ABSTRACT

YINGXI, GENG. The Effect of Inhibitors on Butyric Acid Fermentation by *Clostridium Tyrobutyricum*. (Under the direction of Dr. Wenqiao Yuan).

Lignocellulosic biomass can be used by some microorganisms to produce butyric acid after pretreatment and hydrolysis. However, in addition to sugars such as glucose and xylose, the pretreatment process could also produce other chemicals. These chemicals might cause negative effects on cell growth and metabolic activities of microorganisms and thus inhibit the production of butyric acid. The overall goal of this research is to understand the effects of potential inhibitors on butyric-acid fermentation by *Clostridium tyrobutyricum*. According to existing literature, six potential inhibitors from three general categories of chemicals were chosen. The inhibitors included furfural and hydroxymethylfurfural (HMF) (furan derivatives), formic acid and levulinic acid (organic acids), and vanillin and syringaldehyde (phenolic compounds). The inhibitors were added into the standard xylose culture medium at various concentrations. With the composite of xylose-culture medium and inhibitors, *Clostridium tyrobutyricum* was cultured at 37 ^{o}C under anaerobic conditions in the batch mode. The pH was adjusted to 6.4 by HCl or NH_4OH every 12 hours. During the fermentation, cell growth was measured by a spectrophotometer. Moreover, the concentrations of xylose, organic acids and inhibitors were measured by High Performance Liquid Chromatography (HPLC). The results are summarized as follows:

1. Furan derivatives: Furfural was the most toxic among the six inhibitors, since only 1.2 g/L of furfural was able to completely inhibit cell activities. At low concentrations, such as 0 to 0.9 g/L, furfural had no significant effects on cell replication or butyric-acid yield although the lag phase was prolonged at 0.6 and 0.9 g/L. HMF was less toxic to cells than furfural. Although up to 2.4 g/L of HMF had mild effects on cell growth, with

the increasing concentration of HMF, both yield and productivity of butyric acid decreased. In the medium with 1.8 and 2.4 g/L of HMF, the accumulation of butyric acid was not observed, which indicated that high concentrations of HMF would inhibit the microorganism from producing butyric acid. Furfural and HMF were fully consumed by *C. tyrobutyricum* during fermentation.

2. Organic acids: *C. tyrobutyricum* was tolerant of weak acids. With increased concentrations of formic acid (from 0 to 4.8 g/L), the yield of butyric acid was not affected. The maximum cell concentration mildly decreased to 85.8% of control when the concentrations of formic acid increased from 0 to 4.8 g/L. However, the productivity of butyric acid decreased due to the longer lag and log phases. On the contrary, 2.4 and 4.8 g/L of levulinic acid had no influence on cell activities. For both formic acid and levulinic acid, *C. tyrobutyricum* was not able to metabolize these two weak acids.
3. Phenolic compounds: Low concentration (0.6 g/L) of syringaldehyde and vanillin had no significant effects on cell activities. However, when the concentrations of syringaldehyde and vanillin were increased from 0.6 to 2.4 g/L, both inhibitors gradually decreased the maximum cell concentration, yield, and productivity of butyric acid. In addition, syringaldehyde and vanillin were assimilated by cells.

In this research, we quantified the inhibitory extent of these six inhibitors. Knowledge about the degree of inhibition of these inhibitors contributes to butyric-acid production from lignocellulosic biomass. This study is also the first step in studying the complicated synergistic effects of inhibitors. In addition, this research provides a potential way to monitor the fermentation, which is important for manufacturing butyric acid.

The Effect of Inhibitors on Butyric Acid Fermentation by *Clostridium Tyrobutyricum*

by
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BIOGRAPHY

Yingxi Geng was born in Tianjin, China in 1990. She lived in her hometown for 18 years before she went to Fuzhou University in Fujian. She majored in Vehicle Engineering and completed a Bachelor's degree in 2012. After graduating from college, Yingxi decided to dedicate herself to researching renewable energy. Subsequently, she was admitted to the Department of Agricultural and Biological Engineering in North Carolina State University. She joined Dr. Yuan's research group to pursue her Master's degree from fall semester 2012, until now.

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CHAPTER 1 Introduction and Literature Review

1.1 Introduction

1.1.1 Butyric acid and its applications

Butyric acid is a short-chain fatty acid (C_3H_7COOH). In nature, the esters form of butyric acid exists in animal fat and some plant oil (Liu et al., 2013). Butyric acid has many applications. It can serve as an intermediate to produce potential biofuels, such as butanol, ethyl butyrate, and butyl butyrate (Al-Shorgani et al., 2012; Dwidar et al., 2012; Li et al., 2013; Wu et al., 2010). As carbon source, butyric acid can be converted to butanol, which has proven to reduce the toxicity of alcohol in fermentation broth, and to enhance the theoretical yield of butanol by 67% (Du et al., 2012; Dwidar et al., 2012). Second, in the pharmaceutical industry, butyric acid is used to treat hemoglobinopathies, cancer, and gastrointestinal diseases due to its anticancer effect (Dwidar et al., 2012). Various derivatives of butyric acid are produced as vasoconstrictor drugs and antioxidants. Other potential prodrugs that are produced by butyric acid are still under investigation (Du et al., 2012). Third, one major application of butyric acid in the chemical industry is plastics production, especially, cellulose acetate butyrate plastics. These plastics have better flexibility and greater resistance to light and to cold than traditional plastics (Cao et al., 2011; Dwidar et al., 2012). In addition, plasticizers, surfactants, and textile auxiliaries also use butyric acid as an important ingredient (Liu et al., 2013). Last but not least, in the form of esters and salts, butyric acid can be utilized as fragrance and flavoring agents in foods, beverages, and cosmetics (Dwidar et al., 2012; Liu et al., 2013).

1.1.2 Butyric acid production methods

Generally, there are three methods of producing butyric acid. At the industrial scale, butyric acid is obtained mostly from petroleum feedstocks by a petrochemical method (Jones et al., 1986; Liu et al., 2013). This chemical process is related to the oxidation of butyraldehyde. Butyraldehyde comes from propylene, which is derived from crude oil by oxo synthesis (Cascone et al., 2008). This method is preferred due to its relatively low production cost and the availability of the crude material (Dwidar et al., 2012). The second method is extracting butyric acid from butter directly. The concentration of butyric acid in butter ranges from 2% to 4%, but the extraction process is so complex and expensive that this method is not a promising alternative (Zigová et al., 2000). The third method is a biological method via microbial fermentation (Liu et al., 2013; Jones et al., 1986). Even though the current cost of the biological method is higher than the chemical-synthesis method, the microbial resources are renewable and they are more abundant, compared to limited crude oil (Dwidar et al., 2012). In addition, the demand for bio-based organic and natural products makes the biological method more desirable, especially for food additives, cosmetics and pharmaceuticals (Dwidar 2012; Dwidar et al., 2013; Liu et al., 2013; Zhang et al., 2009).

1.2 Fermentation

1.2.1 Butyric acid prodution microorganisms

In microbial fermentation, a number of gram-positive, obligate anaerobes, such as *Clostridium sp.* and *Butyrivibrio fibrisolnes,* have the ability to produce butyric acid as primary metabolite in significant quantities (Liu et al., 2013; Waites et al., 2009). Among these microorganisms,

Clostridium strains are preferred in industry because of their higher productivities and greater final-product concentrations (Dwidar et al., 2013). The characteristics of these *Clostrium* species are gram-positivity, chemo-organotrophy, strict anaerobiosity and sporeformation (Zigová, 1999). *Clostridum* species can be isolated from soil, waste water, animal digestion-systems, contaminated dairy products, and so on. The optimal cultivation conditions include these attributes: temperature between 35-37 °C, pH between 4.5-7.0, and an atmosphere of pure CO_2 or pure N_2, or a mixture of N_2 and CO_2 in a ratio of 1:9 (Zigová, 1999). *Clostridium tyrobutyricum* is one of the most promising strains due to the ability of producing butyric acid with high selectivity, the tolerance of high concentrations of products (Mohanmmed et al., 2013), and relatively high and stable productions (Liu et al., 2006b; Najafpour et al., 2006; Song et al., 2010; Zigová et al., 2000). This strain is able to utilize some common carbon sources, such as glucose, xylose, fructose, and lactate (Dwidar et al., 2013). *Clostridium butyricum* and *Clostridium thermobutyricum* are also widely studied since they could utilize a larger range of fermentable sugars as carbon source. However, these two strains are not used in industry because their yields of butyric acid are lower than *C. tyrobutyricum* (Fayolle et al., 1990; He et al., 2005).

1.2.2 Biosynthesis pathway of butyric acid during fermentation

Figure 1.1 shows the metabolic pathway of butyrate (the conjugate base of butyric acid) production in *Clostridial* strains. Xylose is used as the carbon source in this pathway. At the first step, xylose is converted to pyruvate via Pentose Phosphate Pathway (PPP) (Jiang et al., 2010; McMillan et al., 1993). Subsequently, depending on the types of strains, pyruvate is

fermented into different products. For the acid-producing strains, the pathway undergoes an acidogenesis phase. Pyruvate is converted to lactate, which is catalyzed by lactate dehydrogenase, whereas pyruvate dehydrogenase catalyzes the transformation of pyruvate into acetyl-CoA. Phosphotransacetylase (PTA) and acetate kinase (AK) are two key enzymes during acetate formation when phosphotransbutyrylase (PTB) and butyrate kinase (BK) act similarly to PTA and AK during butyrate formation (Dwidar et al., 2013). With high cell-growth rate, cells prefer to form acetate rather than butyrate, because four molecules of ATP are released due to acetate production, whereas just three molecules of ATP are released during butyrate formation (Li et al., 2011; Michel-Savin et al., 1990). Furthermore, in order to reduce the toxic chemicals in medium, at the exponential phase, cells transfer acetate into butyrate to reduce the total hydrogen-ion concentration (Zhang et al., 2009).

Those alcohol-producing strains go through the solventogenesis phase after the acidogenesis phase, when cells enter the stationary phase. Therefore, acids, which formed in the first phase, are converted into alcohols (acetone, ethanol, and butanol) (Zigová et al., 2000).

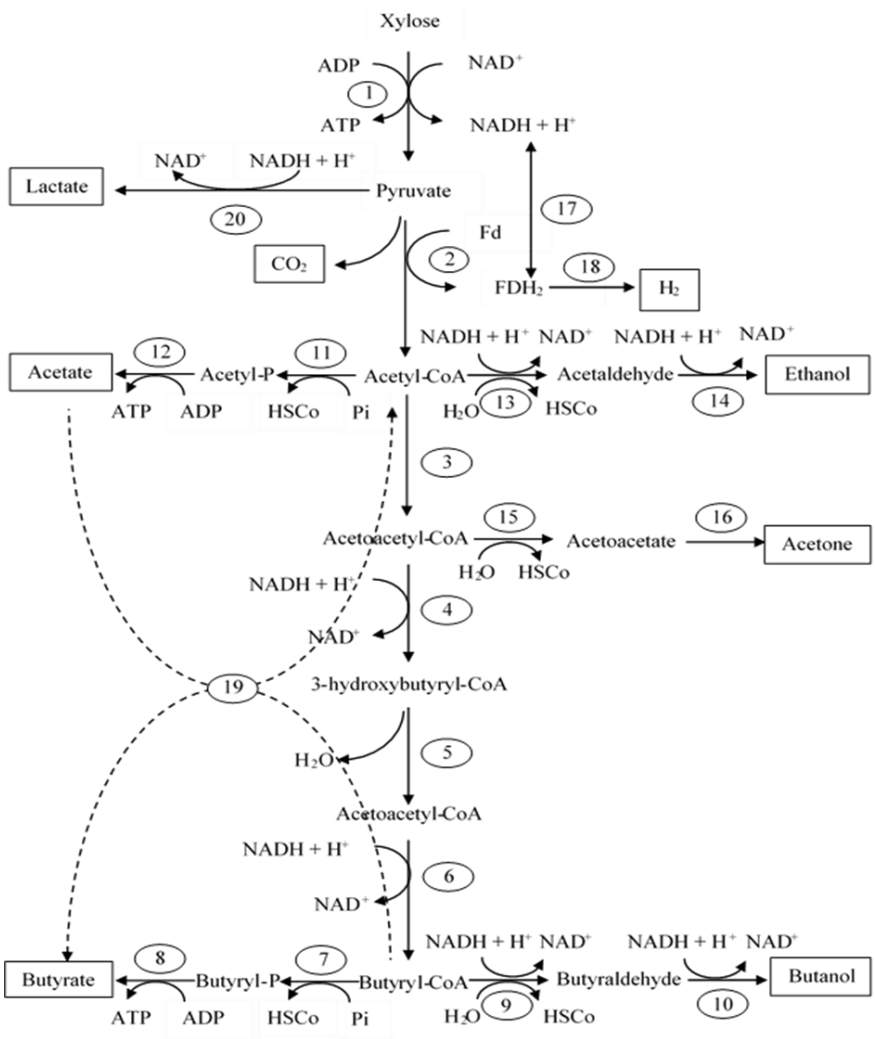

Figure 1.1 Metabolic pathway of butyrate production by *Clostridial* strains (Liu et al., 2004; Mazzoli et al., 2012; Zhu et al., 2004), 1: EMP and PPP pathway, 2: pyruvate-ferredoxin oxidoreductase, 3: acetyl CoA-acetyl transferase (thiolase), 4: beta-hydroxy butyryl CoA dehydrogenase, 5: crotonase, 6: butyryl CoA dehydrogenase, 7: phosphotransbutyrylase, 8: butyrate kinase, 9: butyraldehyde dehydrogenase, 10: butanol dehydrogenase, 11: phosphotransacetylase, 12: acetate kinase, 13: acetaldehyde dehydrogenase, 14: ethanol dehydrogenase, 15: CoA transferase, 16: acetoacetate decarboxylase, 17: ferredoxin-NAD(P)+reductase, 18: hydrogenase, 19: butyryl CoA-acetate transferase (proposed enzyme), 20: lactate dehydrogenase. The dashed lines indicate a possible reaction catalyzed by CoA transferase to produce acetate in the absence of PTA and AK (Liu et al., 2006a).

1.2.3 The differences between glucose and xylose on metabolic pathway when C. tyrobutyricum utilizes them as carbon sources

C. tyrobutyricum could feed on a broad range of carbon sources, such as glucose, xylose, fructose, and so on (Song et al., 2010). Among these carbon sources, glucose and xylose are commonly used because they are main soluble sugars in hydrolysates of lignocellulosic biomass (Zhu et al., 2004). The equations for converting glucose and xylose to butyric acid by *C. tyrobutyricum* are shown below (Equation 1.1 and 1.2) (Dwidar et al., 2012). The theoretical (per carbon unit) yield of butyric acid is equal for both sugars.

$$\text{Glucose} + 3\ \text{ADP} \rightarrow \text{butyric acid} + 3\text{ATP} \quad (1.1)$$

$$6\text{Xylose} + 15\text{ADP} \rightarrow 5\text{butyric acid} + 15\text{ATP} \quad (1.2)$$

Nevertheless, the metabolic pathways are different when *C. tyrobutyricum* utilizes glucose or xylose. First, the pathway from glucose/xylose to pyruvate is different (process 1 in Fig. 1.1). The glucose is converted into pyruvate through Embden-Meyerhof-Parnas (EMP) pathway, whereas xylose is metabolized via Pentose-Phosphate (PP) pathway (Jiang et al., 2010). Although the process 1 is not the same for glucose and xylose, the yield (per carbon unit) of pyruvate is theoretically identical for these two sugars (Equation 1.3 and 1.4) (Jiang et al., 2010). After these two sugars are transferred into pyruvate, the rest of the pathway is the same for xylose and glucose.

$$\text{Glucose} + 2\text{ADP} \rightarrow 2\ \text{Pyruvate} + 2\text{ATP} \quad (1.3)$$

$$3\text{Xylose} + 5\text{ADP} \rightarrow 5\ \text{Pyruvate} + 5\text{ATP} \quad (1.4)$$

Second, extra energy is needed to transport xylose across the cell membrane (Jiang et al., 2010). The transport of xylose is energized by a high-energy phosphate compound, which is implied

to be ATP. It was reported that one mol of ATP is required for the transport of one mol of xylose into the cells of *C. butyricum* LMF 1213t_1 (Heyndrickx et al., 1991). Glucose is transported into cells by means of a phosphoenolpyruvate (PEP) -dependent phosphotransferase system without requiring extra energy (Jiang et al., 2010). So, the net ATP yield from metabolizing xylose is lower than that from metabolizing glucose.

1.3 Lignocellulosic biomass

It is considerably costly to apply a pure carbon source in the fermentation. As an abundant and inexpensive renewable resource for organic carbon, plant biomass has gained great attention (Messmer et al., 2013; Mohan et al., 2006). In addition to biofuels, biomass can also produce petrochemical products, such as butyric acid (Nhien et al., 2015). Among varieties of biomass, the non-edible ligocellulosic biomass is more favorable due to worldwide food crises (Patel et al., 2015). The majority components of lignocellulosic biomass are lignin, cellulose, and hemicellulose (Mohan et al., 2006). The minority components include organic extracts and inorganic minerals (Mohan et al., 2006). In the micro-structure of biomass, cellulose and hemicellulose are combined together to form a carbohydrate polymer matrix, and this matrix is cross-linked with lignin (Jørgensen et al., 2007; Toquero et al., 2014). The percentages of these three components in plants vary depending on the species. Generally, cellulose comprises 33-51% of lignocellulosic biomass. Hemicellulose accounts for 19-34% of lignocellulosic biomass. Lignin constitutes the rest (Almeida et al., 2007).

1.3.1 Cellulose

Cellulose provides the strength of wood due to beta-linked glucopyranose residues that stabilize the chair structure and reduce flexibility (Mohan et al., 2006). As an unbranched homopolymer, the long cellulose chain, containing 5000-10000 glucose units, is constituted by polymerized glucoseanhydride. Cellulose is insoluble in water and the crystalline structure of cellulose makes it more resistant to thermal decomposition than hemicellulose (Agbor et al., 2011; Mohan et al., 2006). Cellulose starts to degrade when temperatures reach 240-350 °C (Mohan et al., 2006).

1.3.2 Hemicellulose

Hemicellulose is the second major constitute in lignocellulosic biomass and consists of different kinds of polymerized monosaccharides, including glucose, xylose, arabinose, mannose, and so on. Compared to 5000-10000 glucose units in cellulose, only 100-150 units of saccharide monomers are contained in a hemicellulose molecule. (Mohan et al., 2006). Unlike the long-chain structure of cellulose, some short and side chains are attached to the main chain in hemicellulose. Due to its branched short lateral chains and lower molecular weight, it is easier and faster for hemicellulose to be decomposed under thermal conditions (200-260 °C), and to be hydrolyzed by diluted acid or alkaline (Agbor et al., 2011; Avci et al., 2013; Mohan et al., 2006; Yang et al., 2006).

