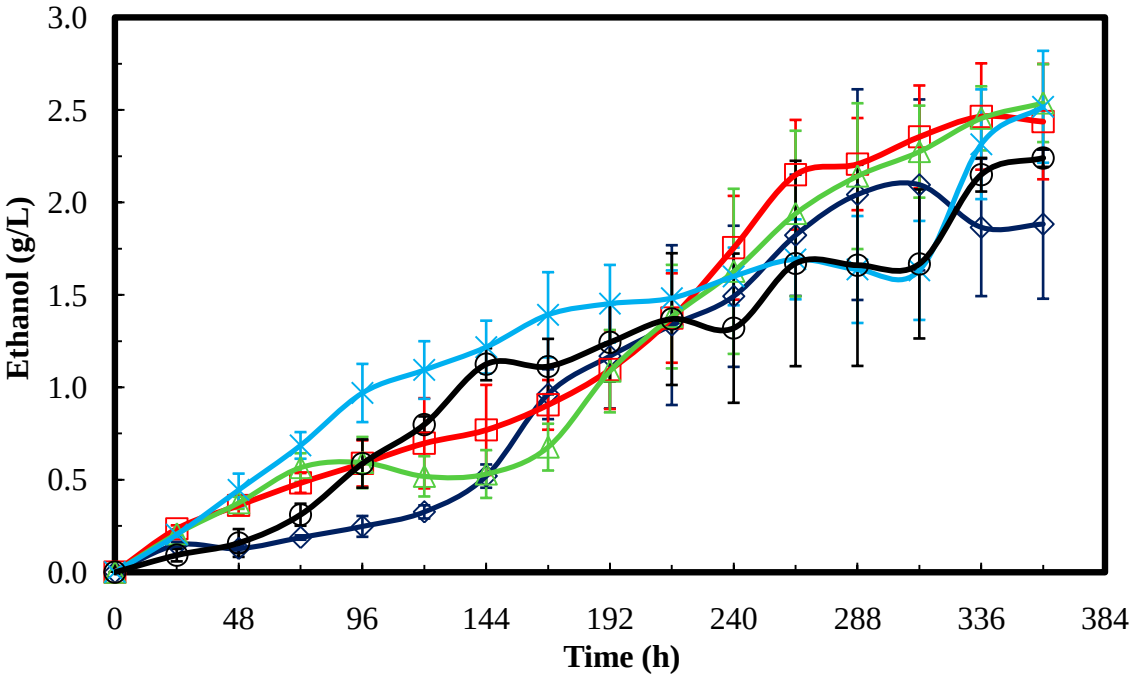

Figure 4.12 Kinetics of ethanol production in 10 g/L corn steep liquor with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The ethanol yield in the presence of 10 g/L DTT was 16.24 g ethanol/g cells, which is nearly 300% greater than the ethanol yield in control treatment (5.31 g ethanol/g cells) (Table 4.2). However, the increase in ethanol yield was due to lower concentration of cells in the 10 g/L DTT medium compared to the control treatment.

Table 4.2 Specific growth rates and ethanol yields in 10 g/L corn steep liquor media with simulated syngas and various dithiothreitol (DTT) concentrations using *Clostridium* strain P11.

DTT (g/L)	Initial cells (mg/L)	Specific growth rate, μ (h^{-1})	Ethanol yields[a] (g/g)
0.0	38.5	0.042	5.31
2.5	38.5	0.078	8.33
5.0	37.0	0.074	9.16
7.5	30.4	0.077	15.08
10.0	29.5	0.043	16.24

[a] Values are calculated after 360 h in g ethanol/g cell mass

The profile for butanol production is shown in Figure 4.13. More butanol production was observed using 10 g/L CSL media than in the 1 g/L YE media. In CSL media, butanol concentration was 0.33 g/L in control at 360 h (Figure 4.13), but greater butanol concentrations were observed in media that had DTT. The maximum butanol concentration observed was 0.79 g/L in 2.5 g/L DTT medium after 360 h of fermentation, which is nearly 240% more butanol than what was produced in control treatment (0.33 g/L after 360 h). In contrast, DTT had no effect on butanol production in the YE media.

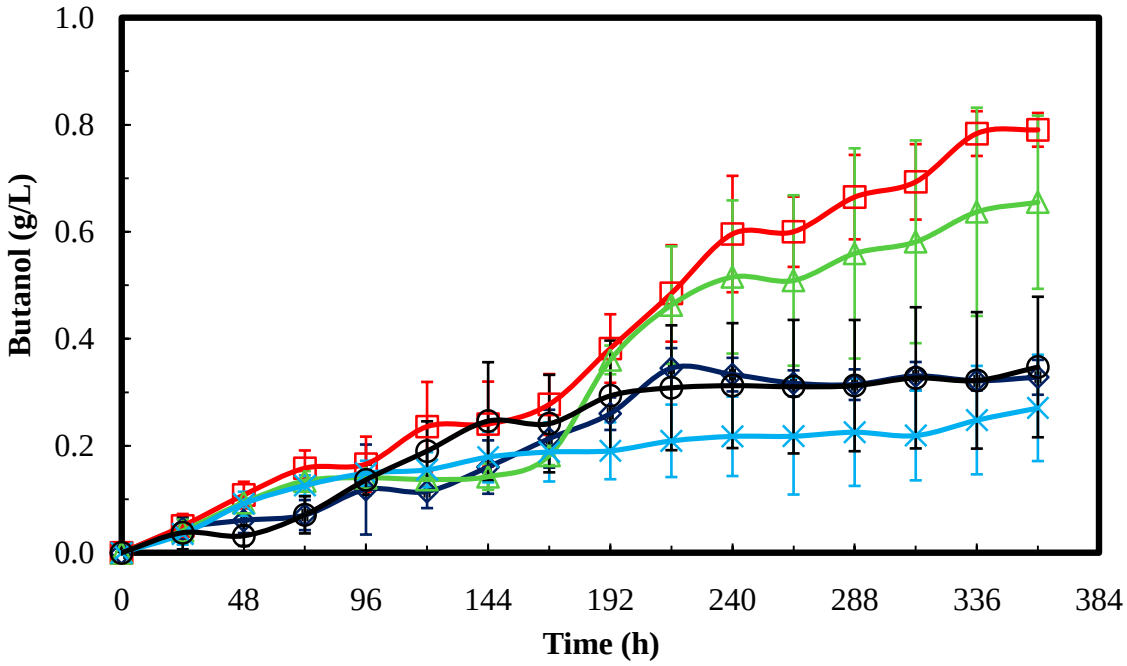

Figure 4.13 Kinetics of butanol production in 10 g/L corn steep liquor with simulated syngas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The effect of DTT on ethanol production by strain P11 in CSL media was lower compared to its effect in YE based media (Figure 4.14). This could be due to the increased production of butanol in presence of DTT in CSL media. Production of butanol from butyryl−CoA is a two step reduction reaction involving the intermediate, butyraldehyde. The production of butanol from butyryl−CoA is similar to the production of ethanol from acetyl−CoA and the reaction involves oxidation of two molecules of NADH to NAD^+. In CSL media, the reducing power from DTT was used for the production of butanol, which led to a 240% increase in butanol production and just a 35 % increase in ethanol production in the presence of either 2.5 or 5 g/L DTT. Also, unknown components in CSL may have favored the routing of reducing power from DTT for more butanol production.

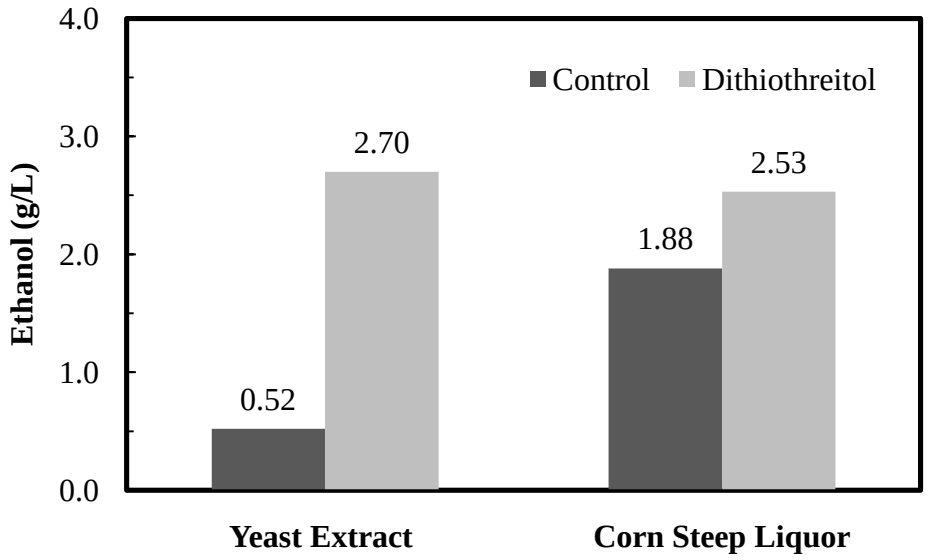

Figure 4.14 Ethanol produced after 360 h of fermentation in presence of 0 g/L, 10 g/L dithiothreitol in 1.0 g/L yeast extract medium and 0 g/L, 5 g/L dithiothreitol in 10 g/L corn steep liquor medium using simulated syngas.

4.2 Biomass Syngas (Producer Gas)

4.2.1 Yeast Extract Medium and Dithiothreitol

4.2.1.1 Cell Growth

Growth profiles of *Clostridium* strain P11 in YE media containing various concentrations of DTT using producer gas are shown in Figure 4.15. The producer gas generated from gasifying switchgrass was composed of 8.24% H_2, 14.21% CO_2, 15.38% CO, 56.9% N_2 and 5.21% methane (by volume). This produced gas composition was different from the simulated biomass syngas, which was composed of 5% H_2, 15% CO_2, 20% CO and 60% N_2 (by volume). Similar growth profiles were noticed in all media except when DTT concentration was 10 g/L. The addition of 10 g/L DTT in the YE medium resulted in about 48 h of lag phase (Figure 4.15). This lag phase was not

observed in YE media with lower DTT concentrations with producer gas or in YE media and simulated syngas (Figure 4.2). This suggests that strain P11 needed more time to adapt in YE media with 10 g/L DTT and producer gas. Cell growth was noticed in the first 96 h for all media except with 10 g/L DTT. Cells grew in the YE medium with 10 g/L DTT between 48 h and 168 h. The final cell concentration in all YE media was similar (0.1 g/L). About 50% fewer cells were produced in YE media with producer gas (Figure 4.15) compared to simulated syngas (Figure 4.2). The specific growth rate for strain P11 in YE media using producer gas was 0.028 h^{-1} when DTT concentrations were below 10 g/L (Table 4.3). The specific growth rate in the medium with 10 g/L DTT was 29% lower than in the control medium. Generally, slightly higher specific growth rates were obtained in YE media with simulated syngas (Table 4.1), which was likely due to the higher CO concentration than in the producer gas. After 24 h of fermentation with producer gas, YE media with 7.5 g/L and 10 g/L DTT produced less cell mass compared to the control ($p < 0.05$).

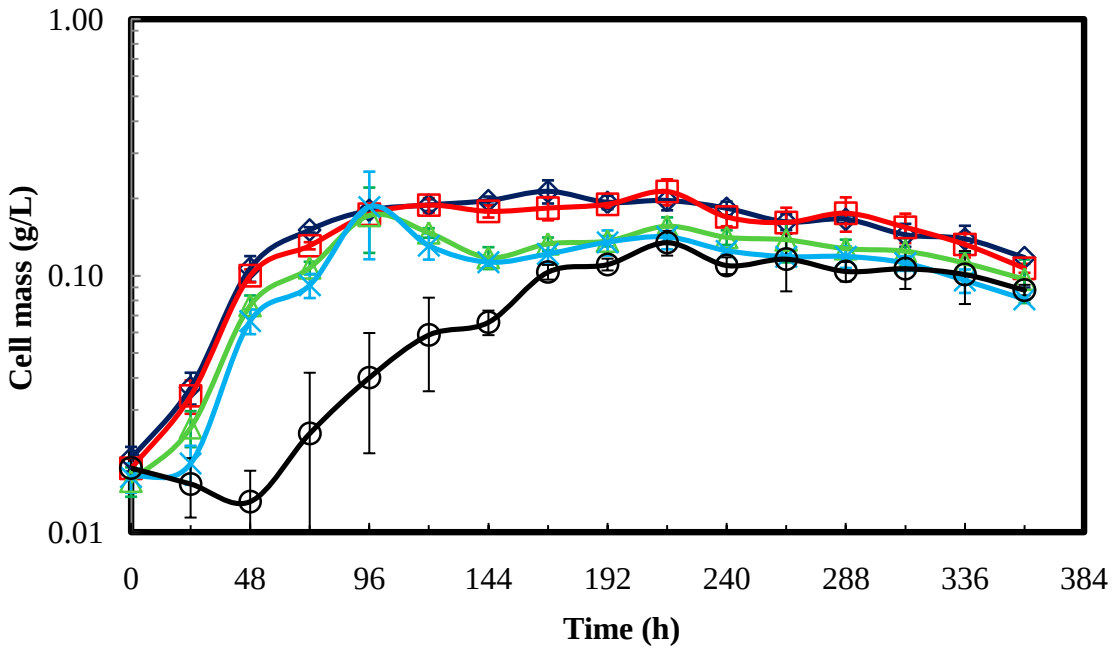

Figure 4.15 Kinetics of cell mass production in 1.0 g/L yeast extract media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

Table 4.3 Specific growth rates and ethanol and isopropanol yields in 1.0 g/L YE media with producer gas and various dithiothreitol (DTT) concentrations using *Clostridium* strain P11.