For different types of hemicelluloses, hardwood hemicelluloses contain larger amounts of xylan (a polymer made from xylose) than of glucomannan. But, the percentage of xylan and

glucomannan in softwood hemicelluloses is quite the opposite (Mohan et al., 2006). Xylan is easily extracted in an acid or alkaline environment, while the extraction of glucomannan requires a strong alkaline environment (Agbor et al., 2011).

1.3.3 Lignin

Lignin is the third major component. Lignin is the rigid and impermeable part of biomass because the polysaccharides in lignin is three-dimensional and cross-linked (Agbor et al., 2011; Mohan et al., 2006; Tenkanne et al., 1997; Yang et al., 2006). This recalcitrant polymer prevents microbial destruction and oxidative stress on cellulose and hemicellulose (Vanholme et al., 2010). Due to the complex inner structure of lignin, it is harder to dehydrate and thermally decompose lignin than cellulose and hemicellulose. Lignin starts to decompose when the temperature is 280-500 °C (Mohan et al., 2006).

1.4 Pretreatment and hydrolysis

Because of the complicated structure of lignocellulosic biomass and the limitation of corresponding enzymes in cells, microorganisms cannot directly utilize biomass. Therefore, before utilizing lignocellulosic biomass in fermentation, the biomass must be pre-treated. The pre-treated process mainly refers to degrade lignocellulosic structure, by breaking down the seal and crystalline structure of lignin, and converting cellulose and hemicellulose into fermentable sugars (Mosier et al., 2005; Naiqi et al., 2009).

Normally, the process contains two steps: pretreatment and hydrolysis (Hamelinck et al., 2005; Yang et al., 2008). Pretreatment methods break down cell structures, separate the components of cellulose, hemicellulose and lignin, and make the components more accessible to hydrolysis (Ren et al., 2009; Saha et al., 2003). Pretreatment can be carried out by physical, chemical, physiochemical, and biological methods (Agbor et al., 2011; Mosier et al., 2005; Ren et al., 2009). Physical pretreatment includes mechanical comminution, steam explosion, and hydrothermolysis. Chemical pretreatment methods can be classified as ozonolysis, acid hydrolysis, alkaline hydrolysis, organic-solvents pretreatment, and ionic-liquid pretreatment (Olsson et al., 1996). In addition, physiochemical methods are those that combine the physical and chemical methods together, such as acid-catalyzed-steam explosion, ammonia-fiber explosion, and CO_2 explosion. Biological methods involve the action of fungi, such as, white- and soft-rot fungi (Olsson et al., 1996). Selection of pretreatment methods should meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by hydrolysis; (2) avoid degradation or loss of carbohydrate; (3) avoid releasing inhibitory byproducts that might influence the subsequent hydrolysis and fermentation processes; and (4) be cost-effective. (Sun et al., 2002). These methods have both advantages and disadvantages. Hence, it is more desirable to have a combination of different methods in order to satisfy all the requirements (Agbor et al., 2011). Although dilute-acid pretreatment has limitations, it is still considered as an effective and widely used technique. (Gurram et al., 2011; Mosier et al., 2005; Yang et al., 2008).

For the second step, hydrolysis converts the compounds into fermentation sugars (Mosier et al., 2005). The hydrolysis process can be carried out by chemical methods (sulfuric or other acids) and by enzymatic methods (cellulases, hemicellulases) (Mosier et al., 2006; Ren et al., 2009).

Acid hydrolysis is commonly used because the acids can penetrate lignin (Lenihan et al., 2010), and convert the cellulose and hemicelluloses into xylose and other sugars (Mosier et al., 2005; Wyman et al., 1994). Therefore acid hydrolysis can accomplish pretreatment and hydrolysis processes in one step (Von Sivers et al., 1995). Acid-hydrolysis methods include dilute or concentrated-acid hydrolysis (Li et al., 2012). Concentrated acid is effective for cellulose hydrolysis, but a high concentration of acid is toxic, corrosive, and hazardous (Sun et al., 2002). Furthermore, the concentrated acid must be recovered after hydrolysis to lower the cost (Von Sivers et al., 1995). Compared to concentrated acid, dilute acid (such as dilute sulfuric acid) hydrolysis, could increase the reaction rate and improve cellulose-hydrolysis efficiency (Sun et al., 2002). Dilute-acid pretreatment was shown to be effective in disrupting the protective lignin barrier (Gurram et al., 2011; Mosier et al., 2005).

However, current hydrolysis processes release a large range of compounds in addition to monosaccharide. These compounds might inhibit the growth and metabolism of cells (Quemeneur et al., 2012). For example, after treated by sono-assisted alkaline pretreatment and acid hydrolysis, sugarcane bagasse gave a high yield of sugar, but the ethanol yield was as low as 0.17 g/g in ethanol fermentation because of inhibitory byproducts (Wei, 2013). These

inhibitors can be classified into three groups according to the generation sources. The first group is furan derivatives, which include furfural and hydroxymethylfurfural (HMF). Furfural and HMF are formed during the degradation of pentose and hexose, respectively. The second group is aliphatic-acid group, including formic acid, levulinic acid, and so on. Formic acid comes from the degradation of furfural and HMF, and levulinic acid is formed by HMF degradation only. The third group is derived from lignin fraction, containing vanillin, syringaldehyde and other phenolic compounds (Mills et al., 2009; Mussatto et al., 2004; Palmqvist et al., 2000; Quemeneur et al., 2012; Ren et al., 2009).

The presence and concentration of inhibitors that are released depend on several factors: hydrolysis methods, hydrolysis conditions, and raw materials (woods, grasses, and so on) (Gurram et al., 2011). Generally, the more severe the hydrolysis reaction becomes, the greater the concentrations of inhibitors that are released (Park et al., 2012). Temperature might affect the hydrolysis efficiency and the content in hydrolysate (Sun et al., 2002). A medium that has extreme pH values without any control or buffer easily form main inhibitors (Landaeta et al., 2013; Toquero et al., 2014). Furfural and HMF could be generated during hydrolysis directly. Formic acid, levulinic acid, and some phenols (vanillin and syringaldehyde) are formed in higher-severity conditions (Gurram et al., 2011; Klinke et al., 2004), and phenols prefer the oxidative acidic conditions (Klinke et al., 2004). For each type of microorganisms, inhibitors might show significant inhibitory effects at different concentrations. (Cao et al., 2010; Delgenes et al., 1996 ; Quemeneur et al., 2012).

Previous studies regarding inhibitors on fermentation mostly focused on alcohol (like ethanol or butanol) fermentation process. A study on butyric acid related to inhibitors is still under investigation.

1.5 The generation and inhibitory effect of single inhibitors

In this paper, three classes of potential inhibitors are discussed: furan derivatives, organic aicd, and phenolic compounds. Their structures and sources are presented in Fig. 1.2. Table 1.1 lists the concentrations of furfural, HMF, formic acid, levulinic acid, syringaldehyde and vanillin in hydrolysates after pretreatment and hydrolysis. Table 1.2 displayed the results of previous studies about the negative effects on cell growth and fermentation caused by different concentrations of inhibitors.

1.5.1 Furan derivatives

Furan derivatives contain furfural and HMF. At high temperature and pressure, pentose and hexose tend to degrade to furfural and HMF during hydrolysis (Dunlop et al., 1948) in a range of 0-3.5 g/L in hydrolysate (Table 1.1). In Table 1.2, the concentrations of furans are about 2-3 g/L when they have negative effects on cell growth and fermentation.

Furfural is a major by-product that is generated in acid-based hydrolysis (Hu et al., 2009). Evidence suggests that 0-2 g/L of furfural can affect cell growth and cell activities during fermentation by decreasing specific growth rate (Boyer et al., 1992). The cell-mass yield on ATP and volumetric fermentation rate are also affected (Banerjee et al., 1981a; Taherzadeh et al., 2000a; Vanerjee et al., 1981; Zauner et al., 1979). Both glycolytic and non-glycolytic

enzymes were directly inhibited by furfural (Ren et al., 2009; Banerjee et al., 1981b). Compared to metabolic activities, cell growth was more affected by furfural (Palmqvist et al., 1998). Landaeta et al. made a similar conclusion: when cell growth was reduced, glucose consumption was maintained (Landaeta et al., 2013). Palmqvist et al. also observed that adding furfural to *Saccharomyces cerevisiae* fermentation decreased cell replication without affecting cell activities (Palmqvist et al., 1998).

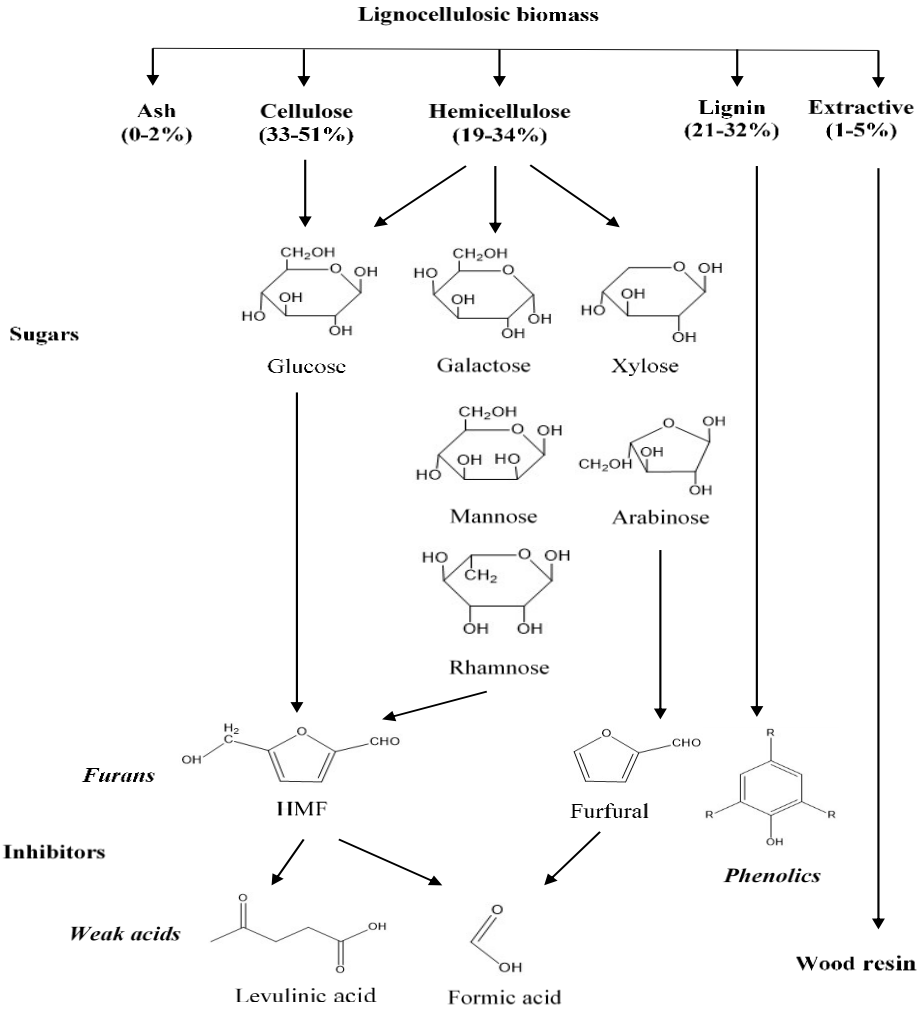

Figure 1.2 Average composition of lignocellulosic biomass and main derived hydrolysis products (Almeida et al., 2007; Smith et al., 2016)

The concentration of HMF is usually lower than furfural in hydrolysates. There are three reasons that can cause the lower production of HMF in hydrolysate. First, HMF is produced during the degradation of hexoses, which are in smaller quantity in hemicellulose than pentose. Second, glucomanna (polysaccharides of hexoses) is more difficult to be released from hemicellulose to hydrolysate than xylan (polysaccharide of pentose), in both acid and alkaline environments. (Hendriks et al., 2009; Mussatto et al., 2004). Third, HMF is of high reactivity (Mussatto et al., 2004). The high reactivity is due to the hyrdoxymethyl group (-H_2-OH), which makes HMF easily undergo reactions, such as oxidaztion and estrification (Biologic effects of exposure, n.d.). HMF was reported as having an inhibitory mechanism that is similar to that of furfural. However, generally, HMF is less toxic than furfural.

During the fermenation, HMF could be metabolized too. The major metabolite was 2,5-bis-hydroxymethylfuran, which is an analog of furfuryl alcohol (Pienkos et al., 2009). HMF might be converted at a lower rate than furfural (Veeravalli et al., 2013). The lower rate is caused by lower membrane permeability, and this lower rate leads to a longer lag phase of cell growth (Palmqvist et al., 2000). Conversely, a shorter lag phase was observed during HMF-added cultures than furfural cultures in hydrogen-producing fermentation (Quemeneur et al., 2012). The authors explained that compared to HMF, furfural has a lower molecular weight. So furfural is easier passing through the membrane of cells. Hence the accumulation of intracellular furfural is faster than HMF. Therefore, it takes cells a longer time to assimilate furfural (Quemeneur et al., 2012). The differences between these two observations might be caused by the different strains that were used.

1.5.2 Aliphalic acids

Formic acid and levulinic acid are major compounds that are classified as aliphatic acids (Palmqvist et al., 2000). Both of them are generated from subsequent degradation of HMF (Tirzh et al., 2009). The concentrations of aliphatic acids are below 3.1 g/L in different kinds of hydrolysates (shown in Table 1.1). However, the concentrations of organic acid differed (from 1 to 20 g/L) on different types of strain when the inhibitors had negative influences on cell growth and fermentation (shown in Table 1.2).

Weak acids can affect microbial growth by penetrating microbial cells at the acid's undissociated form. After dissociating inside cells, weak acids can decrease the intracellular pH, collapse the pH gradients, and thus further inhibit cell growth (Palmqvist et al., 2000; Tirzh et al., 2009). Formic acid was shown to inhibit macromolecule synthesis, and to slow DNA-synthesis processes (Mills et al., 2009). Weak acids were presented to inhibit cell growth rather than to inhibit cell-producing target products (Zaldivar et al., 1999a). Of these two weak acids, formic acid was more toxic due to its higher permeability through cell membranes (Mills et al., 2009).

Table 1.1 The concentrations of inhibitors in hydrolysates of lignocellulosic biomass

Inhibitor	Concentration (g/L)	Biomass	Pretreatment/hydrolysis method	Reference
Furfural	3.5	Birch	Dilute acid	Taherzadehet al., 2000b
	2.72	Eucalyptus	0.65% Sulfuric acid	Carvalho et al., 2006
	2.22	Pine	1% (w/v) Sulfuric acid	Gurram et al., 2011
	1.73	Rice straw	0.6% Nitric acid	Kim et al., 2014
	1.7	Spruce	Dilute acid	Taherzadehet al., 2000b
	1.4	Forest residue	Dilute acid	Taherzadehet al., 2000b
	1.11	Sugar maple	1.5% (wt) Sulfuric acid	Sun et al., 2012
	0.99	Corn stover	3% (w/w) Sulfuric acid	Ruan et al., 2013
	0.77	Corn fiber	0.5% Sulfuric acid	Liu et al., 2013
	0.6	Corn stover	2% (v/v) Sulfuric acid	Cao et al., 2010
HMF	3.1	Forest residue	Dilute acid	Taherzadehet al., 2000b
	2.4	Birch	Dilute acid	Taherzadehet al., 2000b
	1.12	Pine	1% (w/v) Sulfuric acid	Gurram et al., 2011
	1.1	Corn stover	0.5% Sulfuric acid	Liu et al., 2013
	0.27	Rice straw	1% Nitric acid	Kim et al., 2014
	0.25	Corn stover	2% (v/v) Sulfuric acid	Cao et al., 2010
	0.21	Eucalyptus	0.65% Sulfuric acid	Carvalho et al., 2006
	0.12	Sugar maple	1.5% (wt) Sulfuric acid	Sun et al., 2012
	0.1145	Corn stover	1% (w/w) Sulfuric acid	Ruan et al., 2013
Formic acid	3.1	Spruce	-	Taherzadehet et al., 1997
	2.06	Wheat straw	1% (w/w) NaOH	Toquero et al., 2014
	0.69	Sugar maple	1.5% (wt) Sulfuric acid	Sun et al., 2012
	0.24	Peanut shells	2% Sulfuric acid	Martin et al., 2007
	0.2	Bagasse	2% Sulfuric acid	Martin et al., 2007
	0.16	Cassava stalks	2% Sulfuric acid	Martin et al., 2007
	0.15	Rice hulls	2% Sulfuric acid	Martin et al., 2007

Levulinic acid	2.6	Spruce	-	Taherzadehet et al., 1997
Syringaldehyde	0.107	Spruce	-	Taherzadehet et al., 1997
	0.06	Corn stover	2% (v/v) Sulfuric acid	Cao et al., 2010
	0.024	Wheat	-	Taherzadehet et al., 1997
vanillin	0.43	Willow	-	Taherzadehet et al., 1997
	0.12	Spruce	-	Taherzadehet et al., 1997
	0.04	Corn stover	2% (v/v) Sulfuric acid	Cao et al., 2010
	0.032	Wheat	-	Taherzadehet et al., 1997

*The whole pretreatment/hydrolysis process might be a combination of several methods. This table lists only the main methods during processing for each study.

Table 1.2 The effects of inhibitors on cell growth and/or fermentation

Inhibitor	Concentration (g/L)	Inhibition of cell growth	Product	Inhibition of fermentation	strain	Reference
Furfural	2	100%	Ethanol	99%	*Pichia stipitis*	Delgenes et al., 1996; Reborto et al., 1991
	2	100%	CO_2	100%	*Candida guilliermondii*	Sanchez et al., 1988
	1.5	-	Ethanol	90.4%	*Pichia stipitis*	Nigam, 2001
HMF	3	69%	Ethanol	90%	*Pichia stipitis*	Olsson et al., 1996
	2	61.7%	CO_2	0.06%	*Candida guilliermondii*	Sanchez et al., 1988
	0.59	-	Hydrogen	50%	*Seed sludge*	Kumar et al., 2014
Formic acid	10	80%	Ethanol	59%	*Escherichia coli* LY01	Zaldivar et al., 1999
	5.41	100%	Butanol	-	*Clostridium acetobutylicum*	Wang et al., 2015

	1.82	-	Butanol	100%	*Clostridium acetobutylicum*	Wang et al., 2015
	1	-	Total ABE	25%	*Clostridium acetobutylicum*	Cho et al., 2012
Levulinic acid	20	80%	Ethanol	86%	*Escherichia coli* LY01	Zaldivar et al., 1999
	1.55	-	Hydrogen	50%	*Seed sludge*	Kumar et al., 2014
Syringaldehyde	1.5	-	Hydrogen	78%	*sludge*	Siqueira et al., 2015
	1	51.4%	Hydrogen	54.8%	*Thermoanaerobacterium thermosaccharolyticum W16*	Cao et al., 2010
	1	3%	Butanol	26%	*Clostridium beijerinckii*	Cho et al., 2009
	0.375	100%	Ethanol	100%	*Saccharomyces cerevisiae*	Zaldivar et al., 1999b
vanillin	2	100%	Xylitol	-	*Candida guilliermondii*	Kelly et al., 2008
	1	22%	Butanol	100%	*Clostridium beijerinckii*	Cho et al., 2009
	1.83	100%	Lipid	100%	*Rhodosporidium toruloides*	Hu et al., 2009
	1	-	Hydrogen	64%	*sludge*	Siqueira et al., 2015

1.5.3 Phenolic compounds

Phenolic compounds include vanillin, syringaldehyde, hydroxybenzaldehyde, ferulic acid, and so on. In this research, we focus on vanillin and syringaldehyde. These two compounds come from partial depolymerization of lignin polymers (Barakat et al., 2012). In Table 1.1, the concentrations of phenolic compounds are below 1 g/L in hydrolysates. In Table 1.2, the concentrations of phenolic compounds that might cause negative effects on cell growth and fermentation are about 1-2 g/L.

Phenolic compounds have considerable inhibitory effects on the fermentation process even at low concentrations (usually less than 2 g/L) in the culture, as shown in Table 1.2. In addition, those phenolic compounds with low molecular weight are the most toxic (Mussatto et al., 2004; Parajó et al., 1998). Phenolic compounds can cause partition and loss of integrity of biological membranes, thereby affecting the membranes' ability to serve as selective barriers and enzyme matrices (Palmqvist et al., 2000). However, Zaldivar et al. show that damaged membrane-integrity by aldehydes-targeting was not the main mechanism for *E. coli* LY01 (Zaldivar et al., 1999b). According to the research of Fitzgerald et al., (1997), although vanillin had interactions with membrane-related proteins that lead to damaged membrane-integrity, a large number of cells still had functional membranes. Syringaldehyde and vanillin at 1 g/L were more toxic on butanol production than on the cell growth of *C. beijerinckii* (Cho et al., 2009). The authors presumed that the low butanol-production was caused by the phenolic-compound interference with the pathway from acetyl-CoA to butyryl-CoA (Cho et al., 2009). According to Ezeji et al., 1 g/L of syringaldehyde had little effect on cell growth, whereas as little as 0.3 g/L of

syringaldehyde sharply inhibited ABE production (Ezeji et al., 2006). The authors suggested that syringaldehyde was able to interfere with the glycolytic pathway and to affect the alcohol-dehydrogenase enzyme secretion (Ezeji et al., 2006). During the fermentation of *C. guilliermondii*, vanillin was more toxic than syringaldehyde at 1 g/L. Syringaldehyde and vanillin could be assimilated by several strains. For example, 1.5 g/L of syringaldehyde and 2 g/L of vanillin were consumed by *S. cerevisiae, C. shehatae,* and *P. stipites* in the research of Delgenes et al., (1996).

1.5.4 Comparison of inhibitors

According to Kelly et al., the effects of furfural, vanillin, and syringaldehyde on the growth of *Candida guilliermondii* and xylitol accumulation were compared (Kelly et al., 2008). The results showed that vanillin at 1 g/L was the compound that was most-toxic to cell growth at 1 g/L, followed by syringaldehyde and furfural (Kelly et al., 2008). The hydrophobicity order of aldehydes is vanillin (1.21), syringaldehyde (0.99), furfural (0.41) and HMF (-0.37) (Zaldivar et al., 1999b). Hu et al. stated that the more hydrophobilic the aldehyde is, the stronger the inhibitory effects are (Hu et al., 2009). Their experiment results agreed with the conclusion that the inhibitory effect order was PHB, vanillin, and syringaldehyde (Hu et al., 2009). The same conclusion was reported by Zaldivar et al. that the inhibitory order on the growth of *E. coli* was vanillin, syringaldehyde, furfural, and then HMF (Zaldivar et al., 1999b). However, the inhibitory effects of organic acid do not depend on their hydrophobicity. Based on the research of Zaldivar et al., formic acid was more toxic than levulinic acid whereas the

hydrophobicity of formic acid (-0.54) is lower than that of levulinic acid (-0.49) (Zaldivar et al., 1999a).

1.5.5 Detoxification methods

There are several methods, including adsorption, vacuum membrane distillation, electrodialysis, and nanofiltration, for removing or reducing the inhibitors from hydrolysates (Yi et al., 2015). In addition to these methods, changing the fermentation conditions also causes differences in inhibitory effects on cell growth. Zaldivar et al. reported that increasing inoculum size reduced the toxicity of furfural and vanillin (Zaldivar et al., 1999b). Increasing temperature and pH value in the fermentation medium also decreased the toxicity of aromatic aldehydes and HMF (Zaldivar et al., 1999b).

1.6 Objectives

In this research, we aimed to study the inhibitory effects of six potential inhibitors (furfural, HMF, formic acid, levulinic acid, syringaldehyde, and vanillin) on cell performance in butyric-acid fermentation by *C. tyrobutyricum*. If the concentrations of these inhibitors in hydrolysates are not great enough to be harmful to cell activities, then it is unnecessary to apply the detoxification methods to remove the inhibitors. The specific objectives are listed below:

1. Understand the effects of furan derivatives (furfural and HMF) on cell growth, yield, and productivity of butyric acid by *C. tyrobutyricum*.
2. Investigate the inhibitory effects of organic acids (levulinic acid and formic acid) on cell growth, yield, and productivity of butyric acid by *C. tyrobutyricum*.

3. Understand the potential effects of phenolic compounds (syringaldehyde and vanillin) on cell growth, yield, and productivity of butyric acid by *C. tyrobutyricum*.

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CHAPTER 2 The Inhibitory Effects of Furan Derivatives on *C. Tyrobutyricum* During Butyric-acid Fermentation

Abstract The potential inhibitory effects of two furan derivatives, furfural and hydroxymethylfurfural (HMF) on *Clostridium tyrobutyricum* were studied in this chapter. Various concentrations (0, 0.3, 0.6, 0.9 and 1.2 g/L) of furfural and (0, 0.6, 1.2, 1.8 and 2.4 g/L) of HMF were added into the growth medium separately. The optical density of cells was detected by a microplate reader at 620 nm every 12 hours. The concentrations of xylose, butyric acid, and inhibitors were tested by HPLC every 12 hours. The pH value was adjusted to 6.4 by adding NH_4OH or HCl. The results show that 0.3 g/L of furfural had no significant effects on cell activities, whereas the 0.6 and 0.9 g/L of furfural prolonged the lag phases of cell growth and metabolism. While 1.2 g/L of furfural completely inhibited cell activities. For HMF, 0.6 g/L had no effect on cell replication or production of butyric acid. With increasing HMF concentrations, the lag phase of cell growth and butyric acid production were prolonged and maximum cell concentration, yield, and productivity of butyric acid were decreased. Furfural and HMF were both metabolized by *C. tyrobutyricum* during fermentation, although the metabolites were unknown.

2.1 Introduction

Furfural and HMF, derived from furan, are considered to have high potentials for producing chemicals and fuels (van Putten et al., 2013). Furfural ($C_5H_4O_2$), known as 2-furaldehyde or furfuraldehyde, is a colorless to amber-colored liquid. It is easy for furfural to synthesize with other chemicals due to furfural's dominant groups (CHO and C=C=C=C) (Morone et al.,

2015). Furfural is widely applied in multiple areas, such as a selective extraction agent, a solvent, and a flavoring agent in food and beverage products. Also, furfural has been utilized to manufacture pharmaceuticals, cosmetics, fragrances and flavors (Peleteiro et al., 2016). Hydroxymethylfurfural (HMF, $C_6H_6O_3$), also known as 5-(hydroxymethyl)-2-furaldehyde, is a yellow-colored solid (Morone et al., 2015). A number of important furanic compounds (alkoxymethylfurfurals, 5-hydroxymethylfuroic acid, and so on) and non-furanic compounds (levulnic acid, adipic acid, caprolactam, and so on) can be formed from HMF (van Putten et al., 2013) due to its aldehyde and alcohol functional groups (Morone et al., 2015). HMF is also considered a versatile chemical intermediate for the synthesis of plastics, pharmaceuticals, fine chemicals, and liquid fuel (Zhang et al., 2015).

Furfural and HMF are two important potential inhibitors that exist in the lignocellulosic biomass hydrolysate. During lignocellulosic biomass pretreatment in acidic conditions, the inhibitors are usually produced from the sugar degradation under high temperature and pressure (Mills et al., 2009). Furfural is generated from pentose (xylose, etc.), whereas HMF is formed from hexose (glucose, etc.).

Furfural is treated as a key inhibitor because of furfural's high toxicity and synergistic effects with other inhibitors (Zaldivar et al., 1999). Furfural is known to interfere with the activities of crucial enzymes, such as alcohol dehydrogenase, pyruvate dehydrogenase, and aldehyde dehydrogenase, and so on (Ravindran et al., 2016; Banerjee et al., 1981b, Sanchez et al., 1988).

Therefore, furfural can cause growth inhibition and lag-phase prolongation by impacting these enzymes. It was reported that furfural can affect the growth and metabolism of several microorganisms, such as *Candida tropicalis* and *Saccaromyces cerevisiae* (Sanchez et al., 1988). For *Candida tropicalis*, furfural was found to strongly inhibit protein and RNA synthesis (Banerjee et al., 1981a). For yeast, furfural generally impacts energy redirection, which is used to fix the damage that is caused by furfural and by reduced intracellular ATP and NAD(P)H levels (Almeida et al., 2007). Furfural also causes membrane leakage of *E. coli* (Hu et al., 2009; Almeida et al., 2007). The inhibitory mechanism of HMF is similar to furfural (Banerjee et al., 1981b), but HMF is less toxic than furfural at the same concentration (Almeida et al., 2007). The severity of inhibitory effects also depends on the furan concentration and the types of strain that is used (Almeida et al., 2007).

Some researchers also reported that furfural and HMF can be consumed by cells. *For S. cerevisiae*, the major metabolites for furfural and HMF were furfuryl alcohol and 2,5-bis-hydroxymethylfuran (the analog of furfuryl alcohol), respectively (Pienkos et al., 2009). Furfuryl alcohol was proven toxic to *E. coli* cells by causing significant membrane leakage as well (Zaldivar et al., 2000).

Furfural and HMF were studied as potential inhibitors for a very long time. However, their inhibitory effects on butyric-acid fermentation by *C. tyrobutyricum* were not fully investigated.

In this chapter, the effects of furfural and HMF were studied on fermentation process, separately.

2.2 Materials and methods

2.2.1 Strain and cultivation

Clostridium tyrobutyricum ATCC 25755 was purchased from American Type Culture Collection (ATCC) and maintained on an agar plate (ATCC medium 2107: Modified Reinforced Clostridial Agar Medium) at 4 °C anaerobically. *C. tyrobutyricum* is an obligate anaerobe (Jiang et al., 2010), so that it cannot survive with the existence of oxygen. Hence, the condition must be strictly anaerobic for cells to grow. The pre-cultured seed was activated by inoculation into a 300 ml growth medium in a 500 ml flask. The growth medium contained 30 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 3 g/L $(NH_4)_2SO_4$, 1.5 g/L K_2HPO_4, 0.6 g/L $MgSO_4 \cdot 7H_2O$, 0.03 g/L $FeSO_4 \cdot 7H_2O$ (Jiang et al., 2010), and distilled water. The pH value of the medium was adjusted to 6.4 by adding NH_4OH or HCl. Subsequently, the medium was sterilized in an autoclave at 121 °C for 15 minutes. After inoculation, the pre-cultured flask was kept at 37 °C for approximately two days to the exponential phase of the cells. Afterward, 20 ml of the bacteria in pre-cultured flask was inoculated into a 180 ml xylose growth-medium. Because glucose is the preferred substrate for *Clostridial* strains (Ezeji et al., 2007), a glucose medium was used for pre-culture in experiments, so that bacteria went into a log phase faster than using xylose medium. The seed could then be transferred into a xylose medium because xylose is released in hydrolysate more than is glucose (Mohan et al., 2006). The xylose growth

medium included 30 g/L of xylose to replace glucose, and the other components in the growth medium were as described above. Furfural, at 0, 0.3, 0.6, 0.9 and 1.2 g/L, and HMF, at 0, 0.6, 1.2, 1.8 and 2.4 g/L, were added into the medium separately. The concentrations of furfural and HMF were chosen based on Table 1.1 and 1.2. In Table 1.1, the concentration of furans in hydrolysates was below 3.5 g/L, and about 2 g/L of furans caused almost 100% of cell death (Table 1.2). Therefore, we chose a median concentration, 2.5 g/L, to be the maximum concentration. Since the experiment for each inhibitor was designed for testing at five concentration gradients, including control, the maximum cell concentration was altered from 2.5 g/L to 2.4 g/L. The five concentrations of furans to be studied were 0, 0.6, 1.2, 1.8, and 2.4 g/L. In the preliminary experiment, furfural completely inhibited cell growth when the concentration of furfural was higher than 1.2 g/L. Thus, the concentrations of furfural were changed to 0, 0.3, 0.6, 0.9, and 1.2 g/L. The concentrations of HMF were not necessary to be changed.

Each experiment was conducted in triplicate. The cultivation was performed in Forma Anaerobic System 1025 from Thermo Scientific (Grand Island, N.Y., U.S.) with a stable temperature at 37 °C and obligated anaerobic conditions. After substrates were used up (cells were in declined phase), data collection was ceased. The fermentation was performed in batch mode in order to avoid microorganisms' adaption to the inhibitors, which would cause influences on both growth and production rate (Panagiotopoulos et al., 2011).

2.2.2 Analytical methods

The concentration of cells and organic compounds were measured every 12 hours. The cell growth was analyzed by measuring the optical density of the cells by a microplate reader at a wavelength of 620 nm (OD_{620}). One unit of OD_{620} corresponded to 0.797 g/L of cell dry weight for cells grown in the xylose medium (Zhu et al., 2004). The concentrations of glucose, xylose, butyric acid, furfural and HMF were measured by a High Performance Liquid Chromatography (HPLC) instrument that was purchased from Shimadazu (Kyoto, Japan). The column that was used in HPLC was an Aminex HPX-87H, 300*7.8 mm column (Bio-Rad). The mobile phase was 0.005 N of sulfuric acid and the flow rate was 0.6 mL/min. The temperature for the column was set at 35 ˚C. A refractive index detector was used for all chemicals, except that furfural and HMF were measured by a UV detector (at 254 nm). The pH value was tested by an AP85 portable waterproof pH/conductivity meter that was provided by Fisher Scientific (Pittsburgh, P.A., U.S.). The pH was adjusted by NH_4OH or HCl every 12 hours.

2.2.3 Statistical analysis

After collecting data, multiple one-way analysis of variance (ANOVA) were performed using SAS software to test for significant cell-growth and metabolism differences between different concentrations of each inhibitor. The optical density of cells, xylose consumption, yield, and productivity of butyric acid were designated as response variables. Tukey's method was used to calculate pairwise differences at an alpha=0.05 level (Zhang et al., 2012). In the graphs below, bars that have the same letter are not significantly different from each other.

2.2.4 Calculation equations for butyric-acid yield and productivity

Industries are pursuing the maximum yield and productivity of product. Yield and productivity of butyric acid were two significant factors to be determined.

Yield refers to the mass of product per gram of carbon source at final harvest. Butyric-acid yield was calculated via Equation 2.1.

$$\text{Yield} = (BA_f - BA_0)/(XY_0 - XY_f) \quad (2.1)$$

BA_f is the final concentration of butyric acid. BA_0 is the initial concentration of butyric acid. XY_0 represents the initial concentration of xylose and XY_f is the final concentration of xylose.

Productivity is the overall rate of producing product during fermentation. Equation 2.2 is used to compute butyric-acid productivity.

$$\text{Productivity} = (BA_t - BA_0)/t \quad (2.2)$$

BA_t is the maximum concentration of butyric acid at hour t. BA_0 is the initial concentration of butyric acid. t represents the time when butyric acid achieved its maximum concentration.

2.2.5 Modeling the relationship between optical density and its corresponding butyric-acid concentration

In this paper, we employed figures and graphs to represent the relationship between optical density and its corresponding concentration of butyric acid. Optical density is the variable in the horizontal axis, while the concentration of butyric acid is the response in the vertical axis. After plotting the results of the control treatment, we found that the data points approximated an exponential distribution. Therefore, we computed a natural-log transformation of butyric-acid concentration. We used SAS to fit a linear regression to model the transformation as a

function of optical density. We used SAS to obtain the intercept, slope, and R-square of the linear regression. And then we exponentiated the parameter estimates to obtain a model in the un-transformed scale.

2.3 Results and discussion

2.3.1 The effect of furfural on the activities of C. tyrobutyricum

2.3.1.1 Cell growth

Cell growth of *C. tyrobutyricum* in the presence of furfural (0-1.2 g/L) is shown in Fig. 2.1. A low concentration of furfural (0.3 g/L) did not affect the cell-growth curve and there was no significant difference between 0.3 g/L of furfural and control (0.0 g/L) on maximum cell concentration (shown in Fig. 2.2). For the medium that was treated by 0.6 and 0.9 g/L of furfural, the lag phase was 12h longer than control (from 24h to 36h). This delay was probably caused by the assimilation or degradation of furfural (Parajó et al., 1998). In addition, the action of furfural on several key glycolytic enzymes such as triose-phosphate dehydrogenase and alcohol-dehydrogenase also might be the reason for the longer lag phase (Sanchez et al., 1988). Except for the extended lag phase, the maximum cell concentration was not influenced by 0.6 and 0.9 g/L of furfural (shown in Fig. 2.2). When cell growth recovered from furfural, furfural was converted into its corresponding metabolites. The accumulation of these metabolites was not toxic enough to inhibit the cell growth (Liu et al., 2004). Similarly, the most significant effect of 0.5-2 g/L of furfural on *D. hansenii* growth was an increase in the lag phase (Duarte et al., 2005). 1.2 g/L of furfural was lethal to *C. tyrobutyricum* so that no cell growth was observed. Except for key enzymes, furfural might also cause membrane leakage (Almeida et

al., 2007; Hu et al., 2009) and damage on the synthesis of RNA and protein (Banerjee et al., 1981a).