DTT (g/L)	Initial cells (mg/L)	Specific growth rate μ (h^{-1})	Ethanol yields[a] (g/g)	Isopropanol yields[b] (g/g)	Total alcohols produced[c] (g/L)
0.0	19.3	0.028	13.95	6.74	2.44
2.5	17.7	0.028	16.20	8.61	2.65
5.0	15.8	0.027	14.80	10.00	2.40
7.5	16.4	0.029	16.33	13.49	2.41
10.0	17.7	0.020	10.29	11.65	1.98

[a] Values calculated at 360 h in g ethanol/g cell mass
[b] Values calculated at 360 h in g isopropanol/g cell mass
[c] Both ethanol and isopropanol

4.2.1.2 pH and Pressure Profiles

The pH in all media decreased during the course of fermentation as shown in Figure 4.16. The pH dropped from an initial value of 6 to 4.5 in 288 h in the control and 2.5 g/L DTT media. After this, the pH increased to 4.7 (at 360 h), due to consumption of acetic acid, which could have been used for ethanol production. However, the pH values during fermentation in YE media with DTT concentration above 2.5 g/L were slightly higher than in the control due to lower acetic acid production in these media. After 24 h of fermentation, the pH difference in YE media with 10 g/L DTT was significantly higher compared to the control ($p < 0.05$).

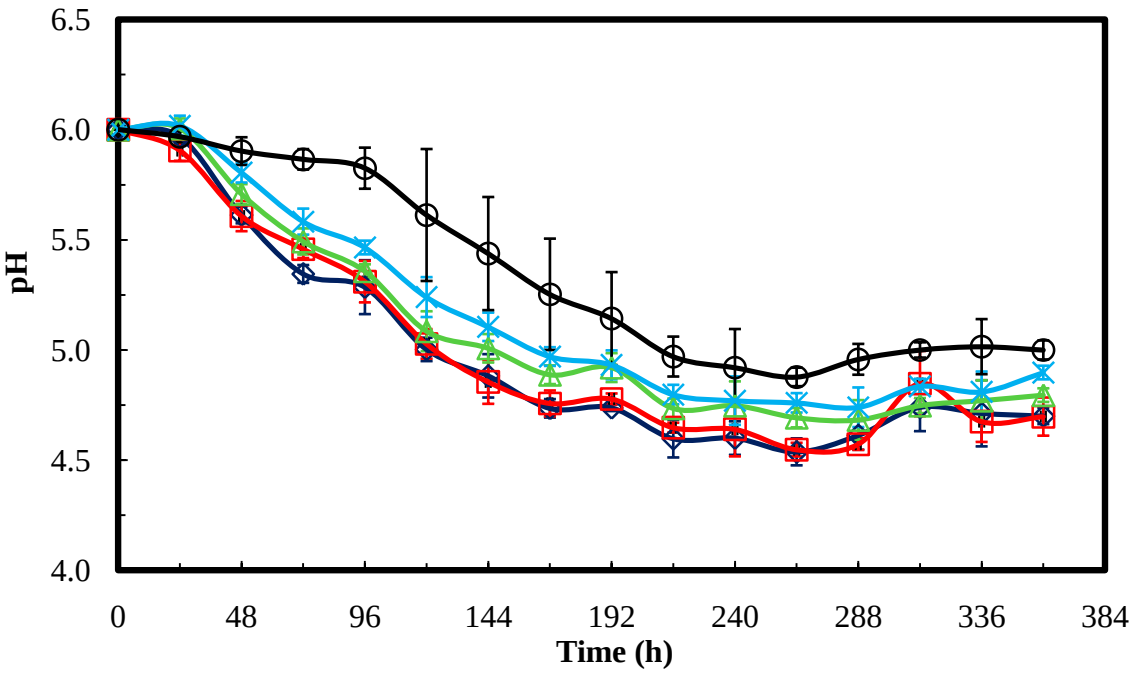

Figure 4.16 pH profile during syngas fermentation in 1.0 g/L yeast extract media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The change in headspace pressure in the serum bottles containing YE media during the course of fermentation with producer gas is shown in Figure 4.17. The initial pressure was set to 239 kPa (absolute) by filling the head space with fresh producer gas every 24 h after taking the liquid samples. Consumption of producer gas started after 24 h in YE media with 7.5 g/L of DTT or lower. Producer gas consumption started after 48 h in the medium with 10 g/L DTT due to the lag phase (Figure 4.15). Overall producer gas consumption was higher in the YE media with 7.5 g/L DTT or lower. There was no consumption of producer gas in these media after 312 h. The YE medium that contained 10 g/L DTT consumed the least amount of producer gas; hence it produced the least amount of cells (Figure 4.15). After 96 h of fermentation, there was no significant difference between pressure profiles with and without DTT ($p < 0.05$).

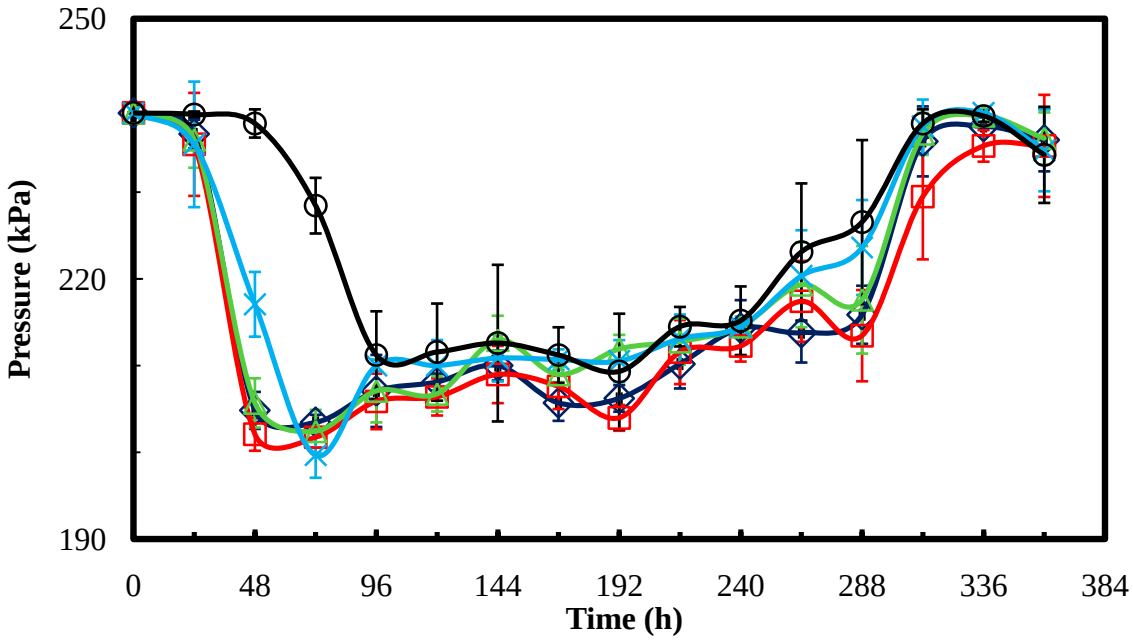

Figure 4.17 Pressures profile during syngas fermentation in 1.0 g/L yeast extract media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

4.2.1.3 Product Profile

The primary products of the fermentation with producer gas in YE media are ethanol, acetic acid and isopropanol. There was very low butanol production in all the media (concentration less than 0.1 g/L). Acetic acid production was noticed during growth and stationary phases (Figure 4.18). About half of the acetic acid formed during fermentation was noticed in the stationary phase in the YE media with 7.5 g/L of DTT or lower. Only 33% of total acetic acid production in YE medium was during stationary phase. Generally, more acetic acid was produced in YE media with simulated gas (Figure 4.5) compared to producer gas (Figure 4.18). This was due to availability of more CO for strain P11 in the simulated syngas as discussed in section 3.4. In the control treatment, the maximum acetic acid concentration was 3.78 g/L at 264 h, after which it decreased with time. The acetic acid concentration in the control at the end of 360 h of fermentation was 2.9 g/L, implying that the cells consumed 24% of the acetic acid present in the fermentation medium assuming there was no acetic acid production after 264 h.

In all media with DTT, the maximum acetic acid concentrations were less than the control and the percentages of acetic acid consumed by the P11 cells were lower when DTT concentration was above 2.5 g/L. The general observed trend was that acetic acid concentration decreased with increasing DTT concentration. In 10 g/L DTT medium, the maximum acetic acid concentration was about 2.4 g/L at 264 h. The acetic acid concentrations (after 24 h) in the DTT 7.5 g/L and DTT 10 g/L media were significantly less than the control medium ($p < 0.05$).

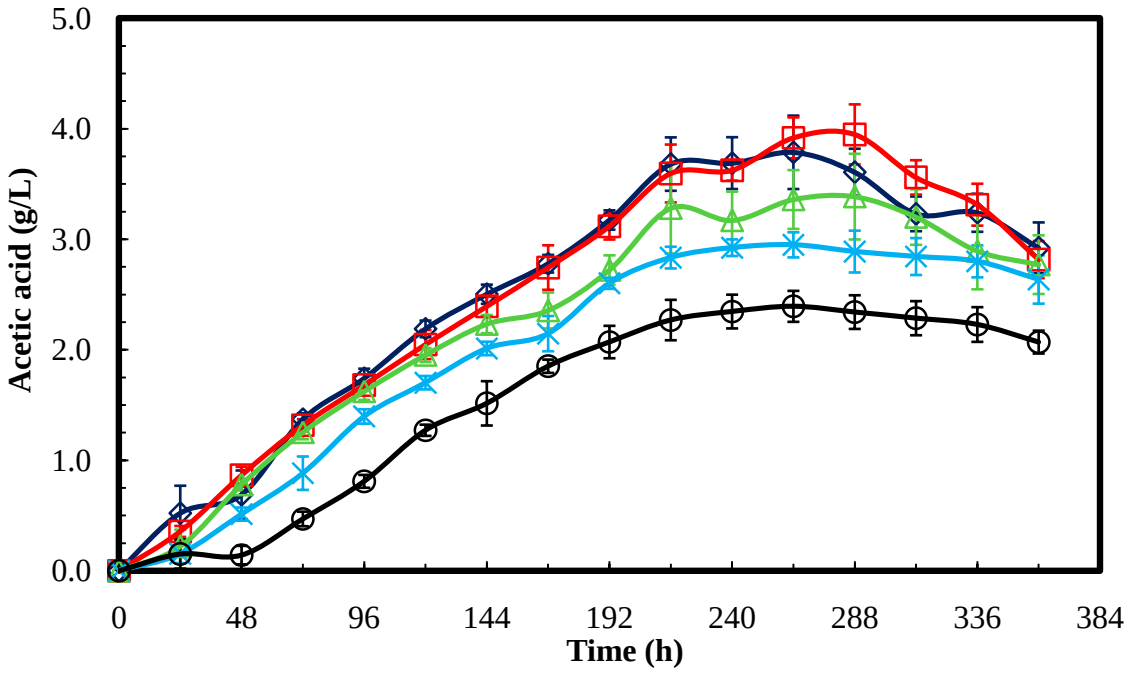

Figure 4.18 Kinetics of acetic acid production in 1.0 g/L yeast extract media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The profile for ethanol production in media with different concentrations of DTT and producer gas in YE media is shown in Figure 4.19. Ethanol production started to increase after 144 h during the stationary phase. The maximum ethanol concentration (1.73 g/L) was obtained in the YE medium with 2.5 g/L DTT. This was comparable to the amount of ethanol produced in the control medium (1.64 g/L). Less ethanol was produced with the increase in the concentration of DTT above 2.5 g/L. This is opposite of what was observed in YE media with simulated syngas (Figure 4.6), in which more ethanol was produced as the concentration of DTT was increased. This could be due to the presence of impurities such as acetone, ammonia and hydrogen sulfide in the

producer gas that reduced DTT efficiency as a reducing agent or diverted its reducing effect to form metabolites other than ethanol. The concentration of ethanol at 360 h in media DTT 7.5 g/L and DTT 10 g/L were significantly lower than the control ($p < 0.05$).