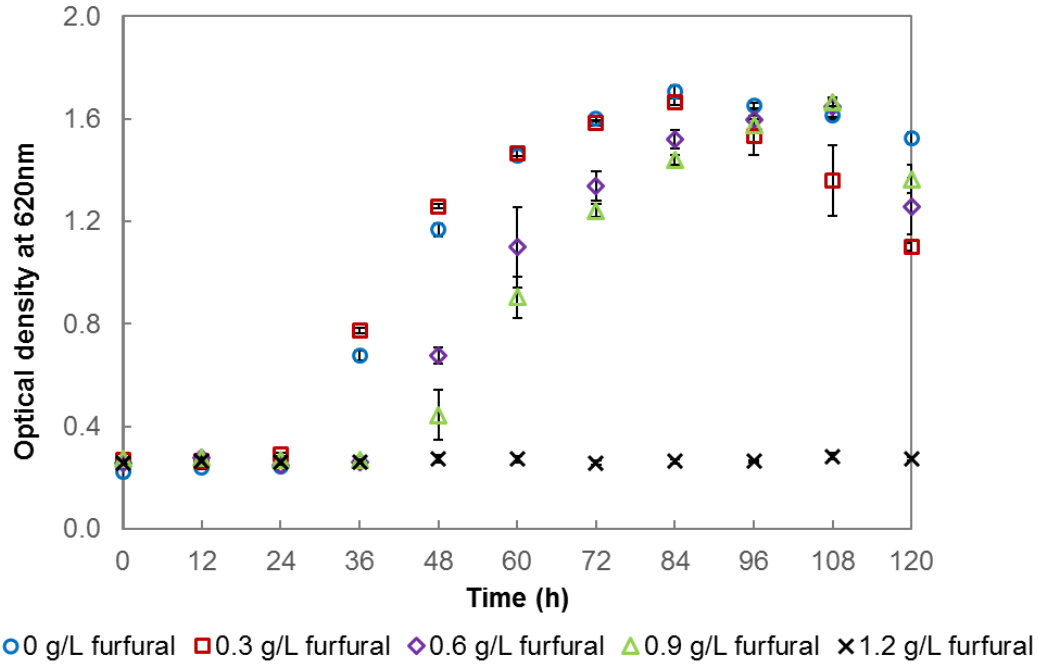

Figure 2.1 The cell growth curve of *C. tyrobutyricum* at five concentrations of furfural

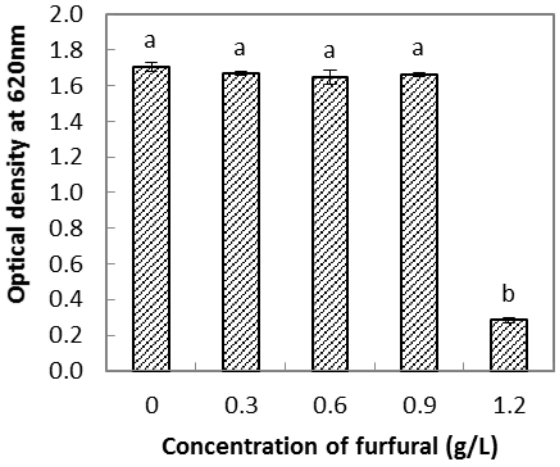

Figure 2.2 The maximum cell concentration of *C. tyrobutyricm* at each concentration of furfural

2.3.1.2 Cell metabolism

Three characteristics of *C. tyrobutyricum* cell-metabolism were investigated: inhibitors metabolization, xylose consumption, and butyric-acid production.

In a previous study, furfural was metabolized by microorganisms into less-inhibitory metabolites when the initial concentration of furfural was within a threshold inhibitory-level (Boyer et al., 1992; Pienkos et al., 2009; Sathyanarayanam et al., 2013). It was reported that during anaerobic growth, furfural could be metabolized into furfuryl alcohol by *S. cerevisiae* with a sacrifice of NADH (Sanchez et al., 1988; Hu et al., 2009). Also, during an experiment performed by Veeravalli et al., (2013), furfural degradation products, furoic acid and furfuryl alcohol, were detected in fermentation broth. This experiment aimed at producing hydrogen by the species of *Clostridium sp.* and *Flavobacterium sp*. In our experiment, 0.3, 0.6 and 0.9 g/L of furfural were convertible by *C. tyrobutyricum*, whereas 1.2 g/L of furfural was beyond the threshold level for consumption in this experiment (Fig. 2.3). Furfural was probably reduced into furfuryl alcohol although the metabolites from furfural were not specified.

The metabolism of furfural can supply some energy for the growth of microorganisms (Sanchez et al., 1988). This statement could explain the phenomenon that in our experiment, when furfural was decomposing, cells began to duplicate. Duplication (36h before cells started to consume furfural and replicate) was especially obvious when the concentration of furfural was at 0.6 and 0.9 g/L.

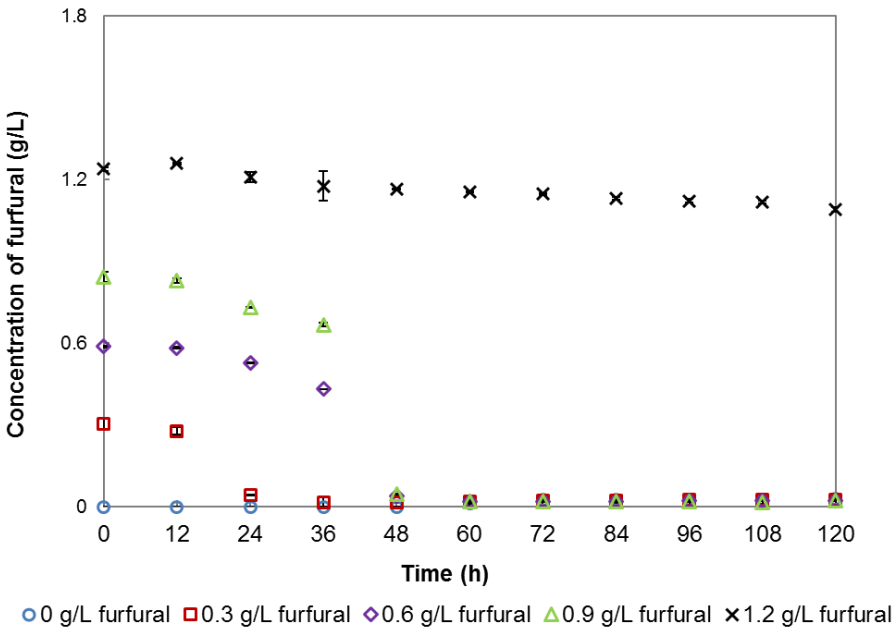

Figure 2.3 The concentration of furfural over fermentation period

Fig. 2.4 and 2.5 indicate the xylose consumption and butyric acid production by *C. tyrobutyricum* treated by five concentrations of furfural. 0.3 g/L of furfural did not affect cell growth. As with cell growth, the lag phase for xylose consumption and butyric production was prolonged 12h by 0.6 and 0.9 g/L of furfural. The butyric-acid yield in the medium that was treated by 0.3, 0.6, and 0.9 g/L of furfural was not significantly different (Fig. 2.6 a)). The accumulation of furfural-associated metabolite in the medium appeared to not inhibit final butyric-acid yield (Liu et al., 2004). However, in the medium that treated by 0.6 and 0.9 g/L of furfural, the productivity of butyric acid decreased due to the longer lag phase (shown in Fig. 2.6 b)). In the medium that was treated by 1.2 g/L of furfural, no butyric acid was produced because cells were completely inhibited.

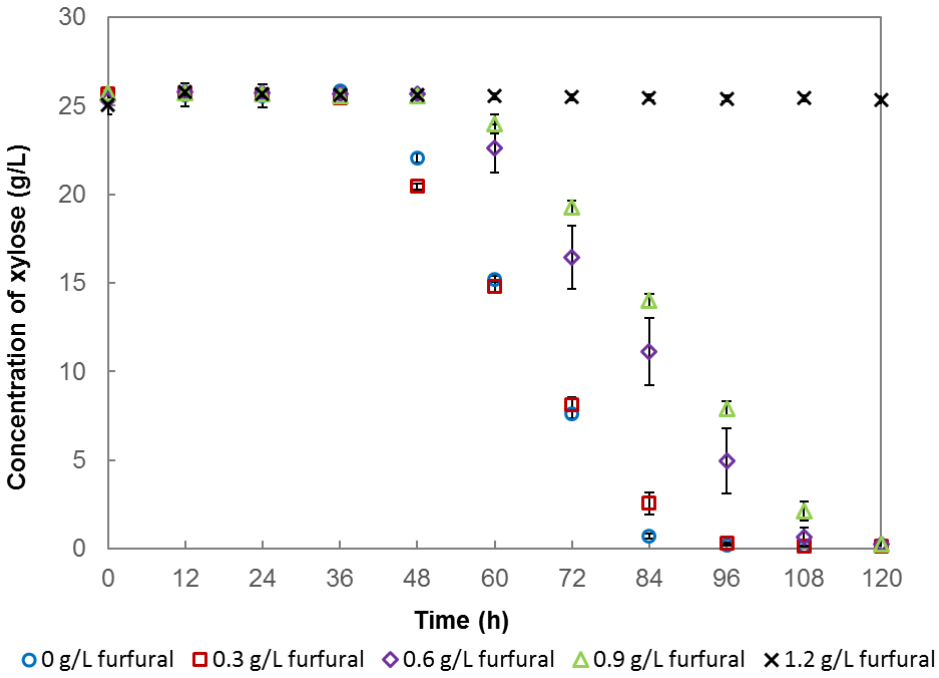

Figure 2.4 The consumption of xylose by *C. tyrobutyricum* during fermentation at each concentration of furfural

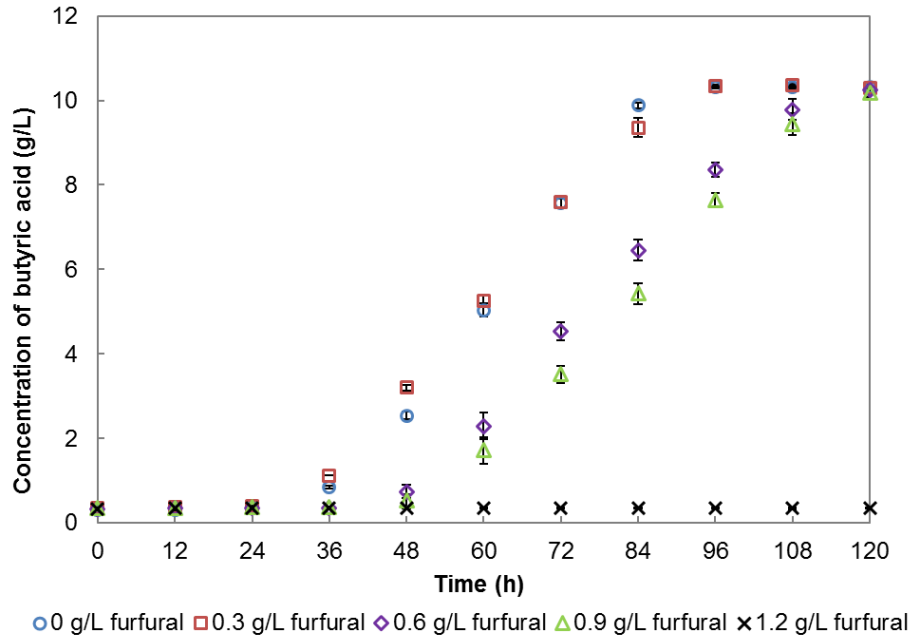

Figure 2.5 The production of butyric acid by *C. tyrobutyricum* at each concentration of furfural

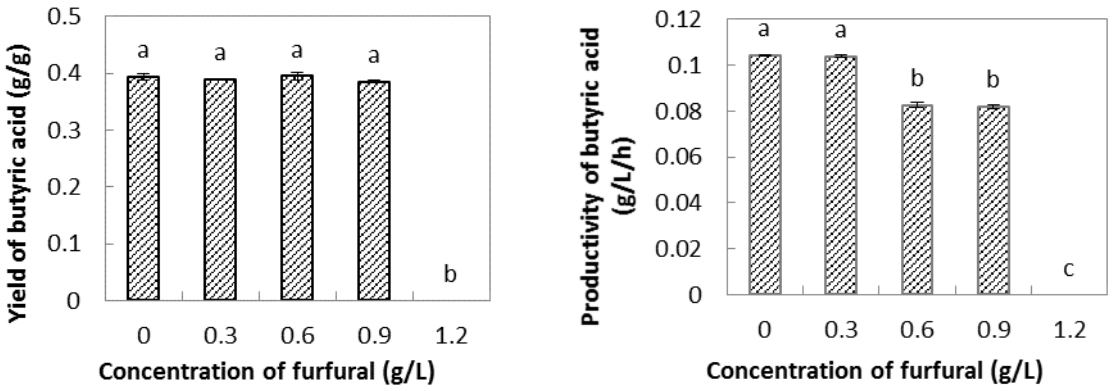

Figure 2.6 a) The yield of butyric acid of *C. tyrobutyricum* at each concentration of furfural; b) The productivity of butyric acid by *C. tyrobutyricum* at each concentration of furfural

2.3.1.3 Relationship between optical density and its corresponding butyric-acid concentration, treated by furfural

Fig. 2.7 displays a scatter plot that shows a relationship between optical density and its corresponding butyric-acid concentration in furfural groups 0, 0.3, 0.6, and 0.9 g/L. This scatter plot suggests an increasing exponential relationship. Therefore, we modeled a natural-log transformation of the response and obtained a linear regression with a high R-square (R^2=0.994), which indicated a good fit of the data. The equation of the linear regression is y=2.383x-1.698. In the original scale, the equation is $y=0.183e^{2.383x}$.

The exponential regression shows that when optical density increased, more butyric acid was produced per OD unit. There are several possible reasons for the exponential relationship between optical density and butyric-acid concentration. First, typically speaking, larger amounts of biomass could generate more butyric acid. Second, the end product for *C. tyrobutyricum*, butyric acid, is toxic to the cells of this strain. Butyric acid is produced in cells.

Dissociated butyric acid and undissociated butyric acid both exist in cells. The anion and undissociated butyric acid are transported out of cells by carrier protein, with no cost of energy. Nevertheless, the transportation of protons from intracellular to extracellular requires the hydrolysis of ATP to provide energy. In order to maintain the intracellular pH value, extra ATP must be generated (Sanda et al., 2011), which further boosts the butyric-acid production. Third, since part of ATP is utilized to transport protons, less ATP is available for cell formation. However, the live cells are still metabolically active (Waites et al., 2009). The rest of the carbon source in the medium is utilized to produce butyric acid. Fourth, as described in the introduction, extra ATP is required for transporting xylose across the cell membrane (Jiang et al., 2010). Thus, more butyric acid is produced.

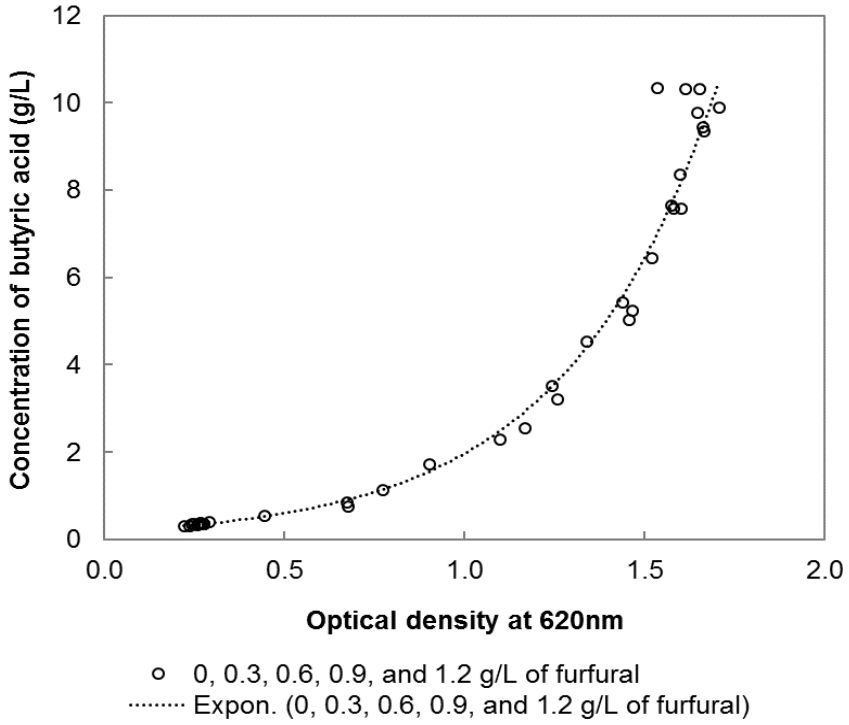

Figure 2.7 The relationship between optical density and its corresponding butyric-acid concentration in the furfural groups 0, 0.3, 0.6, and 0.9 g/L

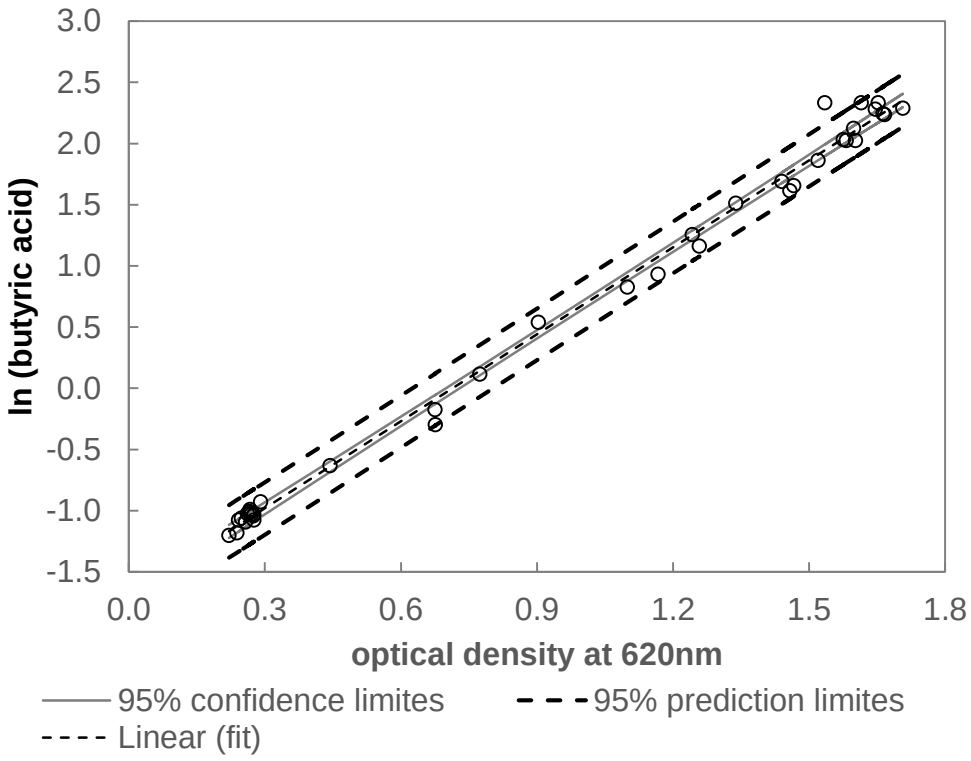

Figure 2.8 The relationship between optical density at 620nm and its corresponding butyric-acid concentration (after natural log response)

2.3.2 The effect of HMF on the cell growth and metabolism of C. tyrobutyricum

2.3.2.1 Cell growth

Fig. 2.9 shows how cell growth responded to five concentrations of HMF. Analyzing from this figure, 0.6 g/L of HMF had no obvious effect on *C. tyrobutyricum* cell growth. The ANOVA analysis that is shown in Fig. 2.10 also suggests that there is no significant difference in cell growth between 0 and 0.6 g/L of HMF. With the increase of HMF concentration from 1.2 g/L to 2.4 g/L, the lag phase of cell growth increased from 48h to 84h (Fig. 2.9). The medium with 1.2 g/L of HMF still achieved the same maximum cell concentration as the control, but with a longer log phase (Fig. 2.10). With 1.8 and 2.4 g/L of HMF, the maximum cell concentration decreased (Fig. 2.10). 2.4 g/L of HMF altered the shape of the growth curve to an irregular

pattern, which indicated that the HMF effect was complicated and unpredictable. Similar to furfural, HMF had inhibitory effects on glucolytic key enzymes, which further caused growth inhibition and a prolonged lag phase (Banerjee et al., 1981b; Sanchez et al., 1988).

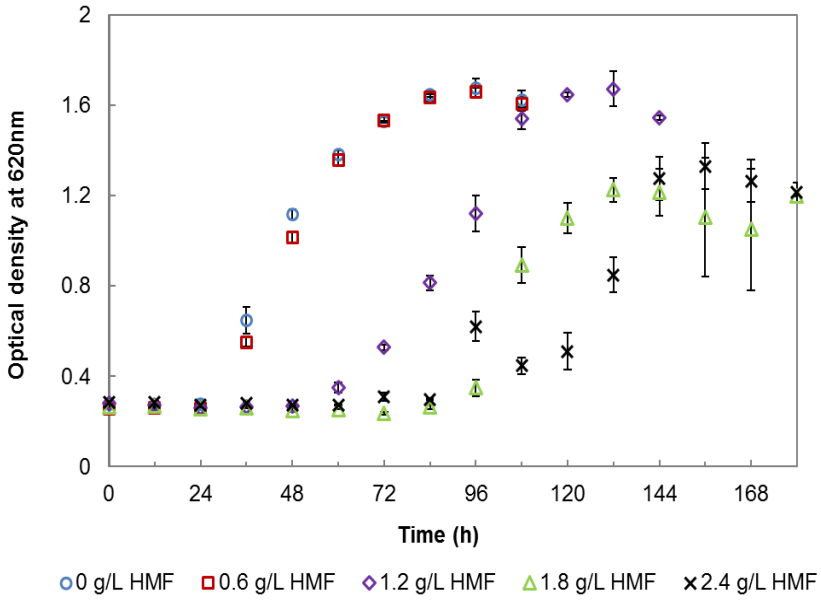

Figure 2.9 The cell growth curve of *C. tyrobutyricum* during fermentation at five concentrations of HMF

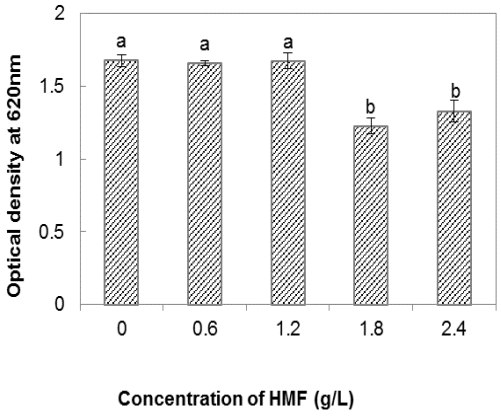

Figure 2.10 The maximum cell concentration of *C. tyrobutyricum* at five concentrations of HMF

2.3.2.2 Cell metabolism

HMF at all concentrations that were added into the fermentation can be consumed by cells, and the HMF concentrations started to drop from 24h, 48h, 84h, and 84h respectively for 0.6, 1.2, 1.8 and 2.4 g/L of HMF (Fig. 2.11). After cells adapted to HMF and started to degrade it, cells began to grow (Figure 2.9). Xylose utilization and butyric-acid production in the presence of HMF did not proceed until nearly all the HMF was converted. This finding was in accordance with the results that were obtained by Liu et al., using *Pichia stipitis* NRRL Y-7124, *S. cerevisiae* NRRL Y-12632, and *S. cerevisiae* ATCC 211239 (Liu et al., 2004; Pienkos et al., 2009). It was postulated that similar to furfural, HMF was metabolized by yeast into the corresponding alcohol, 5-hydroxymethyl furfuryl alcohol, which is a less inhibitory intermediate (Boopathy et al., 1993; Veeravalli et al., 2013). However, Liu et al. investigated that 2,5-bis hydroxymethylfuran, instead of 5-hydroxymethyl furfuryl alcohol, was the product that is converted from HMF by cells (Liu et al., 2004). In their study, the accumulation of 2,5-bishydroxymethylfuran had no influence on cell growth or the final ethanol production (Liu et al., 2004). But in our experiment, both cell growth and metabolism were affected even after HMF being metabolized. A possible reason could be that the target products (ethanol and butyric acid) of these two experiments were different, so that HMF converted into different chemicals. Another reason might be that *C. tyrobutyricum* was more sensitive to 2,5-bis hydroxymethylfuran than was yeast.

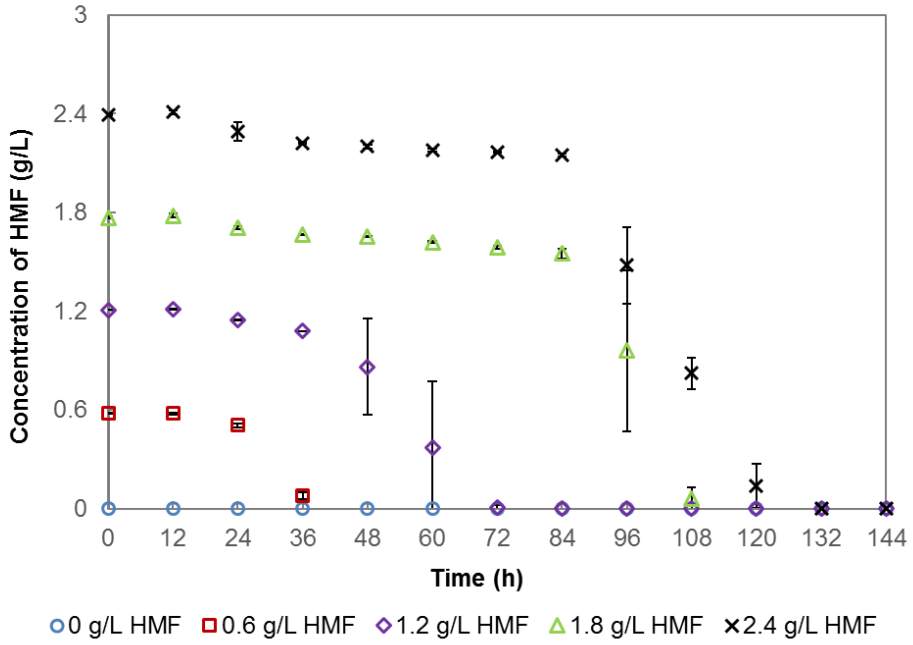

Figure 2.11 The concentration of HMF during fermentation period

With the increasing HMF concentration, a longer lag phase for xylose consumption and butyric acid production were observed from Fig.2.12 and 2.13. There was no significant difference between control and 0.6 g/L of HMF on butyric acid yield and productivity, suggesting that 0.6 g/L of HMF had no effect on cell metabolism (Fig. 2.14a and Fig. 2.14b). Up to 14.7 mM (1.85 g/L) of HMF had little inhibitory effect on lipid production during the fermentation of oleaginous yeast *Rhodosporidium toruloides* (Hu et al., 2009). Our experiment proves that in a low concentration range of HMF (0.6 g/L), cell activities were not affected. Although the butyric acid yield for the group with 1.2 g/L of HMF was similar to the control, it took a longer time to achieve the maximum concentration of butyric acid. Thus, the butyric acid productivity was affected. In the medium with 1.8 and 2.4 g/L of HMF, the xylose was consumed in both groups, but butyric acid was not accumulated. *S. cerevisiae* Tembec T-1 and *S. cerevisiae* Y-

1528 that was treated by 3 and 4 g/L of HMF showed decreased growth rate, ethanol yield, and productivity (Keating et al., 2006), which agreed with the results obtained above.

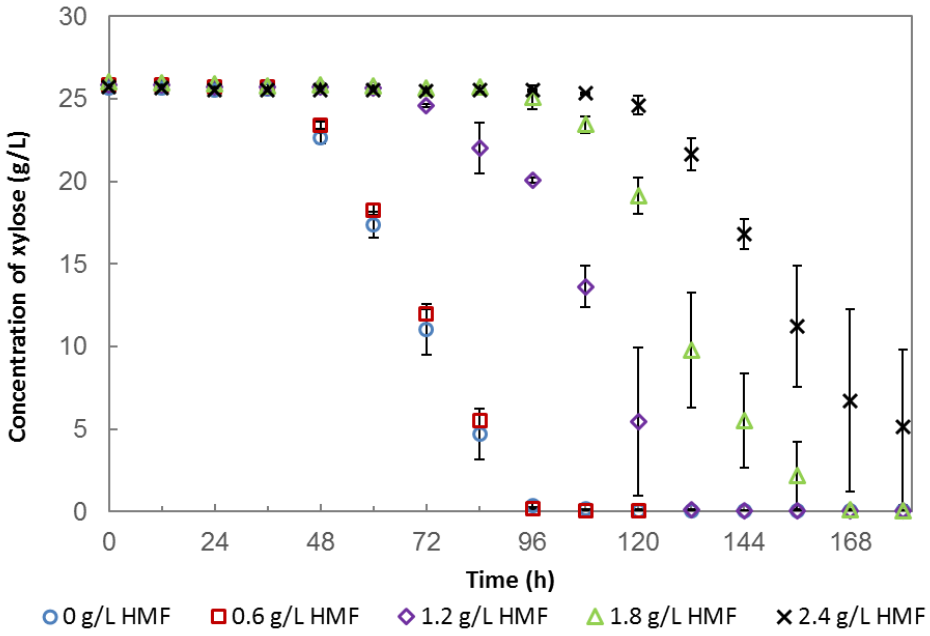

Figure 2.12 The consumption of xylose by *C. tyrobutyricum* during fermentation at five concentrations of HMF

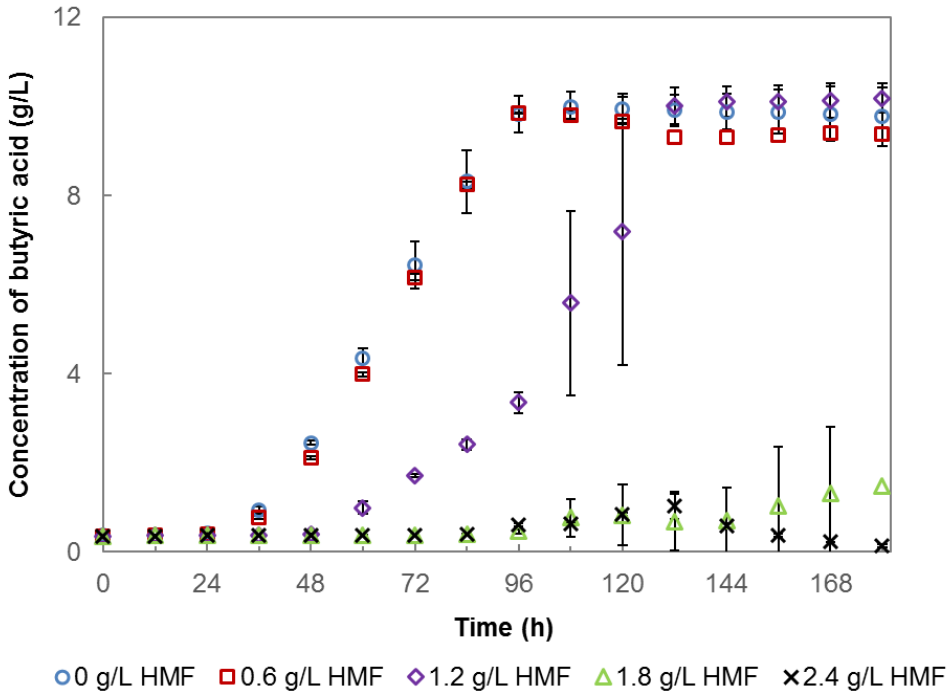

Figure 2.13 The production of butyric acid by *C. tyrobutyricum* at five concentrations of HMF

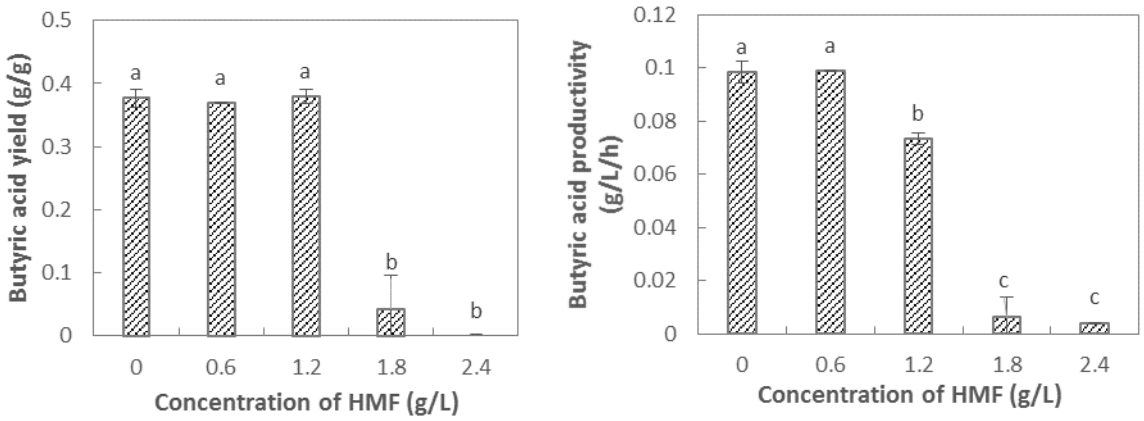

Figure 2.14 a) The yield of butyric acid by *C. tyrobutyricum* at five concentrations of HMF; b) The productivity of butyric acid by *C. tyrobutyricum* at five concentrations of HMF

2.3.2.3 Relationship between optical density and its corresponding butyric-acid fermentation treated by HMF

Figure 2.15 is a scatter plot that shows a relationship between optical density and its corresponding butyric-acid concentration in the HMF groups, 0, 0.6, 1.2, 1.8, and 2.4 g/L. The scatter plot suggests that the data in HMF groups of 0, 0.6, and 1.2 g/L followed exponential distribution. The exponential regression shows that with the optical density increased, more butyric acid was produced per OD unit. In Fig. 2.16, the R^2 of the linear regression is high for these groups (R^2=0.967), which indicates the regression fits the data well. The equation for the linear regression is y=2.186x-1.418. Thus, the equation for the untransformed response is $y=0.242e^{2.186x}$.

The data in the 1.8 and 2.4 g/L HMF groups shows no significant relationship. One explanation is that with 1.8 and 2.4 g/L of HMF, the optical density of cells was affected, but the butyric acid production was totally inhibited. Hence, although the optical density increased, the accumulation of butyric acid was not changed. Figure 2.15 also indicates that butyric-acid production is more sensitive to high concentrations of HMF than is cell growth.

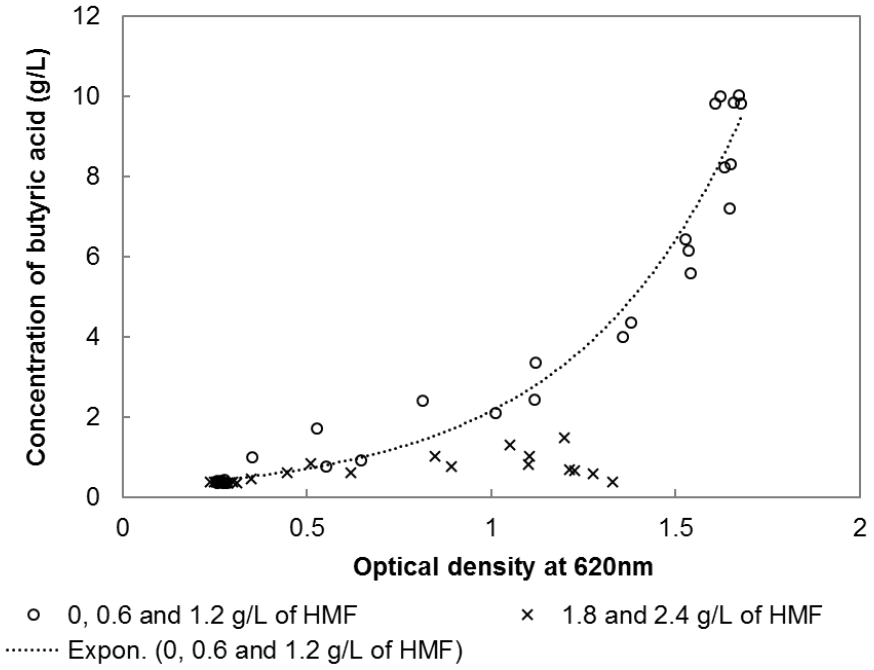

Figure 2.15 The relationship between optical density and its corresponding butyric-acid concentration treated by HMF

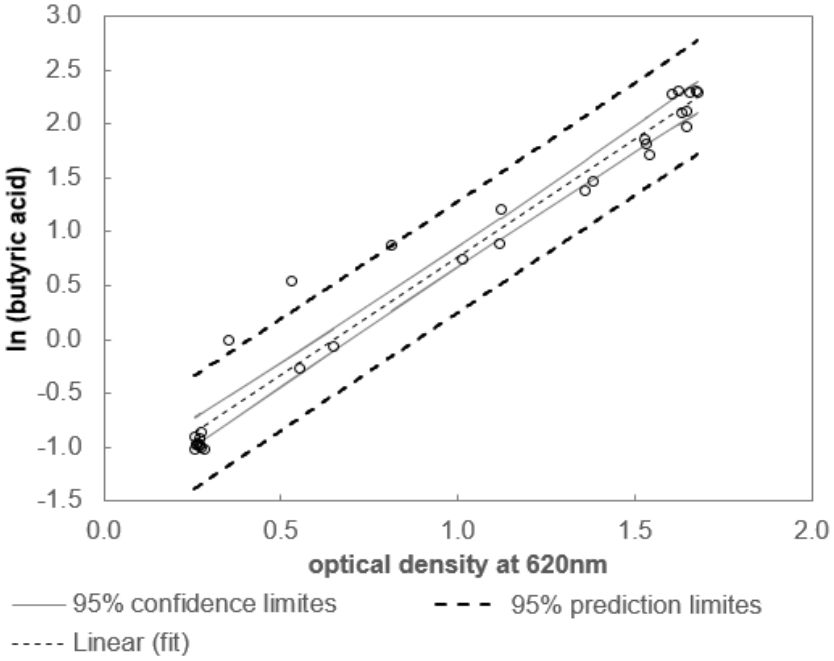

Figure 2.16 The relationship between optical density at 620nm
and its corresponding butyric-acid concentration treated by HMF (after natural log response)

2.3.3 Comparison between furfural and HMF

In the present experiments, although both furfural and HMF at the same concentration (0.6 g/L) were consumed, the rate for cells converting furfural was lower than that for converting HMF. Thus, the lag phase in furfural-added cultures was longer than that for HMF. A similar phenomenon was reported when 1 g/L furfural and HMF were added during *Clostridium beijerinckii* BA101 fermentation (Quemeneur et al., 2012). The longer lag phase was caused by furfaral's lower-than-HMF molecular weight. Thus, furfural went into cells at a higher diffusion rate, which caused cells to take more time to accommodate furfural than HMF (Quemeneur et al., 2012). But for *Candida guilliermondii* 1241, furfural was assimilated in the first 15 minutes, whereas it took 1 hour to consume HMF (Sanchez et al., 1988). The researchers provided the possibility that the enzymes for consuming furfural are constitutive enzymes, whereas those for HMF are not (Sanchez et al., 1988).