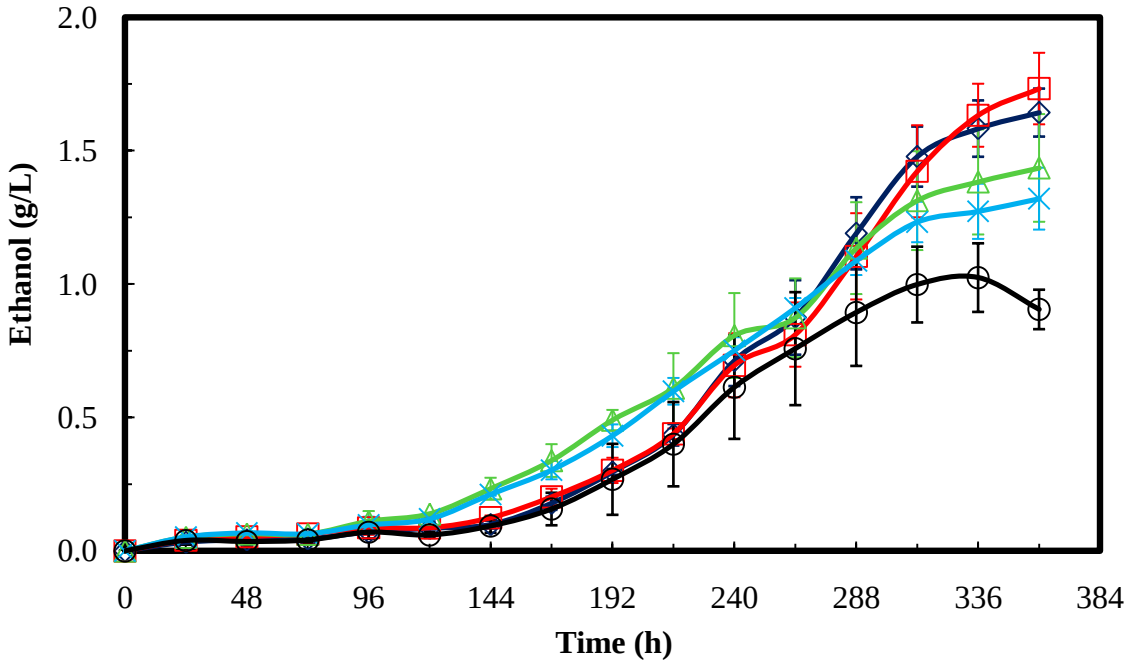

Figure 4.19 Kinetics of ethanol production in 1.0 g/L yeast extract media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The ethanol yield in the presence of 10 g/L DTT was 10.29 g ethanol/g cells, which is nearly 26% lower than the ethanol yield in control treatment (13.95 g/g) (Table 4.3). Ethanol yield decreased with the increase in DTT concentration in YE media above 5 g/L. Although, lower ethanol production was observed in the medium with 7.5 g/L DTT compared to the control medium, the ethanol yield (16.33 g/g) in the medium with 7.5 g/L DTT was 17% higher than in the control due to lower cell concentration in the medium with DTT.

The producer gas used in the present study contained 0.4% acetone (by volume). Acetone is miscible with water (major component of strain P11 medium) so it transferred from the gas phase (producer gas) to the liquid phase (fermentation medium) and accumulated in the medium because fresh producer gas is fed to P11 every 24 h. The profile of acetone accumulation in YE media during fermentation with producer gas is shown in Figure 4.20. There was much variability in the concentration of acetone in the media, which was below 0.4 g/L in all YE media.

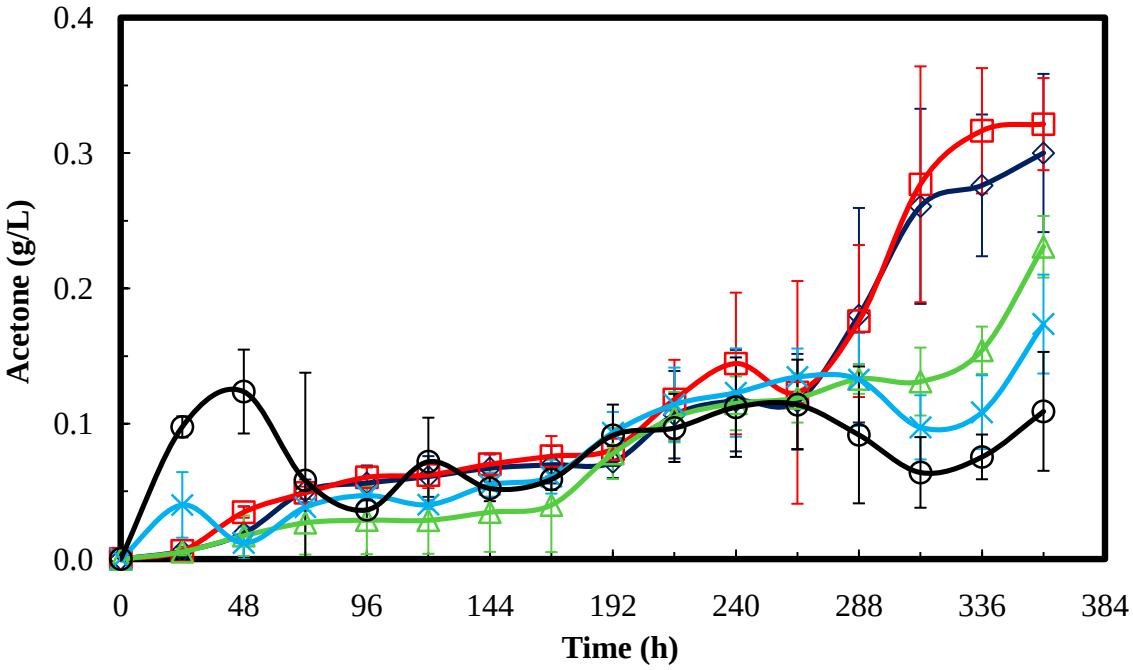

Figure 4.20 Acetone profile in 1.0 g/L yeast extract media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

In addition, isopropanol production by strain P11 was also noticed in all YE media during fermentation of producer gas (Figure 4.21). The concentration of isopropanol in the YE media increased gradually to about 1.0 g/L. The final concentration of isopropanol increased with the increase in DTT concentration. However,

there was no statistical difference in the final concentrations of isopropanol in the control and media contained 2.5 and 5 g/L DTT. The concentration of isopropanol between 288 h and 360 h in media with DTT 7.5 g/L and DTT 10 g/L were significantly higher than the control ($p < 0.05$). Generally, isopropanol yields increased with the increase in DTT concentrations in the fermentation media (Table 4.3). Ethanol yields were higher than isopropanol yields in the media with DTT below 10 g/L.

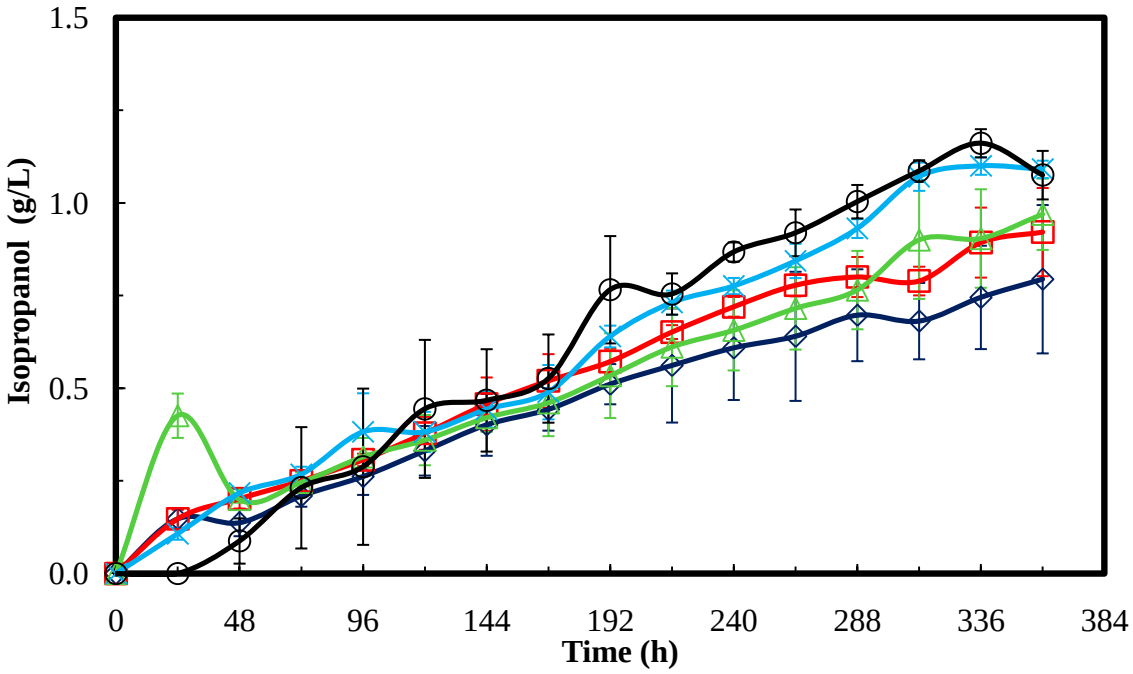

Figure 4.21 Kinetics of isopropanol production in 1.0 g/L yeast extract media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

Strain P11 produced 9.25 g/L isopropanol after 576 h of fermentation using producer gas (Kundiyana et al. 2010). This was due to the presence of acetone in the producer gas. It is believed that strain P11 can directly reduce acetone to isopropanol. This was recently confirmed by our research group in an experiment with YE medium

that only contained acetone in the liquid phase and N_2 was the only gas in the headspace (unpublished data).

In addition, several microbial species such as *Clostridium beijerinckii* (Ismaiel et al. 1993), *Burkholderia sp.* (Isobe and Wakao 2003) were able to reduce acetone to isopropanol. Production of isopropanol from acetone was observed in recombinant *E. coli* strain (Subbian et al. 2008). Acetone is reduced to isopropanol according to the following reaction:

$$\text{Acetone} + \text{NADPH} + H^+ \rightarrow \text{Isopropanol} + NADP^+ \quad (4.1)$$

Acetone can be formed from acetyl–CoA according to the following reactions (Subbian et al. 2008):

$$\text{Acetyl–CoA} \rightarrow \text{Acetate} + \text{CoA} \quad (4.2)$$

$$2\ \text{Acetyl–CoA} \rightarrow \text{Acetoacetyl–CoA} + \text{CoA} \quad (4.3)$$

$$\text{Acetoacetyl–CoA} + \text{Acetate} \rightarrow \text{Acetoacetate} + \text{Acetyl–CoA} \quad (4.4)$$

$$\text{Acetoacetate} \rightarrow \text{Acetone} + CO_2 \quad (4.5)$$

The reduction of acetone to isopropanol involves oxidation of NADPH to $NADP^+$. The producer gas used in the present study contained 0.41% acetone (by volume), which accumulated in the fermentation media. The addition of DTT could have helped in the regeneration of NADPH from $NADP^+$ to produce isopropanol from acetone than for the production of ethanol from acetyl–CoA. This could explain why higher isopropanol concentrations were obtained in the YE media with higher concentrations of

DTT compared to ethanol, which was not enhanced as the concentration of DTT increased.

4.2.2 Corn Steep Liquor Medium and Dithiothreitol

4.2.2.1 Cell Growth

Growth profiles of strain P11 during fermentation using producer gas in CSL media containing various concentrations of DTT are shown in Figure 4.22. The producer gas was composed of 8.76% H_2, 14.36% CO_2, 13.67% CO, 60% N_2, 3.19% methane and 0.41% acetone (by volume). This producer gas has a slight variation in its composition from the producer gas used in the YE media because it was made from different gasification of switchgrass. Minor changes in the gasifier operating conditions would change the producer gas composition.

Similar growth profiles were noticed in all CSL media, which suggests no effect of DTT on growth of strain P11 in these media. No lag phase was noticed in CSL media (Figure 4.22) unlike the media with YE (Figure 4.15). This showed that strain P11 grows faster in CSL compared to YE media. Cells grow exponentially in the first 24 h, after which, growth slowed down. Cells reach stationary phase after 72 h of fermentation. The final cell concentration in all CSL media was 0.15 g/L. The specific growth rate for strain P11 in CSL media using producer gas was 0.041 h^{-1} (Table 4.4). Higher specific growth rates were obtained in CSL media compared to YE media with producer gas (Table 4.3), which could be because CSL is rich in more nutrients than YE.