In the previous studies, furfural was shown to be a more efficient inhibitor than HMF in cell growth on ethanol-producing yeasts (Sakai et al., 2007). A similar conclusion was reached in this study: cell growth and metabolism on *Clostridium tyrobutyricum* was affected more severely by furfural than by HMF.

2.4 Conclusion

In this chapter, the potential inhibitory-effects of (0, 0.3, 0.6, 0.9 and 1.2 g/L) of furfural and (1, 0.6, 1.2, 1.8 and 2.4 g/L) of HMF on *Clostridium tyrobutyricum* during butyric-acid

fermentation were studied. According to the results and discussion above, some conclusions were drawn:

1. A low concentration of furfural (0.3 g/L) and HMF (0.6 g/L) had no significant influence on cell growth and butyric acid production.
2. With 0.6 and 0.9 g/L of furfural, the lag phases of cell replication, xylose consumption, and butyric-acid production were prolonged.
3. Cell growth was completely inhibited by 1.2 g/L of furfural.
4. The lag and log phase of cell growth and butyric acid production were prolonged with an HMF concentration increasing from 1.2 g/L to 2.4 g/L.
5. 1.2 g/L of HMF had no significant influence on maximum cell-concentration of butyric-acid yield.
6. 1.8 and 2.4 g/L of HMF inhibited butyric-acid production, but had relatively minor effects on cell density.

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CHAPTER 3 The Inhibitory Effects of Carboxylic Acids on *C. tyrobutyricum* During Butyric-acid Fermentation

Abstract Formic acid and levulinic acid act as potential inhibitors on cell activities during fermentation. In this chapter, their inhibitory effects on *C. tyrobutyricum* were studied. Formic acid, at the concentrations of 0, 1.2, 2.4, 3.6, 4.8 g/L, and levulinic acid, at the concentrations of 0, 2.4, 4.8 g/L, were separately added into the culture medium. The cell growth was estimated by optical density that was tested by a microplate reader at 620nm. The concentrations of xylose, butyric acid, and inhibitors were detected by HPLC. The results showed that within 4.8 g/L, the main inhibitory effect of formic acid was on the lag phase of cell growth, xylose consumption, and butyric-acid production. Increasing formic acid concentration decreased the maximum cell concentration to 93.3% (1.2 g/L), 93.6% (2.4 g/L), 88.8% (3.6 g/L), and 85.8% (4.8 g/L) of control , whereas the final butyric-acid yield was not affected. Levulinic acid of 2.4 and 4.8 g/L had no influence on cell growth or butyric-acid production at all. During the fermentation, formic acid and levulinic acid were not metabolized by cells.

3.1 Introduction

In this chapter, we conducted research in two carboxylic acids, formic acid and levulinic acid. Formic acid is a pungent, caustic, and flammable compound that has been widely applied in many areas, such as medicine, pesticide, organic synthesis, and the tannery industry. Particularly, formic acid was reported as an effective hydrogen carrier for hydrogen production (Lu et al., 2016). Levulinic acid ($C_5H_8O_3$) is a five-carbon short-chain fatty acid (Morone et

al., 2015). The ketone carbonyl group (C=O) and acidic carboxyl group (COOH) of levulinic acid make levulinic acid capable of reacting with various functional groups and forming derivatives (Morone et al., 2015). Levulinic acid and its derivatives are applied in different areas (Bozell et al., 2000; Long et al., 2015), such as chiral reagent, biologically active materials, polymers, adsorbents, batteries, and so on (Bozell et al., 2000).

Formic acid and levulinic acid are potential inhibitors commonly found in hydrolysates (Almeida et al., 2007). Generally, they are formed from the degradation of hydroxymethylfurfural (HMF) during the pretreatment process on lignocellulosic biomass. Furthermore, in acidic conditions with elevated temperature, formic acid also can be formed from furfural (Larsson et al., 1999). The amount of formic acid that is released in hydrolysates varies from 0.5 to 5 g/L due to different conditions of pretreatment (Park et al., 2012; Thomsen et al., 2009). The concentration of released levulinic acid is also 0.5 to 5 g/L, but usually lower than that of formic acid during the same pretreatment process (Mills et al., 2009; Park et al., 2012).

It was reported that formic acid and levulinic acid were toxic to many organisms (Kumar et al., 2014), primarily on cell formation (Zaldivar et al., 1999). But, previous research indicated that formic acid and levulinic acid inhibited cell formation and ethanol yields on yeast fermentation at the same time (Larsson et al., 1999). The dissociated form of weak acids can diffuse from medium into cells through plasma membranes, and then dissociate to release the anion and proton (Almeida et al., 2007; Mills et al., 2009). This reaction lowers the high

intracellular pH, which is approximately 7.8 for *E. coli* (Roe et al., 1998), and 6.0 for *C. tyrobutyricum* when the pH of the medium was 6.5 (Jiang et al., 2010). The decrease of pH inside cells is compensated by the ATP depletion, a process that pumps protons out of the cells (Sanda et al., 2011). Hence, less ATP is available at lower pH for cell formation, and thus cell growth and production yield are effected (Almeida et al., 2007). The anion that is released by acids also had an inhibitory effect on cell formation through accumulating and affecting the turgor pressure of cells (Mills et al., 2009). Sometimes, under anaerobic conditions, a low concentration of acids was proved to stimulate the production of ATP during ethanol production (Almeida et al., 2007).

Most studies about these two inhibitors focused on their inhibitory effects on biofuel production (ethanol, butanol, H_2, and so on) (Cho et al., 2012; Hasunuma et al., 2011). However, the research regarding their inhibition on butyric-acid production was inadequate. Therefore, in this study, the toxic effects of formic acid and levulinic acid on *C. tyrobutyricum* were investigated, including cell growth, butyric acid production, and the variation of inhibitors' concentrations.

3.2 Materials and methods

The *C. tyrobutyricum* strain, culture medium, and fermentation conditions that were applied in this chapter were the same as described in Chapter 2. Formic acid was applied at concentrations 0, 1.2, 2.4, 3.6, and 4.8 g/L, and levulinic acid was used at concentrations 0, 2.4, and 4.8 g/L. The concentrations of these two acids were chosen based on Table 1.1 and Table 1.2. In these

two tables, the concentrations of organic acids were below 3.1 g/L. But the concentrations for organic acid to be inhibitory fell in a large range (1 to 20 g/L). According to Park et al., (2012) and Thomsen et al., (2009), the concentrations of organic acid that are released in hydrolysates were 0.5 to 5 g/L. Thus, 5 g/L was selected as the maximum concentration for formic acid and levulinic acid in this experiment. In order to be consistent with Chapter 2, the maximum concentration of organic acid was altered to 4.8 g/L. Thus, for formic acid, the concentrations to be studied were 0, 1.2, 2.4, 3.6, and 4.8 g/L. Based on the results of the formic acid experiments, formic acid below 4.8 g/L had mild effect on cell growth and fermentation. It was known that levulinic acid was less inhibitory than formic acid. Therefore, only 0, 2.4, and 4.8 g/L of levulinic acid were studied, for saving time and energy. The results showed that even 4.8 g/L of levulinic acid had no effect on cell growth and fermentation, so no more concentrations of levulinic acid were added to the experiment. These two inhibitors were separately added into the medium before autoclaving.

The optical density of the cells, the concentrations of xylose, butyric acid, and inhibitors, were also detected by the same methods as described in Chapter 2. The data was analyzed using Tukey's method in SAS (introduced in Chapter 2).

3.3 Results and discussion

3.3.1 The effect of formic acid on the activities of C. tyrobutyricum

3.3.1.1 Cell growth

The cell growth curves of *C. tyrobutyricum* with different concentrations of formic acid in the medium are shown in Figure 3.1. With an increase of formic-acid concentration, the lag phase was prolonged from 24h (0 g/L of formic acid) to 60h (4.8 g/L of formic acid). Moreover, higher concentrations of formic acid led to longer times for *C. tyrobutyricum* to achieve the maximum cell concentrations: it took 60h, 72h, 72h, 96h, and 132h under the conditions of 0, 1.2, 2.4, 3.6, and 4.8 g/L of formic acid, respectively. Furthermore, the maximum cell-concentration mildly decreased to 88.76% and 88.78% of control when the concentrations of formic acid were 3.6 and 4.8 g/L (Fig. 3.2, $P<0.05$).

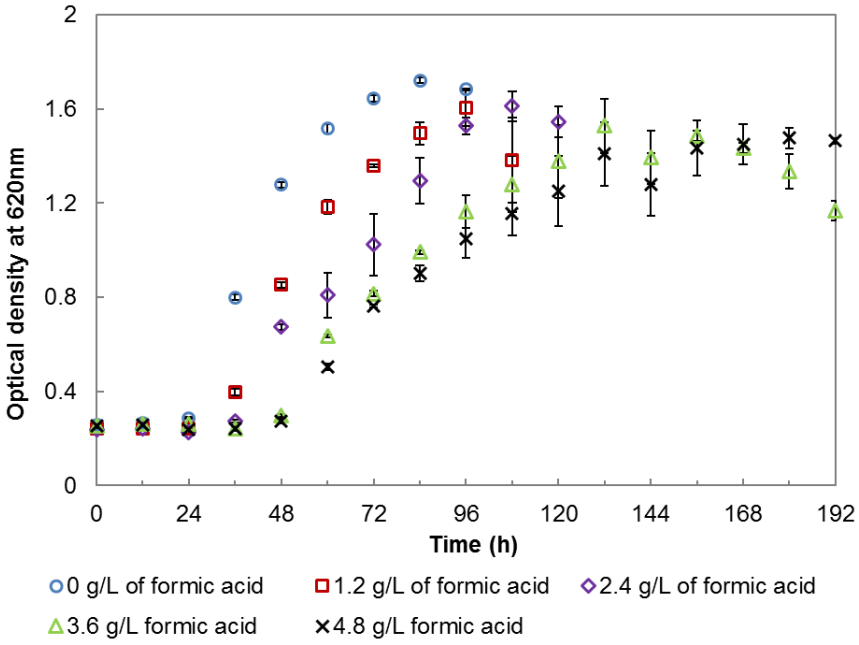

Figure 3.1 The cell growth curve of *C. tyrobutyricum* at five concentrations of formic acid

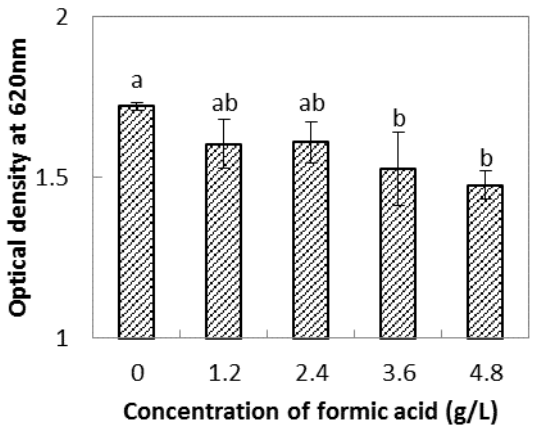

Figure 3.2 The maximum cell concentration of *C. tyrobutyricum* at each concentration of formic acid

3.3.1.2 Cell metabolism

The concentration of formic acid in the medium did not vary much during the cultivation process, which indicates that formic acid was not consumed by *C. tyrobutyricum* cells (shown in Figure 3.3). Unlike furan derivatives, which can be depleted by cells at first several hours (discussed in Chapter 2), formic acid existed in the medium and affecting cells throughout the fermentation process. It was reported that several strains, such as *C. beijerinckii*, and *C. carboxidivorans*, contain formate dehydrogenase (FDH). These two strains belong to the same genus (*Clostridial*) with *C. tyrobutyricum*, so they share the same metabolic pathway (Fig. 1.1) with *C. tyrobutyricum*. So *C. tyrobutyricum* might contain FDH too. FDH is an enzyme that exists on the cell membrane and is able to consume formic acid by catalyzing the oxidation of formate into carbon dioxide (Calusinska et al., 2010; Cho et al., 2012). But some strains, such as *Saccharomyces cerevisiae,* still cannot consume formic acid even though they have FDH in their cells. Hasunuma et al. suggested that the innate activity of FDH in *S. cerevisiae* was too weak to detoxify formic acid (Hasunuma et al., 2011). In addition, strains like *C. acetobutylicum* do not have the FDH enzyme intracellularly (Cho et al., 2012). It was not clear

whether *C. tyrobutyricum* has FDH in its cell. Even if *C. tyrobutyricum* has this enzyme, the FDH could be too weak to be effective, as stated by Hasunuma et al.

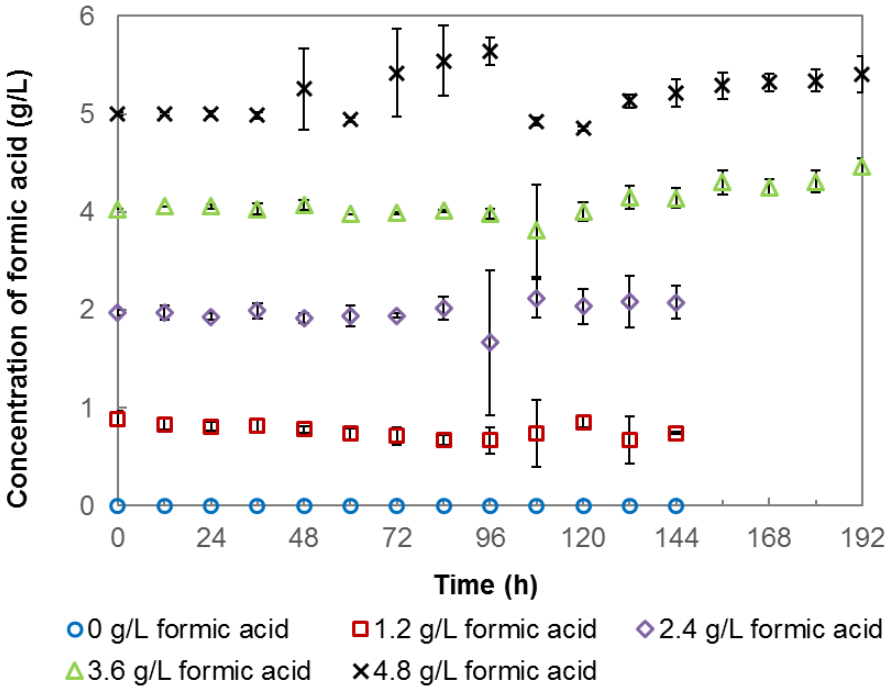

Figure 3.3 The concentration of formic acid over fermentation period

After cultivation for 108h, the concentration of formic acid in groups 3.6 and 4.8 g/L increased a little. For various organisms, formic acid is a kind of one-carbon intracellular-metabolite and can be produced by tetrahydrofolate mediated reactions that occur in mitochondria (Fu et al., 2001; Hasunuma et al., 2011). These reactions lead to an increase of formic-acid concentration during fermentation (Thomsen et al., 2009). This increase could be the potential reason that the concentration of formic acid in the groups 3.6 and 4.8 g/L increased in the late phase.

Regarding xylose consumption and butyric-acid production, the lag phases were extended with increasing formic-acid concentrations (Fig 3.4 and Fig. 3.5). For the control, the lag phase was 24h, which extended to 36h for 1.2 and 2.4 g/L of formic acid, and to 48h for 3.6 and 4.8 g/L of formic acid, respectively. The same phenomenon was reported about H_2 fermentation in the presence of formic acid by Kumar et al., (2014). A possible reason for the increase in lag time could be that cells need more time to acclimate to greater amounts of this inhibitor.

As shown in Figure 3.6 a), 1.2 g/L, 3.6 g/L, and 4.8 g/L of formic acid had no obvious effect on butyric-acid yield. Compared to the control, the butyric-acid yield decreased with 2.4 g/L of formic acid in the medium. As to the butyric-acid productivity (Fig. 3.6 b)), it took a longer time to achieve the maximum butyric-acid concentration with higher concentrations of formic acid in the medium, thus leading to a lower productivity. According to Fig. 3.5, Fig. 3.6 a), and Fig. 3.6 b), within 4.8 g/L, formic acid had only a mild effect on butyric-acid production. The toxicity of this inhibitor depends on the type of strain. The acetone-butanol-ethanol (ABE) production of *C. acetobutylicum* decreased to 77% and 24% with only 0.4 and 1.0 g/L of formic acid in the culture medium (Cho et al., 2012). Similarly, 20 mM (about 0.92 g/L) of formic acid strongly inhibited xylose consumption and ethanol production by *S. cerevisiae* (Tomaya et al., 2011). But in the present study, *C. tyrobutyricum* is an acid-forming bacteria, and the pH value of the medium was monitored (pH=6.4) every 12 hours. Hence, for *C. tyrobutyricum*, although formic acid is able to enter into cells, it will not change intracellular pH values greatly, which is the probable reason for the mild inhibitory-effect of formic acid.

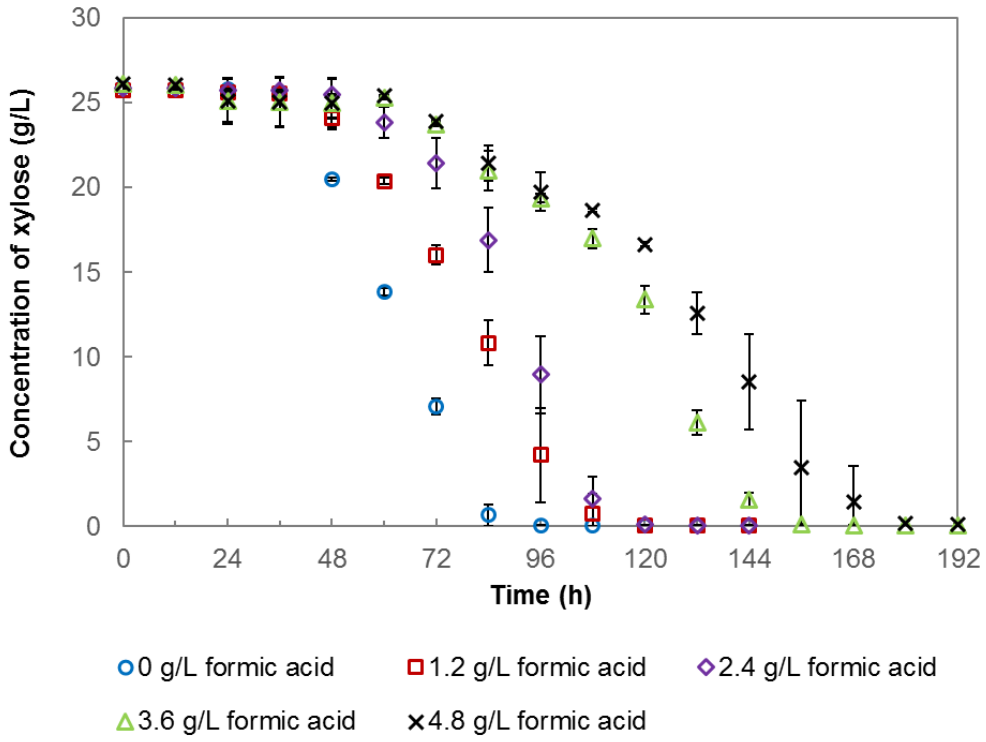

Figure 3.4 The consumption of xylose by *C. tyrobutyricum* during fermentation

Previous studies suggested that formic acid at a lower concentration was able to stimulate the production of ATP, which slightly increased the yield of ethanol under anaerobic fermentation (Almeida et al., 2007). For example, ABE production by *C. acetobutylicum* with 0.2 g/L of formic acid was greater than the control (Cho et al., 2012). However, in this research, the low concentration (1.2 g/L of formic acid) did not increase or decrease cell activities. That stability can be caused by the difference between solventogenic and acidogenic strains.

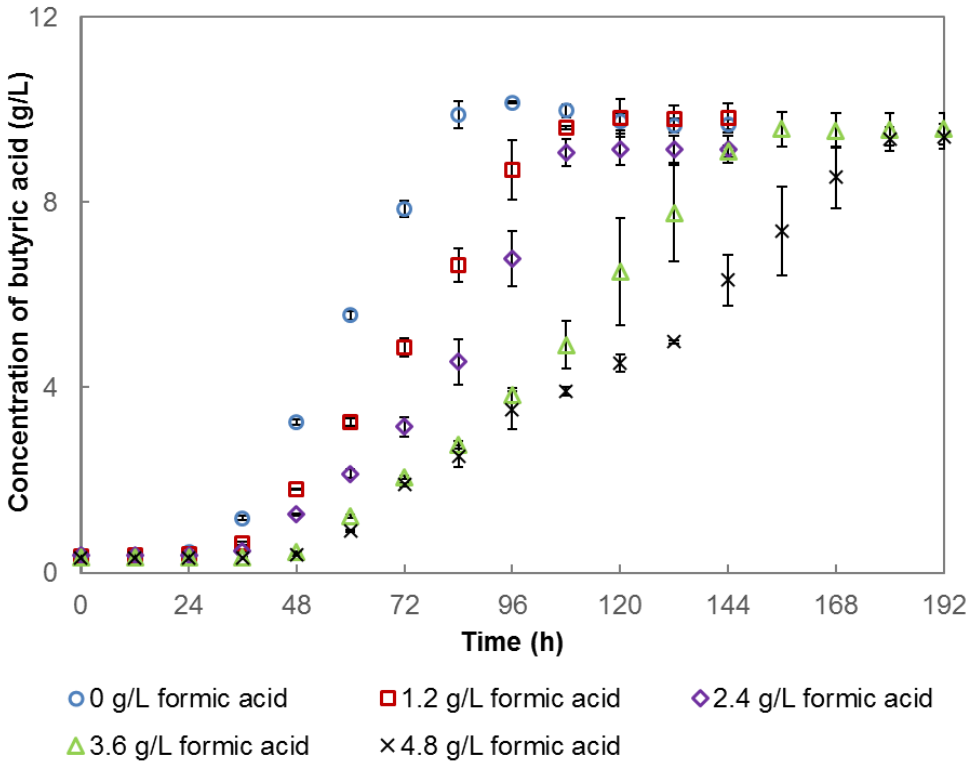

Figure 3.5 The production of butyric acid by *C. tyrobutyricum*

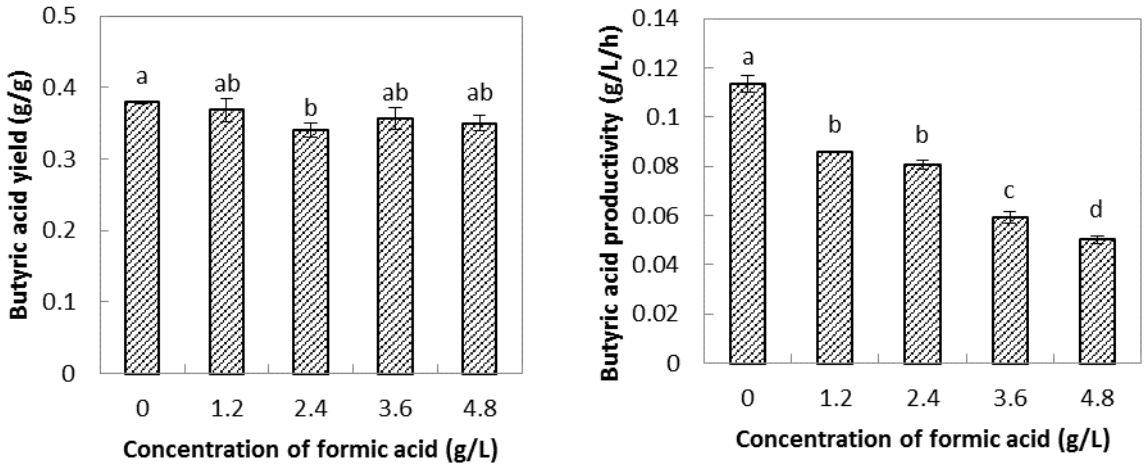

Figure 3.6 a) The yield of butyric acid by *C. tyrobutyricum* at each concentration of formic acid; b) The productivity of butyric acid by *C. tyrobutyricum* at each concentration of formic acid

3.3.1.3 Relationship between optical density and its corresponding butyric-acid concentration treated by formic acid

Fig 3.7 and Fig. 3.8 displays the relationship between optical density and its corresponding butyric-acid concentration (exponential, and linear after transformed) in the formic-acid groups 0, 1.2, 2.4, 3.6, and 4.8 g/L. The exponential regression shows that with the optical density increased, more butyric acid was produced per OD unit. The high R-square of the linear regression (R^2=0.974) demonstrates that the model fits the data well. The equation for the linear regression is y=2.396x-1.539. The equation for the untransformed response is $y=0.215e^{2.396x}$.

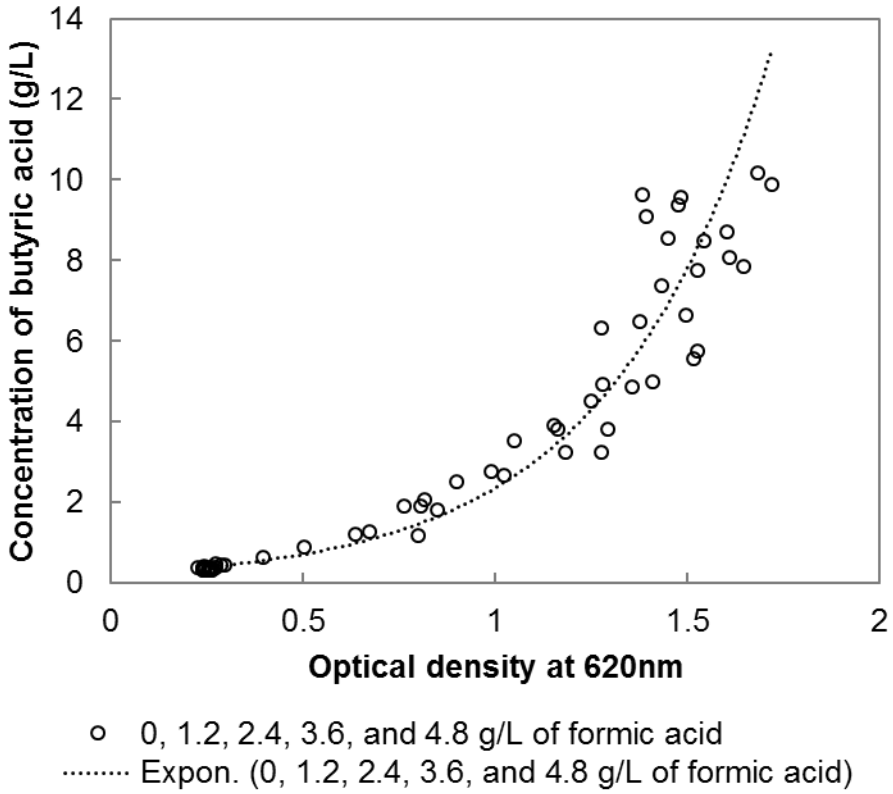

Figure 3.7 Scatter plot and regression fit of optical density and its corresponding butyric-acid concentration treated by formic acid

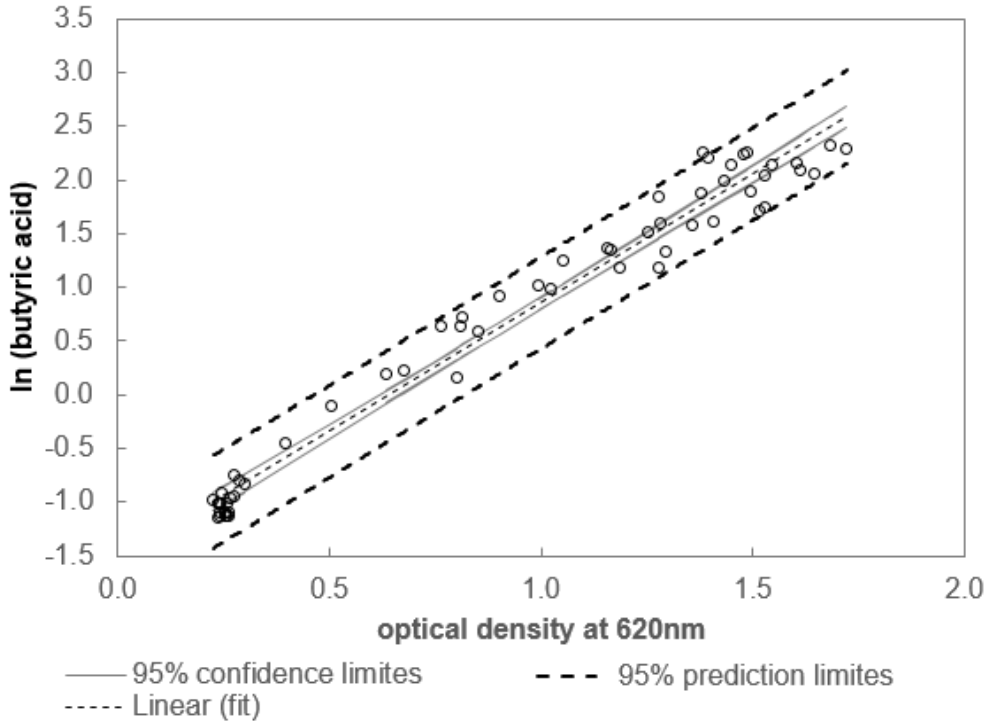

Figure 3.8 The relationship between optical density at 620nm and its corresponding butyric-acid concentration treated by formic acid (after natural log response)

3.3.2 The effect of levulinic acid on the cell growth and metabolism of C. tyrobutyricum

3.3.2.1 Cell growth

Figure 3.9 displays *C. tyrobutyricum* cell growth curves with 0, 2.4, and 4.8 g/L of levulinic acid in the culture medium. No obvious difference in cell growth was observed within these three groups. Further statistical analysis that was conducted using SAS (Fig 3.10) suggested that there were no significant differences between maximum cell-concentration between these three groups. That is, with up to 4.8 g/L, levulinic acid did not affect cell growth.

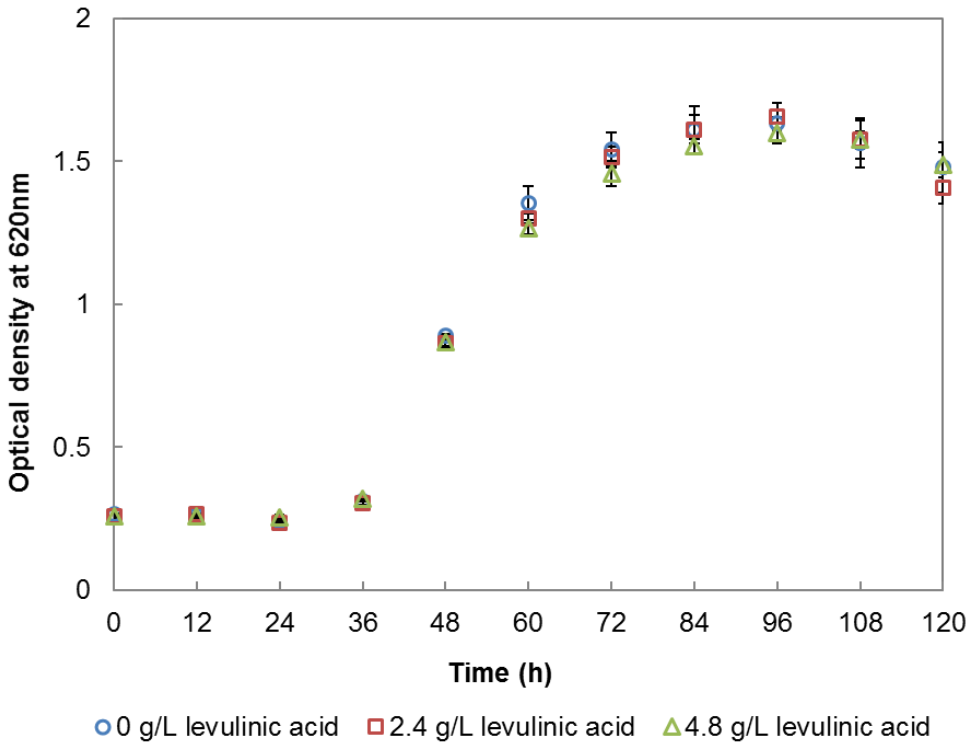

Figure 3.9 The cell growth curve of *C. tyrobutyricum* during fermentation

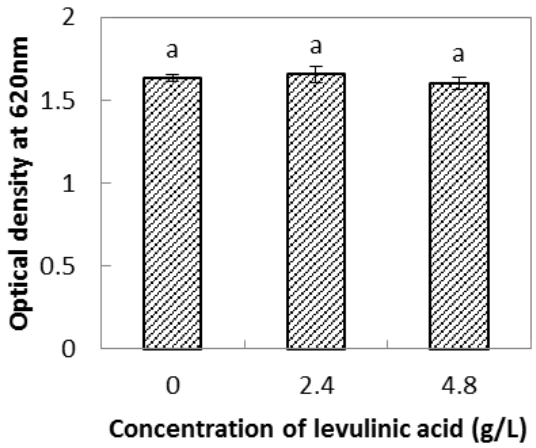

Figure 3.10 The maximum cell concentration of *C. tyrobutyricum* at three concentration of levulnic acid

3.3.2.2 cell metabolism

As with formic acid, levulinic-acid concentration in the culture medium did not vary much during the fermentation process, indicating that levulinic acid was not consumed by cells (Fig. 3.11). The non-consumption might be due to the lack of corresponding enzymes in *C. tyrobutyricum* cells. At the same time, levulinic acid might not be a metabolite in cell activities.

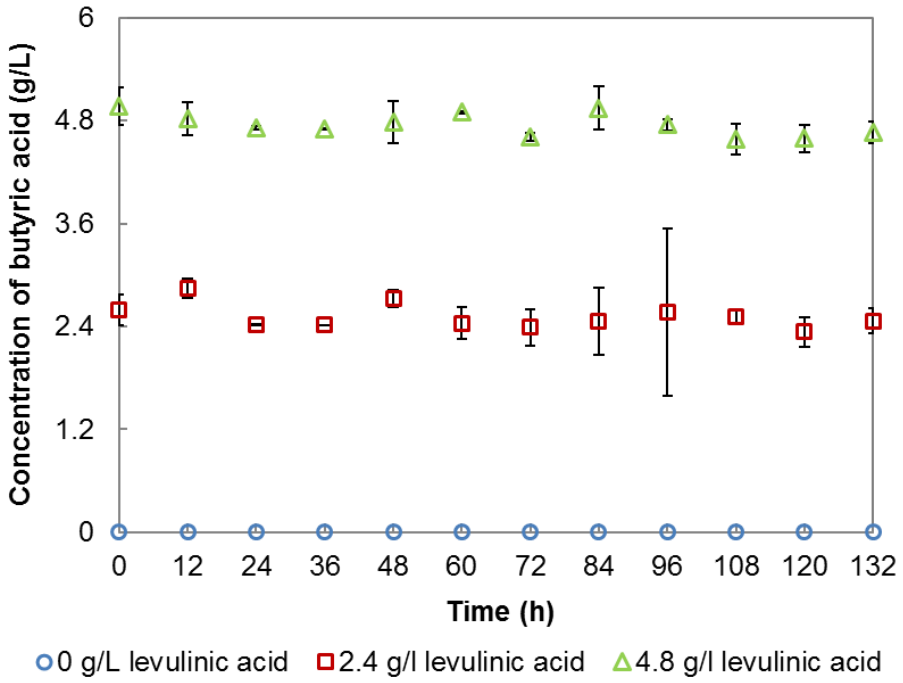

Figure 3.11 The concentration of levulinic acid during fermentation

In Fig. 3.12 and 3.13, xylose consumption and butyric-acid production for each group during fermentation process are displayed. Compared to the control group, no significant variations for either xylose consumption or butyric-acid production were observed when 2.4 g/L of levulinic acid was added into the medium. A relatively lower xylose-consumption rate was observed with 4.8 g/L of levulinic acid in the medium. But, there was no significant difference

between these three groups regarding butyric-acid yield or productivity (Fig 3.14 a) and 3.14 b)). To conclude, for concentrations no greater than 4.8 g/L, levulinic acid had no effect on butyric-acid production.