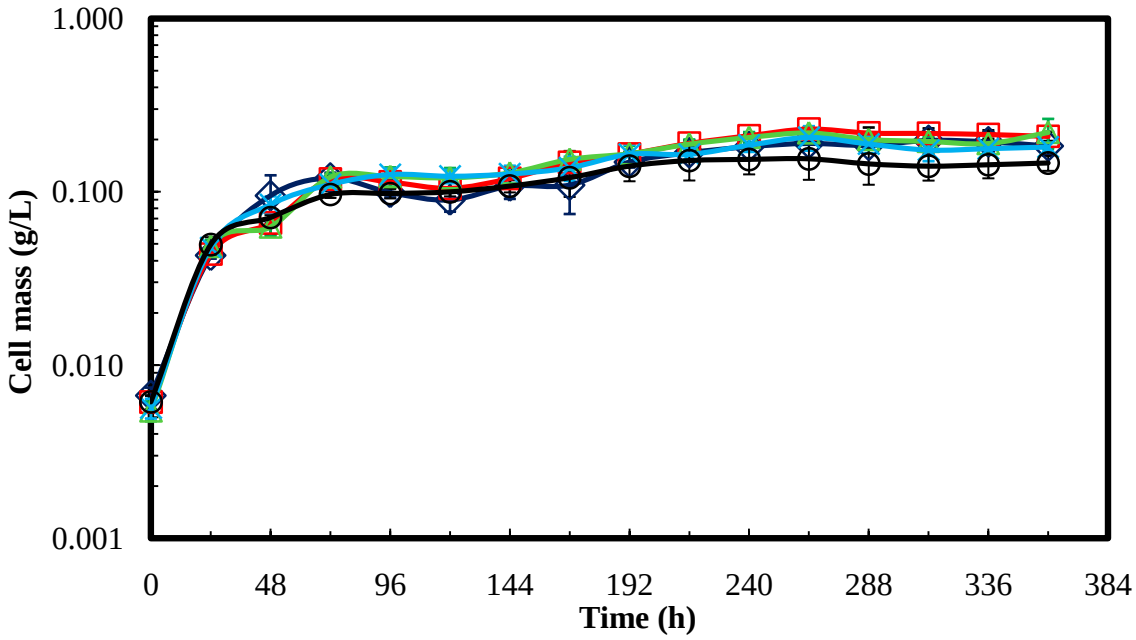

Figure 4.22 Kinetics of cell mass production in 10 g/L corn steep liquor media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

Table 4.4 Specific growth rates and ethanol and isopropanol yields in 10 g/L corn steep liquor media with producer gas and various dithiothreitol (DTT) concentrations using *Clostridium* strain P11.

DTT (g/L)	Initial cells (mg/L)	Specific growth rate μ (h^{-1})	Ethanol yields[a] (g/g)	Isopropanol yields[b] (g/g)	Total alcohols produced[c] (g/L)
0.0	6.66	0.040	8.55	12.74	3.91
2.5	6.10	0.041	7.05	11.07	3.78
5.0	5.44	0.043	6.87	9.50	3.60
7.5	5.46	0.041	6.60	14.31	3.78
10.0	6.12	0.038	7.20	19.00	3.83

[a] Values calculated at 360 h in g ethanol/g cell mass
[b] Values calculated at 360 h in g isopropanol/g cell mass
[c] Both ethanol and isopropanol

4.2.2.2 pH and Pressure Profiles

The pH decreased in all media during fermentation as shown in Figure 4.23. The pH dropped from an initial value of 6 to 4.7 in 240 h and remained fairly constant for all media except 10 g/L DTT. In the 10 g/L DTT medium, the pH decreased to 5.0 in 240 h and then slightly increased to 5.15 by the end of the fermentation. After 264 h of fermentation, the pH difference in CSL media with 10 g/L DTT was significantly higher compared to the control ($p < 0.05$). The pH profile in CSL media with producer gas (Figure 4.23) was different from fermentations with simulated syngas (Figure 4.9).

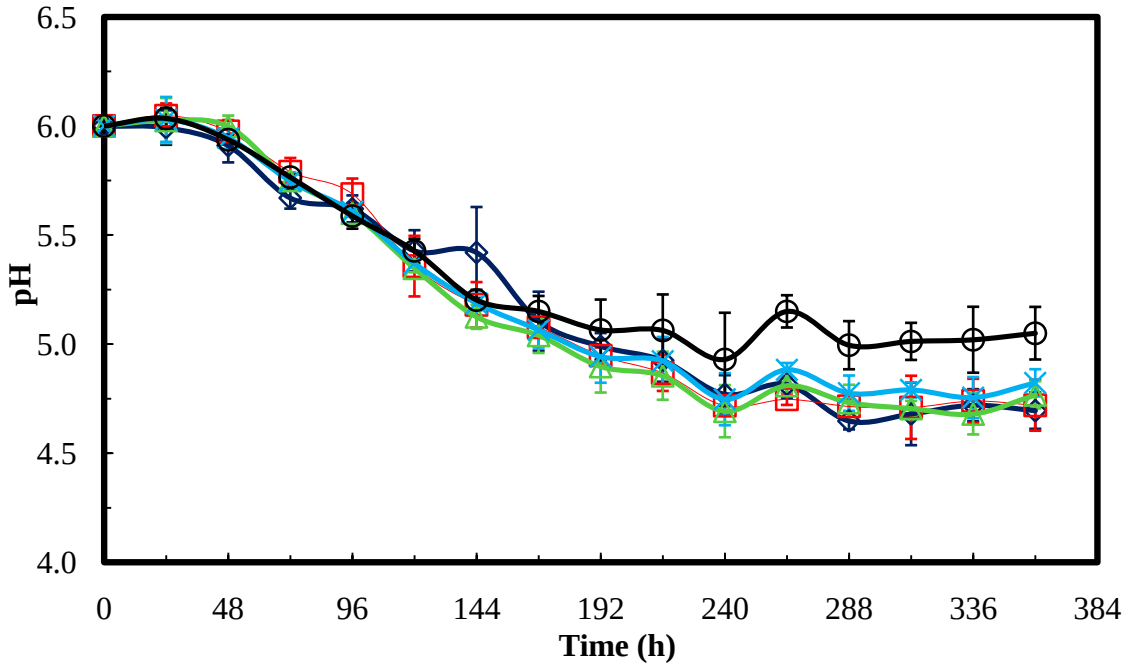

Figure 4.23 pH profile during syngas fermentation in 10 g/L corn steep liquor media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The change in headspace pressure in the serum bottles containing CSL media during fermentation of producer gas is shown in Figure 4.24. The initial pressure was set

to 239 kPa (absolute) by filling the head space with fresh producer gas every 24 h after taking the liquid samples. Consumption of producer gas started after inoculation. Overall producer gas consumption was higher in CSL media with 7.5 g/L DTT or lower. There was less producer gas consumption in these media after 288 h. The CSL medium that contained 10 g/L DTT consumed the least amount of producer gas. This was also measured with the same concentration of DTT in YE medium with producer gas (Figure 4.17) and in CSL medium with simulated syngas (Figure 4.10). The reason for lower gas consumption in media with 10 g/L DTT could be because of presence of high reducing equivalents that reduced strain P11's need for CO or H_2 to get reducing equivalents required in the acetyl-CoA pathway. In addition, measuring the redox potential of the medium and the changes in gas compositions during fermentation will reveal which gases were consumed and which were produced by strain P11 in the medium with 10 g/L DTT. This could also explain why strain P11 with 10 g/L DTT consumed the lowest amount of gas during the course of fermentation.

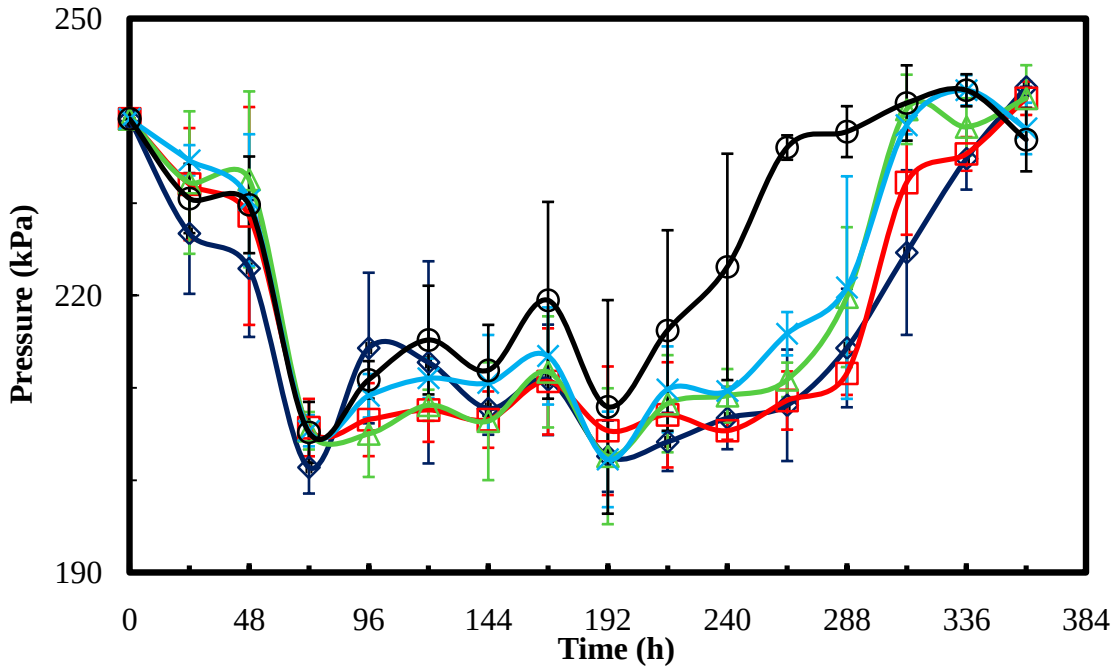

Figure 4.24 Pressure profile during syngas fermentation in 10 g/L corn steep liquor media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

4.2.2.3 Product Profile

Ethanol, acetic acid and isopropanol were produced during the fermentation with producer gas in CSL media. Acetic acid production was noticed during growth and stationary phases (Figure 4.25). More than half of the acetic acid was formed during the stationary phase. Generally, more acetic acid was produced in CSL media with producer gas than in either CSL media with simulated syngas (Figure 4.11) or YE media with producer gas (Figure 4.18) and simulated syngas (Figure 4.5). Comparable amounts of acetic acids were produced in the control and the media with DTT concentration below 7.5 g/L (Figure 4.25). The maximum acetic acid concentration was 6.7 g/L at 312 h in the control medium. As the concentration of DTT increased from 7.5 g/L to 10 g/L, the

amount of acetic acid formed decreased. More acetic acid consumption was measured in the 7.5 g/L and 10 g/L DTT media. The acetic acid concentrations (after 264 h) in the DTT 10.0 g/L medium were significantly less than the control medium ($p < 0.05$).

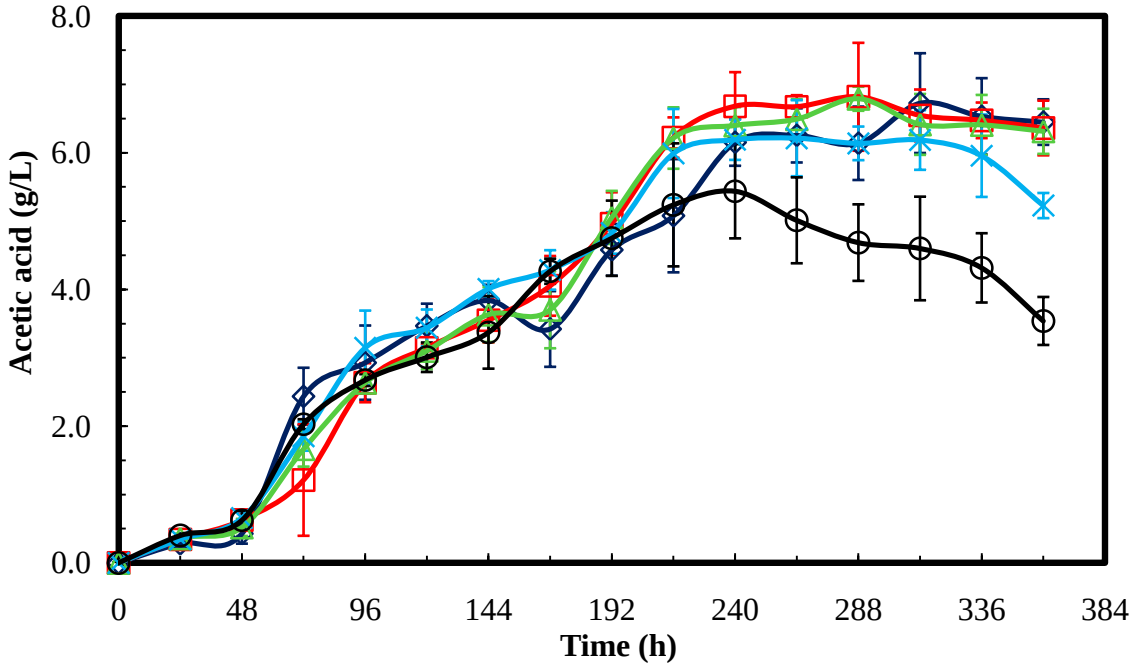

Figure 4.25 Kinetics of acetic acid production in 10 g/L corn steep liquor media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The profile for ethanol production in CSL media with producer gas and different concentrations of DTT is shown in Figure 4.26. Ethanol production started to increase after 144 h during the stationary phase. The maximum ethanol concentration (1.68 g/L) was obtained in the control medium at 312 h. This was about the same amount of ethanol produced in the CSL media with DTT concentrations below 7.5 g/L. However, ethanol production decreased with increasing DTT concentrations in the fermentation medium with producer gas. The amount of ethanol produced at the end of 360 h in DTT 10 g/ L

medium was 33% less than in the control medium. The concentration of ethanol between 336 h and 360 h in treatments with 7.5 g/L DTT and 10 g/L DTT were significantly lower than in the control ($p < 0.05$). In addition, strain P11 produced similar amounts of ethanol with producer gas at the particular DTT concentration in YE media (Figure 4.19) and in CSL media (Figure 4.26). However, ethanol yields (g ethanol/g cells) in CSL media (Table 4.4) were lower than in YE media (Table 4.3). The presence of impurities in the producer gas could have reduced the DTT efficiency as a reducing agent that enhanced ethanol production in YE media with simulated syngas or diverted DTT's reducing power to form metabolites other than ethanol.

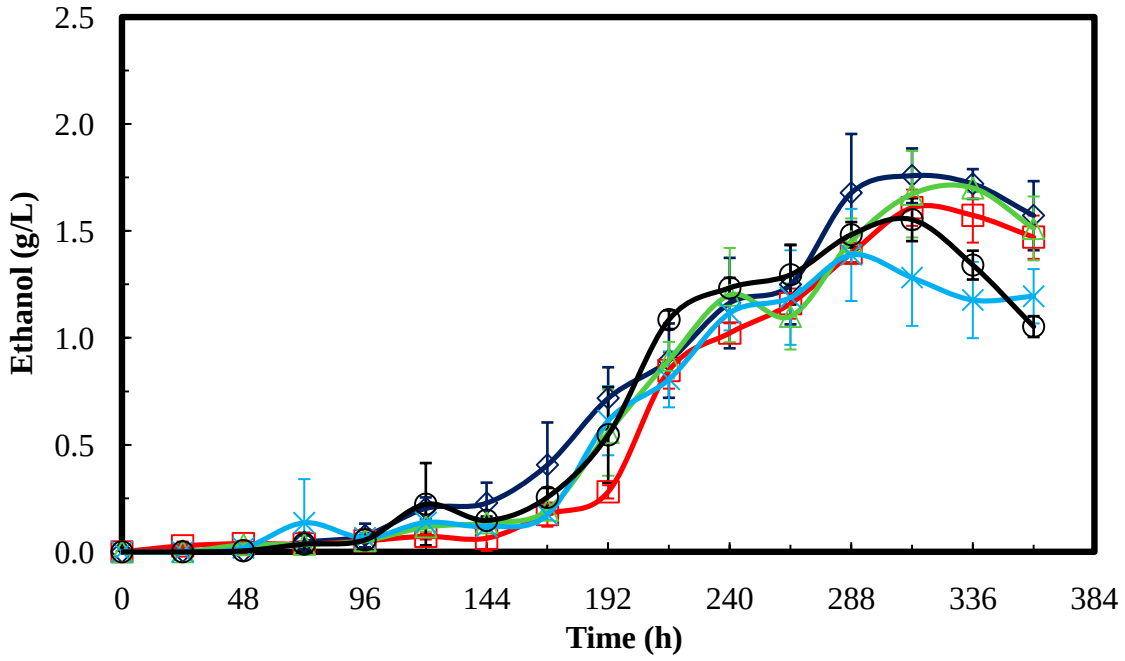

Figure 4.26 Kinetics of ethanol production in 10 g/L corn steep liquor media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

The profile of acetone consumption and accumulation in CSL media during fermentation with producer gas is shown in Figure 4.27. No acetone was detected in any of the media tested in the first 192 h, which means that acetone was reduced to isopropanol as shown in Figure 4.28. About 1.2 g/L of acetone accumulated in the control and CSL media with DTT concentrations below 7.5 g/L (Figure 4.27). Only 0.4 g/L of acetone were left in the CSL media with 7.5 g/L DTT and 10 g/L DTT.

About 1.6 g/L of isopropanol was produced in all media at 192 h (Figure 4.28). This was twofold higher than what was produced in YE media at 192 h (Figure 4.21). Strain P11 continued to produce isopropanol in CSL media after 192 h. A maximum concentration of isopropanol (2.78 g/L) was observed in the CSL medium with 10 g/L DTT after 360 h. This was 19% more than in the control CSL medium. The concentration of isopropanol at 360 h in CSL media with 7.5 g/L DTT and 10 g/L DTT were significantly higher than the control ($p < 0.05$). Generally, isopropanol yields increased with the increase in DTT concentration in the CSL media (Table 4.4). Isopropanol yields were higher than ethanol yields in all CSL media with producer gas. The total amount of alcohols (ethanol and isopropanol) produced in CSL media were between 3.60 g/L and 3.91 g/L (Table 4.4).

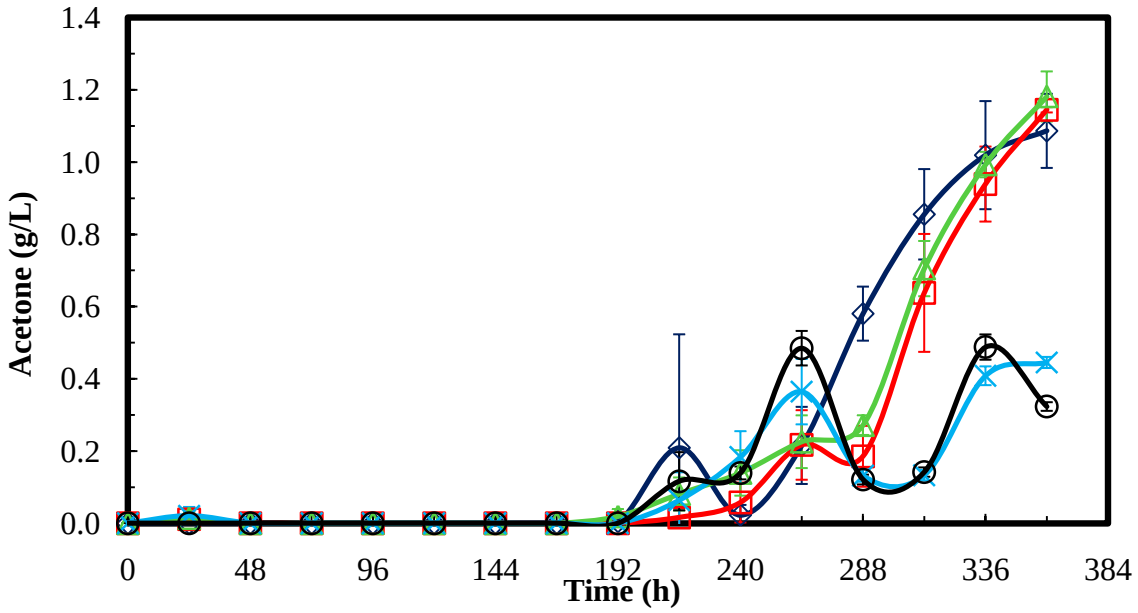

Figure 4.27 Acetone profile in 10 g/L corn steep liquor media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

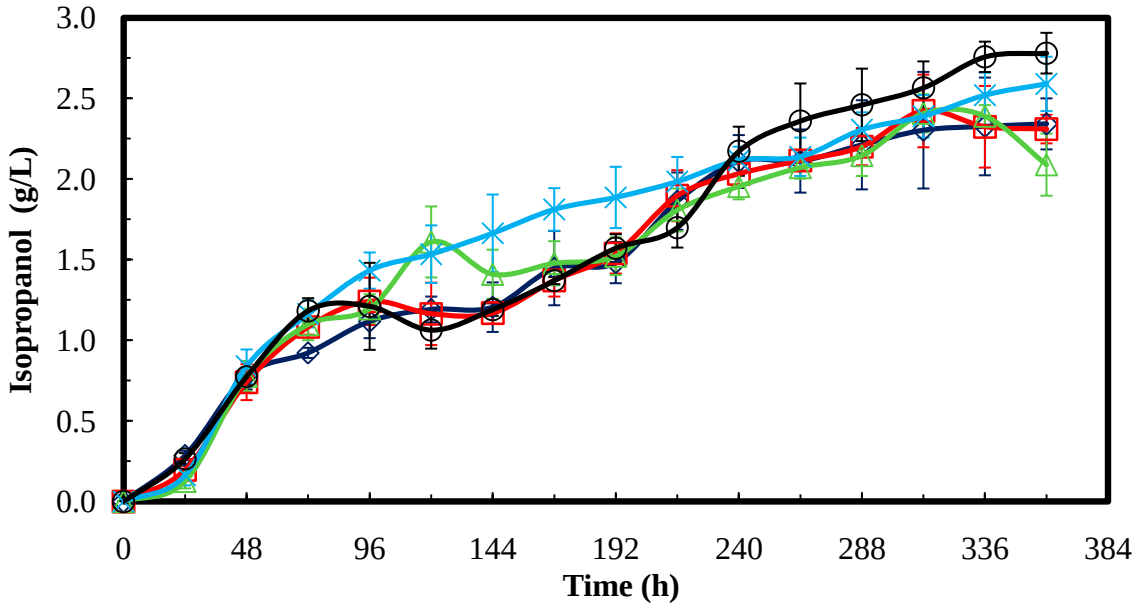

Figure 4.28 Kinetics of isopropanol production in 10 g/L corn steep liquor media with producer gas and various concentrations of dithiothreitol in (g/L): (◇) 0; (□) 2.5; (Δ) 5; (×) 7.5; (O) 10.0.

Table 4.5 Summary of results from experiments in 1.0 g/L yeast extract (YE) medium and 10 g/L corn steep liquor (CSL) medium with simulated and producer syngas.

Media	Syngas	Alcohol influenced	DTT (g/L)	Percentage increase in alcohol production compared to control
YE	Simulated	Ethanol	10.0	500
YE	Simulated	Ethanol	7.5	350
YE	Producer	Isopropanol	10.0	35
YE	Producer	Isopropanol	7.5	37
CSL	Simulated	Ethanol	2.5	35
CSL	Simulated	Butanol	2.5	240
CSL	Simulated	Butanol	5.0	100
CSL	Producer	Isopropanol	10.0	19

Strain P11 produced ethanol and butanol in YE and CSL media with simulated syngas. However, it formed ethanol and isopropanol in the same media with producer gas. Strain P11 reduced the acetone present in the producer gas to isopropanol. The effect of DTT on enhancing alcohol production in YE and CSL media with simulated syngas and producer gas is summarized in Table 4.5. The addition of DTT was more effective in YE media and using simulated syngas. The percentage increase in ethanol production in YE media was 500% with simulated syngas and 10 g/L DTT compared to control (Table 4.5). However, the same DTT concentration did not enhance ethanol production in CSL media. This DTT concentration resulted in lower ethanol formation by strain P11 with producer gas in both YE and CSL media.

CHAPTER V

CONCLUSIONS

The effect of the reducing agent dithiothreitol (DTT) on ethanol and acetic acid production by *Clostridium* strain P11 using simulated syngas and actual biomass producer gas in two different fermentation media was investigated. The simulated syngas was composed of 5% H_2, 15% CO_2, 20% CO and 60% N_2 (by volume). However, the producer gas generated from gasifying switchgrass has a composition of 8.76% H_2, 14.36% CO_2, 13.67% CO, 60.0% N_2, 3.19% methane and 0.41% acetone (by volume). The two fermentation media used were 1 g/L yeast extract (YE) and 10 g/L corn steep liquor (CSL). Various concentrations of DTT between 0 g/L and 10.0 g/L were examined. The following are the conclusions that were reached in this project.

- The addition of DTT in the YE and CSL fermentation media increased ethanol concentration with simulated syngas, whereas, DTT addition increased both ethanol and butanol concentrations in the media with 10 g/L CSL.
- Over 350% increase in ethanol concentration was observed in the 1.0 g/L YE media that contained at least 7.5 g/L of DTT after 360 h of fermentation with simulated syngas compared to the control medium (without DTT).

- The addition of 7.5 g/L DTT or lower increased ethanol concentration in the 10 g/L CSL media when simulated syngas was used. There was about a 35% increase in ethanol concentration with the addition of 5 g/L DTT to the 10 g/L CSL medium with simulated syngas.
- Ethanol yields (g ethanol/g cell mass) increased from 3.48 g/g to 11.42 g/g when the concentration of DTT was increased from 0 g/L to 10 g/L in the 1.0 g/L YE media using simulated syngas.
- More ethanol production and greater ethanol yields were observed in 1.0 g/L YE media with simulated syngas using 10 g/L (64 mM) DTT compared to (0.1 mM) neutral red or (0.1 mM) methyl viologen.
- The addition of DTT to the 10 g/L CSL media with either simulated syngas or producer gas was ineffective in enhancing ethanol production compared to the control medium (without DTT).
- Strain P11 grew faster in the 10 g/L CSL media compared to the 1.0 g/L YE media because CSL contains nutrients such as vitamins, amino acids and minerals that support growth.
- Strain P11 can reduce acetone to isopropanol in both media used when consuming producer gas.
- The addition of DTT to the 1.0 g/L YE and 10 g/L CSL media enhanced isopropanol production over ethanol formation.

CHAPTER VI

FUTURE WORK AND RECOMMENDATIONS

The results showed that effectiveness of dithiothreitol (DTT) varied with the composition of the fermentation medium and the source of syngas used. Based on these results and other findings in this project on the effect of DTT on ethanol and acetic acid production during syngas fermentation with *Clostridium* strain P11, the following recommendations are made for future studies:

- Measure the concentration of reduced DTT remaining in the media during syngas fermentation. This would allow a better understanding of why DTT performed differently in both yeast extract (YE) and corn steep liquor (CSL) media, and with the simulated syngas and producer gas.
- Measure the rate of DTT oxidation during syngas fermentation would suggest the minimum amount of DTT that is required to enhance ethanol production. This could also help in the timing for addition of DTT to the media for maximum effectiveness. The concentration of reduced form of DTT can be determined using Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid) or DTNB).

- Examine the effect of adding DTT when cells enter stationary phase instead of at time zero. This could allow a reduction in the amount of DTT used to enhance ethanol production.
- The effect of DTT in enhancing ethanol production was diminished in CSL medium with simulated syngas. Some of the CSL medium components could have oxidized DTT, thereby reducing its efficiency as a reducing agent. A previous study has shown that Fe^{3+} or Ni^{2+} at a concentration of 0.5 mM could oxidize DTT in 24 h at 25°C (Burmeister et al. 1999). The same study also suggested that the addition of metal chelators such as ethylene glycol tetra acetic acid (EGTA) could significantly improve DTT stability. Elemental analysis of the 10 g/L CSL medium done by another student in our group revealed that the concentration of Fe^{3+} and Ni^{2+} were 1.1 ppm and 0.6 ppm, respectively. The addition of some metal chelators like the EGTA might help improve the stability of DTT during syngas fermentation. However, removing these metals will deprive strain P11 from these important metals for its activity.
- The addition of DTT in concentrations below 5 g/L in the CSL medium improved butanol productivity by over twofold with simulated syngas. Production of butanol is much favored over ethanol from an economical point of view because butanol has 50% more energy density compared to ethanol and can be incorporated into the existing liquid fuel infrastructure. Although the amount of butanol produced by strain P11 was very small, the use of DTT or other reducing agents in the fermentation medium could improve butanol production with strain P11 and other butanol producing microorganisms.

- DTT did not improve ethanol production when producer gas was used. This could be due to the presence of impurities in the producer gas such as trace amounts of oxygen, acetone, ammonia and tar. The effect of these components on oxidation of DTT should be studied before using this reducing agent in fermentation with producer gas. If it is determined that some of the impurities reduce the DTT effectiveness by oxidizing it, then a gas clean up should be employed to remove these impurities from the producer gas prior to fermentation. Producer gas clean up could improve DTT stability and effectiveness during fermentation.
- The effect of low cost reducing agents such as TCEP (tris (2-carboxyethyl) phosphine), sodium formaldehyde sulfoxylatehydrate, sodium thioglycolate and L- ascorbic acid on enhancement of ethanol production should be investigated.
- The use of mutagenic microorganisms that produces less acetic acid and more ethanol should be explored. Nitrosoguanidine (NTG) is effective in mutating the genes that code for the formation of two enzymes phosphotransacetylase and acetate kinase (enzymes involved in acetic acid production) (Rothstein 1986). Using NTG to produce mutants of strain P11 with a potential to produce more ethanol warrants further investigation.
- Examine the possibility for production of isopropanol from simulated syngas with and without the presence of acetone and examine if acetyl–CoA can be used for production of acetone by strain P11.
- Examine the production of isopropanol from producer gas that is scrubbed to remove all acetone and check if impurities in the producer gas have an effect on enhancing isopropanol production over ethanol formation.

- Optimization of the concentrations of media components and DTT using statistical tools like the Plackett-Burman design could reduce the total cost of employing a reducing agent in the fermentation process.

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APPENDIX A

Yeast Extract Medium and Various Reducing Agents

It was previously shown (section 4.1.1.4) that dithiothreitol improved ethanol production by over 500% at a concentration of 10 g/L in the YE medium. Methyl viologen and neutral red were also shown to improve ethanol production (Panneerselvam 2009). Methyl viologen and neutral red enhanced ethanol production when both used at a concentration of 0.1 mM in 1.0 g/L YE media (Panneerselvam 2009). DTT at a concentration of 10 g/L (64 mM) produced the maximum ethanol concentration in 1.0 g/L YE medium in the present study. The effect of the three reducing agents DTT, methyl viologen and neutral red at concentrations of 64 mM, 0.1 mM and 0.1 mM, respectively, on syngas fermentation in 1.0 g/L YE media was evaluated under similar conditions.

Cell Growth

The growth profile of *Clostridium* strain P11 with the three reducing agents is shown in Figure A.1. The cells in the media with neutral red and methyl viologen experienced drop in concentration and lag phase in the first 24 h and 48 h, respectively, after which cells began to grow. However, there was only a lag period of 24 h in growth of strain P11 in the 10 g/L DTT and control media. The cells were in the exponential phase between 24 h and 48 h in the control, DTT and neutral red containing media. However, cells were in the exponential phase between 48 h and 72 h for the methy

viologen medium. The maximum cell concentration in the medium with 64 mM DTT was 0.25 g/L after 240 h, then cells concentration declined. The maximum cell concentration in the media with methyl viologen and neutral red was 0.22 g/L. Cell concentration decline in these two media was slightly lower compared to the control and DTT media.

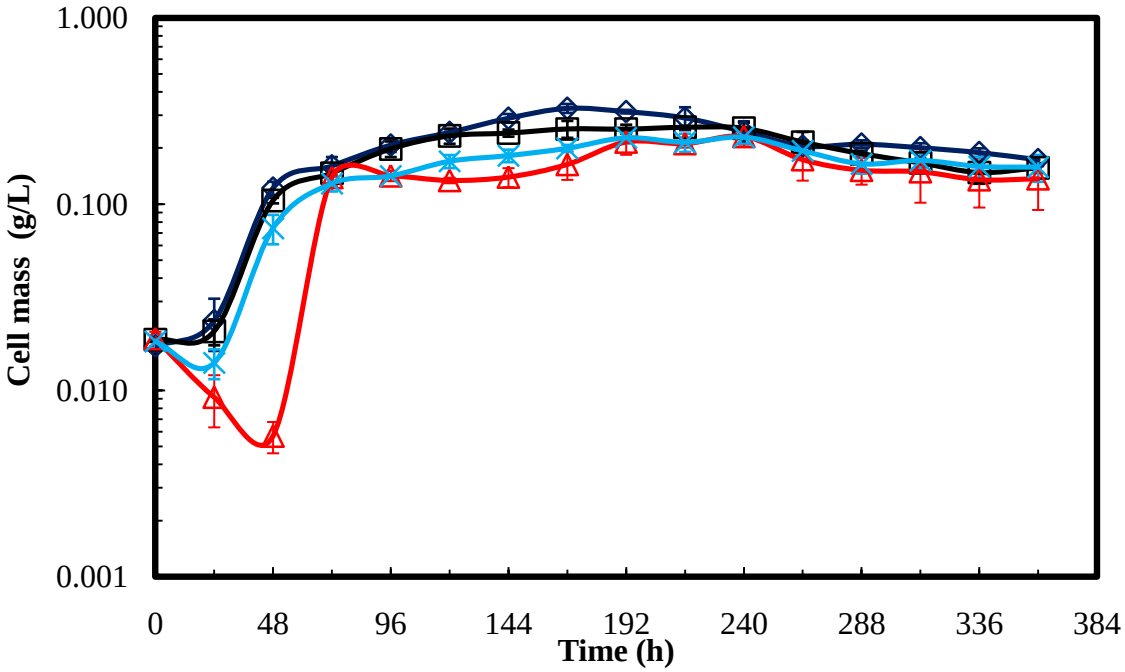

Figure A.1 Kinetics of cell mass production in 1.0 g/L yeast extract media with simulated syngas and different reducing agents: (◇) Control; (□) 64 mM DTT (Δ) 0.1 mM methyl viologen; (×) 0.1 mM neutral red.

pH and Pressure Profiles

The pH profiles of all media were similar, except with methyl viologen (Figure A.2). The pH of the fermentation media decreased from an initial value of 6.0 to about 4.5 after 216 h of fermentation. The pH value leveled off after 216 h, even though there was a small decrease in acetic acid concentration. The pH in the medium with methyl viologen was higher than in the other media. This is primarily due to low acetic acid

production with methyl viologen. The difference in pH profiles in the control, DTT and neutral red media was statistically insignificant ($p < 0.05$). However, the pH in the medium with 0.1 mM methyl viologen was significantly higher than in other media between 72 h and 360 h ($p < 0.05$).

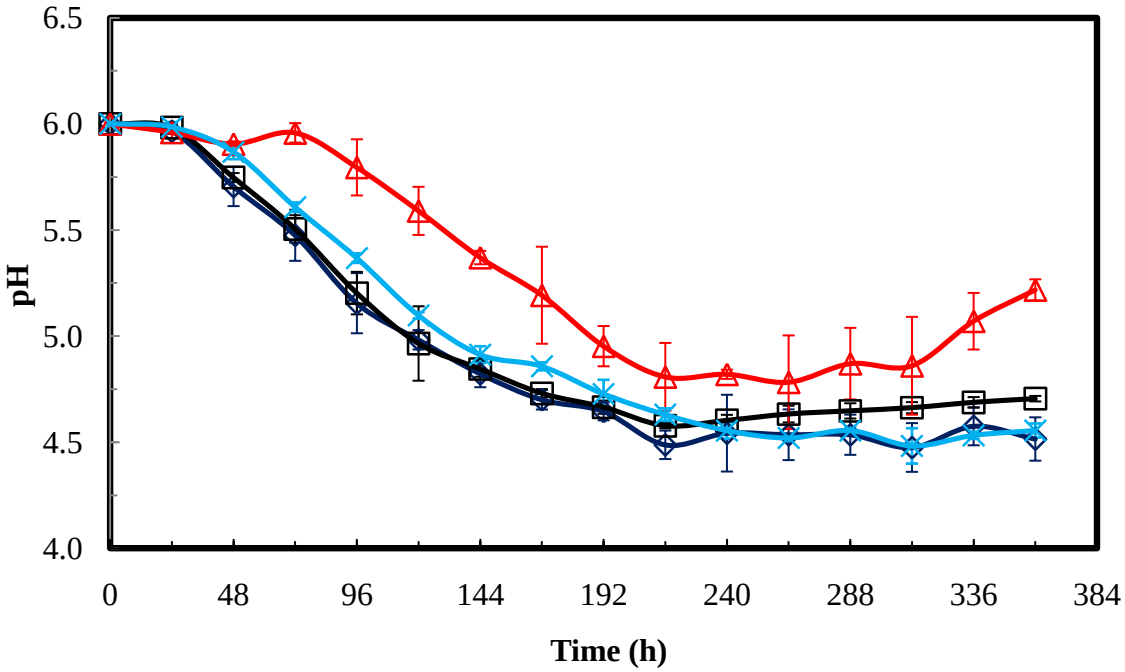

Figure A.2 pH profile during syngas fermentation in 1.0 g/L yeast extract media with simulated syngas and different reducing agents: (◇) Control; (□) 64 mM DTT (Δ) 0.1 mM methyl viologen; (×) 0.1 mM neutral red.

The changes in head space pressure in the fermentation bottles after every 24 h is shown in Figure A.3. It is clear that the gas consumption in the medium with methyl viologen was less compared to other treatments in the first 168 h. Cells in the control medium consumed less gas between 192 h and 360 h compared to other media, possibly due to cell death. The pressure profiles for the DTT and neutral red media were similar. The cells in the medium that contained methyl viologen consumed more gas from 288 h

to 360 h. This was correlated with ethanol production in this medium, which will be discussed in the next section.

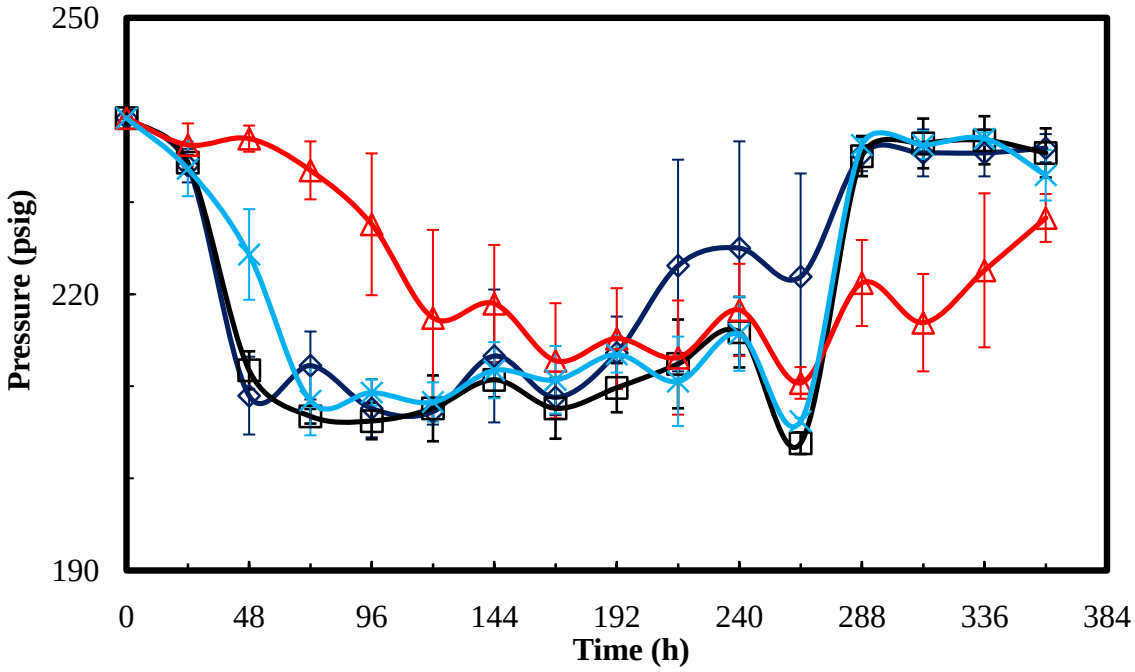

Figure A.3 Pressure profile during syngas fermentation in 1.0 g/L yeast extract media with simulated syngas and different reducing agents: (◇) Control; (□) 64 mM DTT (Δ) 0.1 mM methyl viologen; (×) 0.1 mM neutral red.

Product Profile

The two main products in the fermentation were acetic acid and ethanol. There was no substantial production of butanol (concentrations below 0.1 g/L) in all media. The production of acetic acid was measured during growth and stationary phase upto 240 h of fermentation as shown in Figure A.4. The maximum acetic acid concentration in the control medium was 4.3 g/L (at 240 h). Acetic acid concentration decreased to 3.9 g/L after 360 h of fermentation. The maximum acetic acid concentration in neutral red and DTT media was 3.9 g/L. The medium with methyl viologen produced a maximum of 3.2

g/L acetic acid after 240 h. A previous study also showed that strain P11 produced the least amount of acetic acid in the medium with 0.1 mM methyl viologen among other reducing agents used (Panneerselvam 2009). In the present study, the cells in the medium with neutral red did not consume any acetic acid (Figure A.4). However, acetic acid concentration decreased in the medium with DTT to 3.4 g/L after 360 h of fermentation. The decrease in acetic acid after 240 h corresponded with an increase in ethanol concentration during the same time period, suggesting that acetic acid consumed by the strain P11 cells to produce ethanol. Such a decrease in acetic acid concentration and a corresponding increase in ethanol production was also noticed in another study with strain P11 (Panneerselvam 2009).

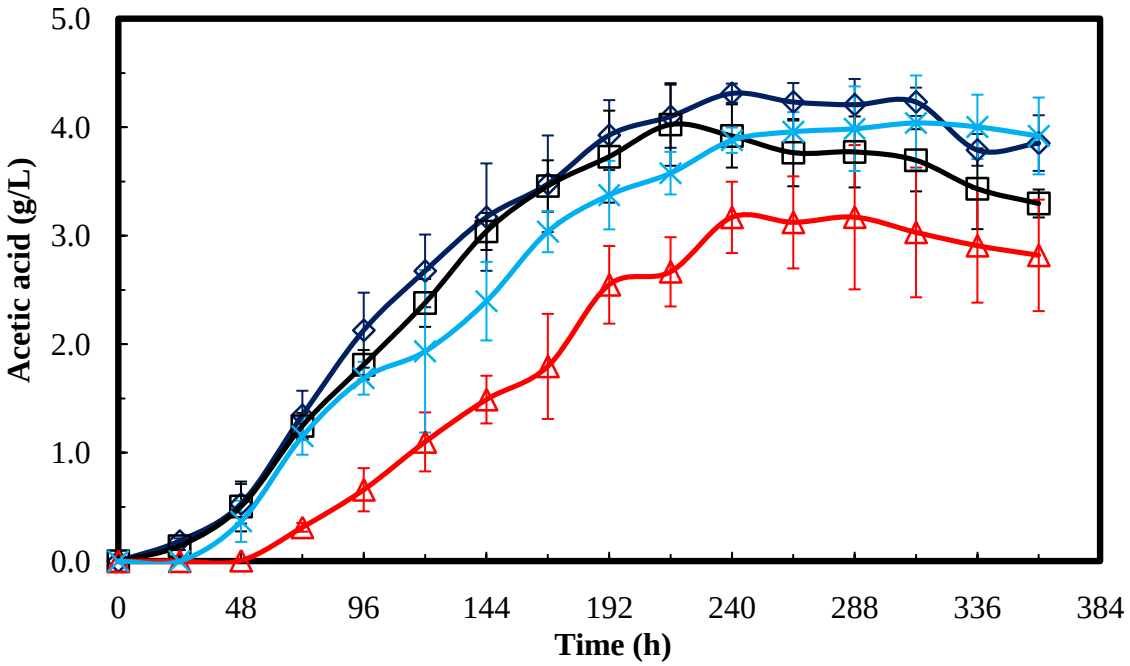

Figure A.4 Kinetics of acetic acid production in 1.0 g/L yeast extract media with simulated syngas and different reducing agents: (◇) Control; (□) 64 mM DTT; (Δ) 0.1 mM methyl viologen; (×) 0.1 mM neutral red.

The kinetics of ethanol production in media with three different reducing agents is shown in Figure A.5. Very small amounts of ethanol were produced in the first 192 h. Ethanol production increased during the death phase of P11 cells. The control medium produced a maximum ethanol concentration of 0.8 g/L after 360 h of fermentation, whereas the medium with 64 mM DTT produced a maximum ethanol concentration of 2.4 g/L after 360 h. The addition of 64 mM DTT enhanced ethanol production by 200%. The maximum amounts of ethanol in the 0.1 mM methyl viologen and 0.1 mM neutral red media were 1.3 g/L and 1.5 g/L, respectively. The increases in ethanol production with the addition of 0.1 mM methyl viologen and 0.1 mM neutral red compared to the control medium were 63% and 88%, respectively.

The ethanol yield in the control and DTT media after 360 h of fermentation were 4.76 g ethanol/g cells and 15.32 g ethanol/g cells, respectively. The addition of 0.1 mM methyl viologen and 0.1 mM neutral red increased ethanol yields by about 100% compared to the control medium. The amount of ethanol produced in presence of DTT, methyl viologen and neutral red were significantly higher ($p < 0.05$) than in the control from 240 h to 360 h. This clearly illustrates that addition of any of these reducing agents enhanced ethanol production, but in different magnitudes.

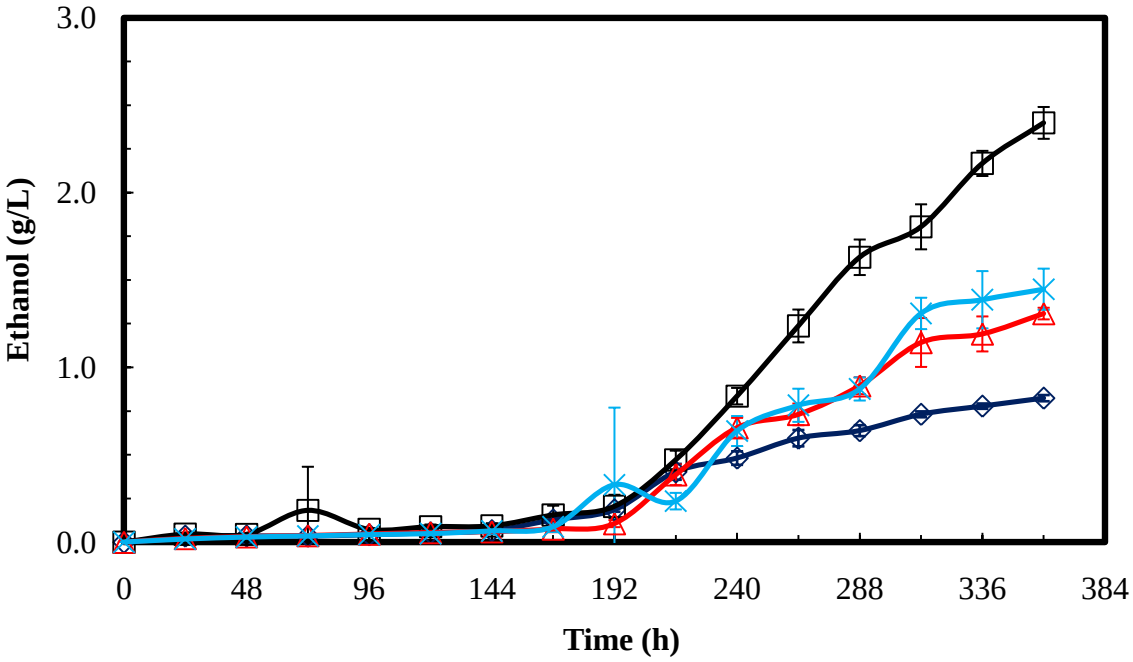

Figure A.5 Kinetics of ethanol production in 1.0 g/L yeast extract media with simulated syngas and different reducing agents: (◇) Control; (□) 64 mM DTT (Δ) 0.1 mM methyl viologen; (×) 0.1 mM neutral red.

There are two main differences between this study and the one conducted by Panneerselvam (2009) . In the earlier study by Panneerselvam (2009), methyl viologen and neutral red were added at 91 h, just when the cells entered stationary phase. Also, the reducing agents were not added in passages 1 and 2 of strain P11 culture, which means the cells were not acclimated to these reducing agents before being inoculated in passage 3.

Because there was no ethanol production during the growth phase, it is clear that there was no effect of any the reducing agents at time 0 h. Therefore, it could be better to add the reducing agents when cells entered stationary phase. This will probably reduce

the amount of reducing agent required to enhance ethanol production during syngas fermentation.

The increase in ethanol production due to the addition of reducing agents could be due to the electrons donated by the oxidation of the reducing agents used. These electrons were probably utilized in the regeneration of NADH from NAD^+. NADH is directly involved in the reduction of acetyl–CoA to acetaldehyde and reduction of acetaldehyde to ethanol. DTT was almost twice as efficient in enhancing ethanol production, compared to methyl viologen and neutral red. However, the concentration of DTT (64 mM) used was 640 times higher than the concentrations of methyl viologen (0.1 mM) and neutral red (0.1 mM).

The amount of ethanol produced after 300 h of fermentation in the presence of 0.1 mM methyl viologen was 1.3 g/L (Panneerselvam 2009). In the present study, the amount of ethanol produced in the presence of 0.1 mM methyl viologen was 1 g/L after 300 h, which is 23% lower than the amount of ethanol produced with 0.1 mM methyl viologen in the previous study (Panneerselvam 2009). However, the ethanol concentration in the medium with 0.1 mM neutral red was 1.15 g/L (after 300 h) in the present study, which is 192% higher than the ethanol produced in an previous study (Panneerselvam 2009) with 0.1 mM neutral red (0.6 g/L after 300h). It is not clear why the addition of neutral red to the fermentation medium at time 0 h in the present study produced more ethanol compared to its addition when cells entered stationary phase (Panneerselvam 2009). However, it is clear that the addition of DTT resulted in the highest ethanol concentration (Figure A.5) and ethanol yield compared to the other two reducing agents (Table A.1). The amounts of acetic acid produced after 300 h of fermentation in Pannerselvam's study

(2009) were similar to the ones obtained in the current study (Table A.2), except in the methyl viologen medium. In addition, higher acetic acid yields were obtained in the medium that contained methyl viologen and neutral red in the present study compared to the previous study as shown in Table A.2.

Table A.1 Ethanol concentration and yields in 1.0 g/L yeast extract media with simulated syngas and dithiothreitol (DTT), methyl viologen and neutral red.

Treatment	Ethanol[a] (g/L)	Ethanol[b] (g/L)	Ethanol yield[a] (g/g)	Ethanol yield[b] (g/g)
Control	0.5	0.7	2.4	3.1
DTT (64 mM)	-	1.7	-	9.7
Methyl Viologen (0.1 mM)	1.3	1.0	7.5	6.1
Neutral red (0.1 mM)	0.6	1.2	2.8	7.7

[a] Values calculated at 300 h (Panneerselvam 2009)
[b] Values calculated at 300 h (present study)

Table A.2 Acetic acid concentration and yields in 1.0 g/L yeast extract media with simulated syngas and dithiothreitol (DTT), methyl viologen and neutral red.

Treatment	Acetic acid[a] (g/L)	Acetic acid[b] (g/L)	Acetic acid yield[a] (g/g)	Acetic acid yield[b] (g/g)
Control	4.6	4.3	21.9	20.2
DTT (64 mM)	-	3.8	-	21.4
Methyl Viologen (0.1 mM)	2.0	3.1	11.4	18.8
Neutral red (0.1 mM)	4.0	4.0	18.2	26.3

[a] Values calculated at 300 h (Panneerselvam 2009)
[b] Values calculated at 300 h (present study)

APPENDIX B

Sample calculation for the determination of specific growth rate of P11

From the Monod equation for cell growth, the maximum specific growth rate equals

$$\mu_{max} = \ln (x / x_0) / (t - t_0)$$

μ_{max} is maximum specific growth rate (h^{-1})

x_0 is the cell mass concentration at time t_0

x is the cell mass concentration at time t

When ln (x/x_0) is plotted versus time, the slope of the line obtained is the maximum specific growth rate (μ_{max} is the growth rate when the cells are in exponential or log phase)

From Figure B, the specific growth rate of strain P11 in the 1.0 g/L yeast extract medium with simulated syngas and without DTT was calculated from the slope of the line to be equal to 0.03 h^{-1}.

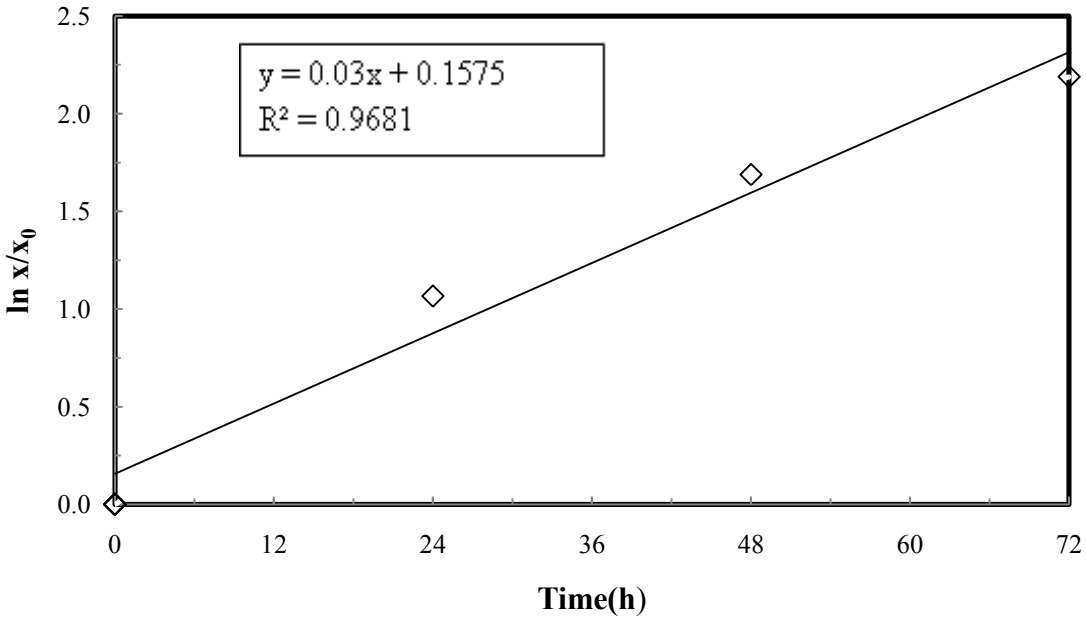

Figure B: Calculation of the maximum specific growth rate of strain P11 in the 1.0 g/L yeast extract medium with simulated syngas and without DTT.

APPENDIX C

Model SAS program for determining least significant difference ($p < 0.05$)

PROGRAM:

Below is the SAS program for determining which treatments produced significantly different amounts of ethanol on day 15 in 1.0 g/L yeast extract medium with simulated syngas.

```
options ls=74 ps=60;
data DTTeth15;
infile "h:\SAS\YEBottledgas\DTTethanolYSI15day.csv" dlm=",";
input trt$ block rep e15;
cards;
run;
proc glm data=DTTeth15; class trt block;
model e15 = trt;
means trt/dunnett('Control');
run;
```

Results: data from all treatments for ethanol production on day 15 (360 h) in 1.0 g/L YE media with simulated syngas.

Table C.1: Statistical analysis for ethanol concentrations in 1.0 g/L YE media with simulated syngas and various DTT concentrations at time = 360 h.

Treatment	Lower limit	Upper limit	Mean	Significantly different
DTT 10 g/L vs Control	1.0294	3.3196	2.1745	YES
DTT 7.5 g/L vs Control	0.6569	2.9471	1.8020	YES
DTT 5.0 g/L vs Control	-0.7841	1.5061	0.3610	NO
DTT 2.5 g/L vs Control	-0.9061	1.3841	0.2390	NO

Discussion:

The treatments with ‘yes’ next to the confidence limit value were significantly different from the control. In this case, the amounts of ethanol produced on day 15 in treatments containing 7.5 and 10 g/L DTT were significantly higher than control treatment in 1.0 g/L yeast extract medium with simulated syngas ($p < 0.05$).

Results: data from all treatments for ethanol production on day 15 (360 h) in 10 g/L CSL media with simulated syngas.

Table C.2: Statistical analysis for ethanol concentrations in 10 g/L CSL media with simulated syngas and various DTT concentrations at time = 360 h.

Treatment	Lower limit	Upper limit	Mean	Significantly different
DTT 2.5 g/L vs Control	0.0107	1.0993	0.5550	YES
DTT 5.0 g/L vs Control	0.1107	1.1993	0.6550	YES
DTT 7.5 g/L vs Control	0.0907	1.1793	0.6350	YES
DTT 10 g/L vs Control	-0.1868	0.9018	0.3575	NO

Discussion:

The treatments with 'yes' next to the confidence limit value were significantly different from the control. In this case, the amounts of ethanol produced on day 15 in treatments containing 2.5, 5.0 and 7.5 g/L DTT were significantly higher than control treatment in 10 g/L CSL medium with simulated syngas ($p < 0.05$).

Results: data from all treatments for ethanol production on day 15 (360 h) in 1.0 g/L YE media with producer gas.

Table C.3: Statistical analysis for ethanol concentrations in 1.0 g/L YE media with producer gas and various DTT concentrations at time = 360 h.

Treatment	Lower limit	Upper limit	Mean	Significantly different
DTT 10 g/L vs Control	-0.9902	-0.4853	-0.7377	YES
DTT 7.5 g/L vs Control	-0.5749	-0.0705	-0.3225	YES
DTT 5.0 g/L vs Control	-0.4599	0.0449	-0.2075	NO
DTT 2.5 g/L vs Control	-0.1624	0.3424	0.0900	NO

Discussion:

The treatments with 'yes' next to the confidence limit value were significantly different from the control. In this case, the amounts of ethanol produced on day 15 in treatments containing 7.5 and 10 g/L DTT were significantly lower than control treatment in 1.0 g/L yeast extract medium with producer gas ($p < 0.05$).

Results: data from all treatments for ethanol production on day 15 (360 h) in 10 g/L CSL media with producer gas.

Table C.4: Statistical analysis for ethanol concentrations in 10 g/L CSL media with producer gas and various DTT concentrations at time = 360 h.

Treatment	Lower limit	Upper limit	Mean	Significantly different
DTT 10 g/L vs Control	-0.7576	-0.2788	-0.5182	YES
DTT 7.5 g/L vs Control	-0.6159	-0.1371	-0.3765	YES
DTT 5.0 g/L vs Control	-0.3399	0.1389	-0.1005	NO
DTT 2.5 g/L vs Control	-0.2989	0.1799	-0.0595	NO

Discussion:

The treatments with 'yes' next to the confidence limit value were significantly different from the control. In this case, the amounts of ethanol produced on day 15 in treatments containing 7.5 and 10 g/L DTT were significantly lower than control treatment in 10 g/L corn steep liquor medium with producer gas ($p < 0.05$).

VITA

Balaji Kubandra Babu

Candidate for the Degree of

Master of Science

Thesis: EFFECT OF THE REDUCING AGENT DITHIOTHREITOL ON ETHANOL AND ACETIC ACID PRODUCTION BY *CLOSTRIDIUM* STRAIN P11

Major Field: Biosystems Engineering

Biographical:

Personal Data: Born in Chennai, Tamil Nadu, India on May 20th 1985. The son of P. Kubandra Babu and K. Chitra. Grew up in the home town and finished high school at Velammal Matriculation Higher Secondary School, Chennai.

Education:

Completed the requirements for the Master of Science in Biosystems and Agricultural Engineering (Minor: Bioprocessing and Biotechnology) at Oklahoma State University, Stillwater, Oklahoma in May, 2010.

Received Bachelor of Technology in Industrial Biotechnology from Anna University (first class with distinction), Chennai, India in May 2006.

Experience:

Final Year Project for Bachelor's degree – Central Leather Research Institute (CLRI), Chennai, India (January 2006 to May 2006).

Graduate Research Assistant – Department of Biosystems and Agricultural Engineering, Oklahoma State University, Stillwater, OK, USA (August 2007 to present).

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Name: Balaji Kubandra Babu Date of Degree: May, 2010

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Title of Study: EFFECT OF THE REDUCING AGENT DITHIOTHREITOL ON ETHANOL AND ACETIC ACID PRODUCTION BY *CLOSTRIDIUM* STRAIN P11

Pages in Study: 110 Candidate for the Degree of Master of Science

Major Field: Biosystems Engineering

Scope and Method of Study:

The objective of this research is to investigate the effect of the reducing agent dithiothreitol (DTT) on enhancing ethanol production from synthesis gas (syngas) using *Clostridium* strain P11 in 250-mL serum bottles. Reducing agents help in regeneration of NADH from NAD^+. NADH is utilized in the production of alcohol from aldehydes. The effect of DTT concentrations from 0 to 10 g/L was studied in 1.0 g/L yeast extract (YE) and 10 g/L corn steep liquor (CSL) media and with simulated syngas and producer gas. Syngas contains mainly carbon monoxide, hydrogen, carbon dioxide and nitrogen. The fermentation process was followed for 360 h. Liquid samples were collected every 24 h to determine cell mass, pH and product concentrations. The experiment was done in quadruplets at each DTT concentration and the results were analyzed for statistical significance using SAS® version 9.2 at 95% confidence level.

Findings and Conclusions:

Results showed that over 350% increase in ethanol concentration was obtained in media that contained at least 7.5 g/L of DTT in the 1.0 g/L yeast extract medium after 360 h of fermentation with simulated syngas compared to the control medium (without DTT). However, only a 35% increase in ethanol production was noticed in 10 g/L corn steep liquor media in the presence of 2.5 and 5.0 g/L of DTT compared to the control medium with simulated syngas. In addition, DTT (at a concentration of 2.5 g/L) produced about 240% more butanol in the 10 g/L CSL medium compared to the control with simulated syngas. The results suggested that the use of small concentrations of DTT in the broth enhances ethanol production from simulated syngas in YE media. When producer gas was used, DTT enhanced isopropanol production instead of ethanol production in both YE and CSL media. The electrons donated by DTT might have been utilized in the reduction of acetone to isopropanol by strain P11 instead of reduction of acetaldehyde to ethanol. The removal of acetone and other impurities from the producer gas could enhance DTT effectiveness as a reducing agent and improve ethanol production in both YE and CSL media.

ADVISER'S APPROVAL: Dr. Hasan K. Atiyeh