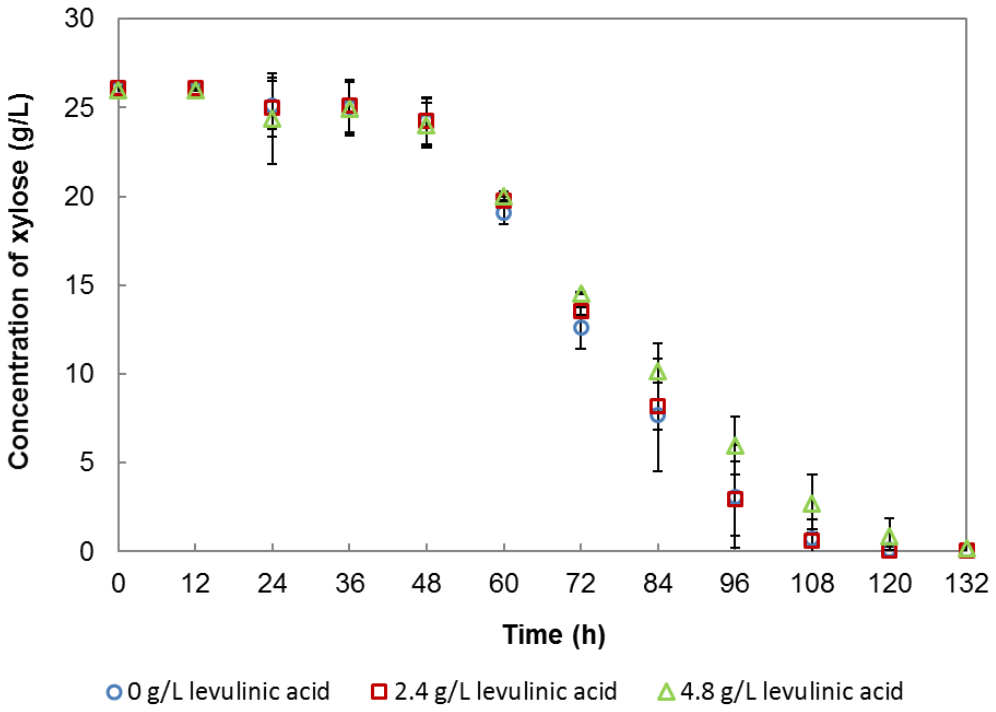

Figure 3.12 The consumption of xylose by *C. tyrobutyricum* during fermentation

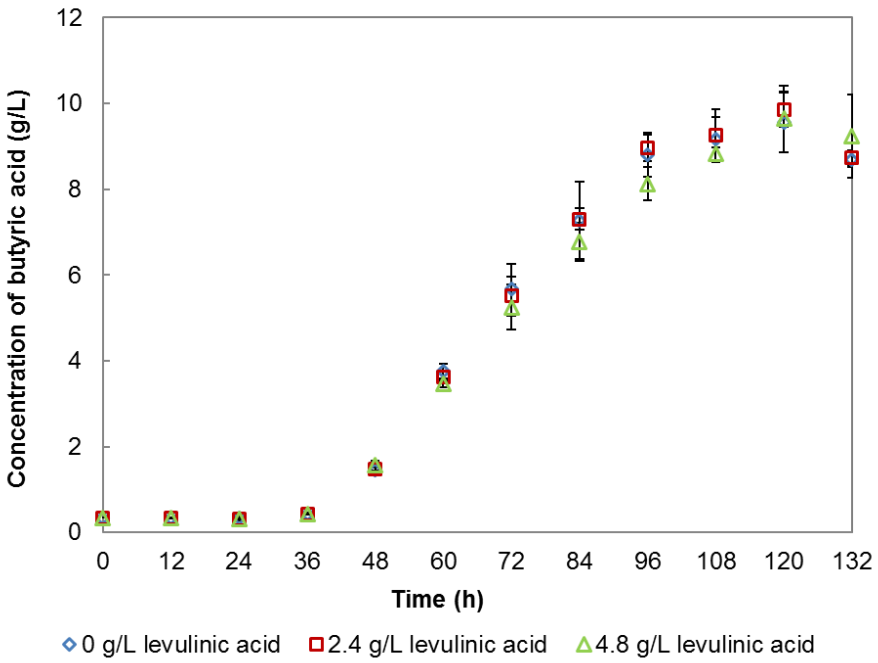

Figure 3.13 The production of butyric acid by *C. tyrobutyricum*

In summary, 2.4 and 4.8 g/L of levuinic acid had no effect at all on cell growth and butyric-acid production. Therefore, if the concentration of levulinic acid is not greater than 4.8 g/L, its negative effects on *C. tyrobutyricum* cells during fermentation can be neglected. Nevertheless, previous studies suggested that levulinic acid could be toxic to other strains, even in a low concentration. The hydrogen-production rate of hydrogen-producing seed sludge decreased to 50% (compared to the control) even with only 1.55 g/L of levulinic acid in the medium (Kumar et al., 2014). 3 g/L of levulinic acid caused a 25% inhibition of *E. coli* LY01 growth (Zaldivar et al., 1999). 2.09 g/L of levulinic acid caused a 50% decrease in the rate of galactose utilization by *S.cerevisiae* ATCC 2341 (Park et al., 2014). As with formic acid, a potential reason for this difference might be that *C. tyrobutyricum* is an acid-formed strain that produces

butyric acid as an intracellular metabolite. In this case, the diffusion of levulinic acid does not cause a sharp decrease of the intracellular pH. It could be possible that levulinic acid has the potential to inhibit the activities of *C. tyrobutyricum* when its concentration is higher than 4.8 g/L. But since the formative-concentration range of levulinic acid in hydrolysate is 0.5 – 5 g/L (Park et al., 2012), 4.8 g/L is about the cutoff threshold.

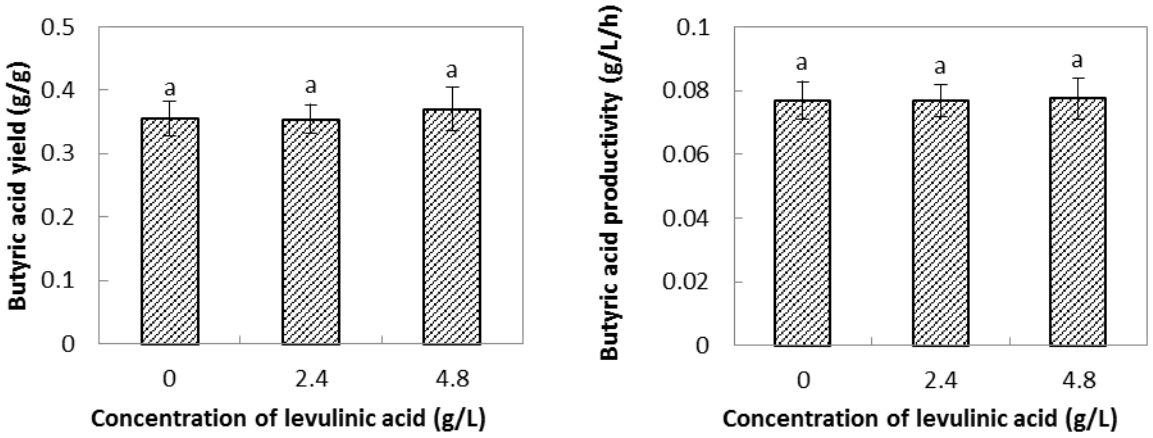

Figure 3.14 a) The yield of butyric acid by *C. tyrobutyricum* at three concentration of levulinic acid; b) The productivity of butyric acid by *C. tyrobutyricum* at three concentration of levulinic acid

3.3.2.3 Relationship between optical density and its corresponding butyric-acid concentration treated by levulinic acid

Fig 3.15 and Fig. 3.16 display the relationship between optical density and its corresponding butyric-acid concentration (pre-transformed, and transformed in levulinic-acid groups 0, 2.4, and 4.8 g/L. Since cell growth and butyric-acid production were not affected by 2.4 and 4.8 g/L of levulinic acid (shown in Fig. 3.9, Fig. 3.10, and Fig. 3.13), all the data for these three groups are modeled well by one exponential regression. The exponential regression shows that

with the optical density increased, more butyric acid was produced per OD unit. The high R-square for the linear regression (R^2=0.995) also supports the conclusion that the regression explains the data. The equation for linear regression is y=2.302x-1.647. So the equation for exponential regression could be calculated as $y=0.192e^{2.302x}$.

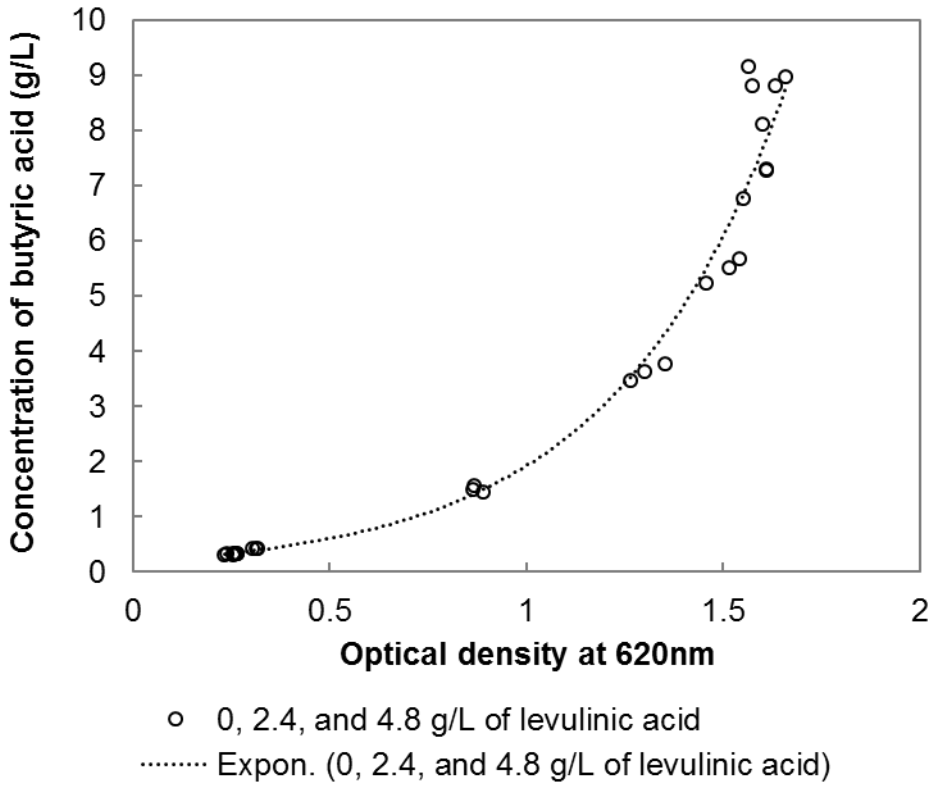

Figure 3.15 The relationship between optical density and its corresponding butyric-acid concentration treated by levulinic acid

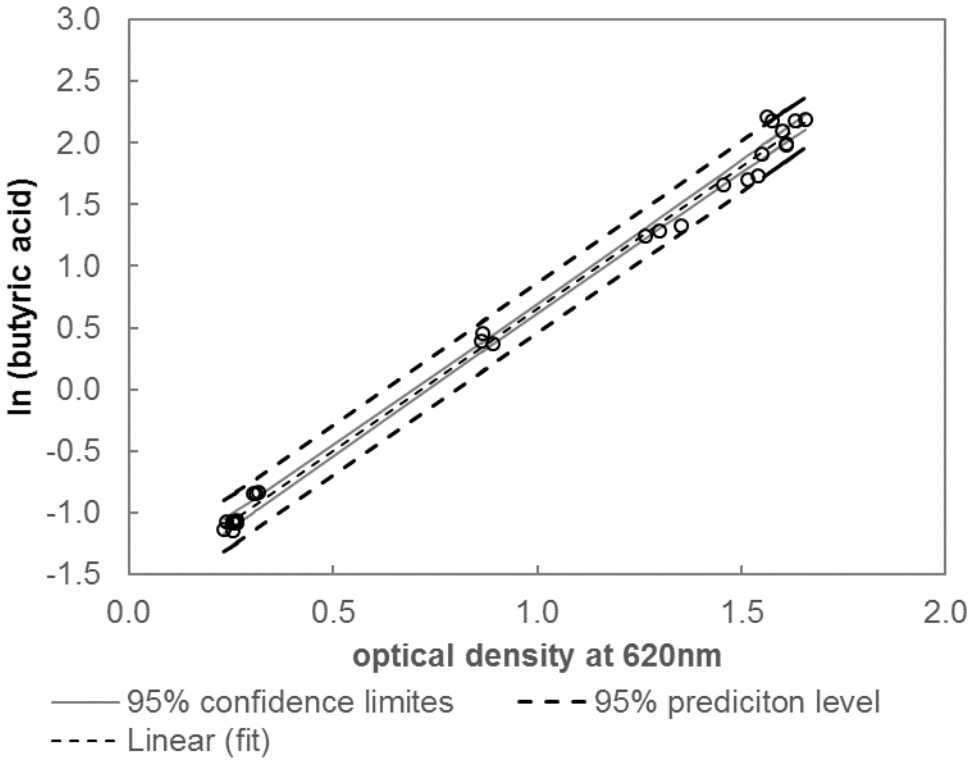

Figure 3.16 The relationship between optical density at 620nm and its corresponding butyric-acid concentration treated by levulinic acid (after natural log response)

3.3.3 Comparison between formic acid and levulinic acid

The inhibition effect of formic acid on *C. tyrobutyricum* growth and butyric-acid production was more obvious than that of levulinic acid at the same concentration. As described in the introduction, a possible reason is that the formic-acid molecule size is smaller than that of levulinic acid. The smaller size facilitates its diffusion into cell membranes (Larsson et al., 1999). The higher anion-toxicity of formic acid (Larsson et al., 1999) and its higher permeability through membranes might also contribute to a higher inhibition effect (Walter et al., 1984).

3.4 Conclusion

In order to study the inhibitory effect of carboxylic acids that are released in hydrolysates, formic acid and levulinic acid were tested on *C. tyrobutyricum* fermentation in this research. 0, 1.2, 2.4, 3.6, 4.8 g/L of formic acid, and 0, 2.4, and 4.8 g/L of levulinic acid were added into broth separately, in triplicate. Some conclusions could be drawn as below:

1. 2.4 and 4.8 g/L of levulinic acid did not affect cell growth and cell activities, which indicated that within 4.8 g/L, levulinic acid was not toxic to *C. tyrobutyricum*.
2. With an increasing concentration of formic acid, the lag phases of cell growth and butyric acid production were affected severely.
3. The maximum cell-concentration decreased with an elevation of formic-acid concentration.
4. Furthermore, the butyric-acid yield was not affected by formic acid, whereas the butyric-acid productivity decreased sharply due to the longer lag phase caused by increased formic-acid concentration.
5. Formic acid and levulinic acid were not consumed by *C. tyrobutyricum* during fermentation.

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CHAPTER 4 The Inhibitory Effects of Phenolic Compounds on *C. Tyrobutyricum* During Butyric-acid Fermentation

Abstract As potential inhibitors, the effects of syringaldehyde and vanillin on *C. tyrobutyricum* were investigated. Concentrations of 0, 0.6, 1.2, 1.8, and 2.4 g/L of syringaldehyde and vanillin were independently added into the growth medium. Cell growth, butyric production, and inhibitor concentration were measured every 12 hours. The results showed that when the concentration was under 0.6 g/L, neither of these two inhibitors had significant effects on cell activities. When the concentration of inhibitors increased from 0.6 g/L to 2.4 g/L, maximum cell-concentration, production, and yield of butyric acid were all decreased gradually. The difference in severity of inhibitory effects between the two chemicals was not as great as expected for this strain. Syringaldehyde and vanillin were both assimilated by cells.

4.1 Introduction

Two kinds of phenolic compounds, syringaldehyde and vanillin, were studied in this chapter. Syringaldehyde (4-hydroxy-3,5-dimethoxy-benzaldehyde) naturally exists within cell walls of plants (Ibrahim et al., 2012). Syringaldehyde could be utilized as antioxidants, antifungal or antimicrobial agents, and anti-tumorigenesis agents (Ibrahim et al., 2012). In this chapter, syringaldehyde is abbreviated as SY. Vanillin, known as 4-hydroxy-3-methoxybenaldehyde, is an important aroma in foods, pharmaceuticals, chemical industries, and cosmetics (Yusof et al., 2014). It can be extracted from seedpods of *Vanilla planifolia* as well as from waste lignin (Yusof et al., 2014). Due to the rapid aqueous-photodegradation of SY and vanillin, they are

capable of providing low volatility, light-adsorbing products (Smith et al., 2015). These two chemicals are commonly found in lignocellulosic hydrolysates (Deniela et al., 2010) and always at higher concentrations than other phenolic compounds, such as ferulic acid and hydroxybenaldehyde (Thomsen et al., 2009). Lignin is one of the three major parts that comprise the lignocellulosic biomass (Li et al., 2014). Lignin is a stable and complex polymer with the following three major phenylpropane units: p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Li et al., 2014; Sun et al., 2013). During the pretreatment of lignocellulosic biomass, SY and vanillin are generated from syringyl residue and guaiacyl residue, respectively, by solubilization, hydrolysis, and oxidation of lignin (Cao et al., 2010; Thomsen et al., 2009). After a diluted-acid pretreatment, the concentration of phenolic compounds is between 0 and 3 g/L (Mills et al., 2009). A recommended concentration-level between 0.5 and 2 g/L was applied to evaluate the effects of phenolic compounds on xylose fermentation (Cortez et al., 2010).

Several researchers proved that phenolic compounds had inhibitory effects on cell growth and cell metabolism (Ravindra et al., 2016). These phenolic compounds were lethal to *Clostridial* even at a low concentration (less than 2 g/L) (Ezeji et al., 2007; Lee et al., 2015). The severity of these inhibitors varied due to the types of microorganisms and the experimental conditions (Cortez et al., 2010). For example, 10 mM of SY was just a weak inhibitor on lipid production by *R. toruloides Y4* (Hu et al., 2009), whereas 0.3 g/L of SY was potent to butanol production by *C. beijerinckii*. The exact inhibitory mechanism of phenolic-compound cell-activities still remains unclear (Ravindra et al., 2016). But, it was noted that for yeast cells, these compounds

can increase the membrane permeability, which leads to partial loss of cellular constituents. Also, phenolic compounds could inactivate essential enzymes or enzyme systems, including those involved in energy production, and synthesis of structural components. Furthermore, the compounds can destroy or functionally inactivate genetic materials, and enhance generation of reactive-oxygen species (H_2O_2, O_2, OH), which can denature enzymes and mutate DNA (Siqueira et al., 2015).

Although many studies have focused on syringaldehyde and vanillin, the effects of those compounds on *C. tyrobutyricum* were not fully investigated. Therefore, in this chapter, the inhibitory effects of these two chemicals on *C. tyrobutyricum* activities, at different chemical-concentrations, were studied.

4.2 Materials and methods

The strain, medium, and fermentation methods that were used in this chapter were the same as in Chapter 2. 0, 0.6, 1.2, 1.8, and 2.4 g/L of SY and vanillin were separately added to the prepared medium ahead of autoclaving. The selected concentrations of SY and vanillin were based on Table 1.1 and Table 1.2. In Table 1.1, the concentration of phenolic compounds was below 1 g/L in hydrolysates. And, the concentrations of SY and vanillin were studied at about 1 to 2 g/L, in Table 1.2. In order to be consistent with Chapter 2, 2.4 g/L was selected as the maximum concentration of SY and vanillin. Thus, 0, 0.6, 1.2, 1.8, and 2.4 g/L of these two inhibitors were set as the concentrations to be studied in experiment.

The optical density of cells, and the concentrations of xylose and of butyric acid were also measured by the same methods as in Chapter 2. SY and vanillin were detected by using the same HPLC and column as well, but these two inhibitors were measured using methods that were different from those of other inhibitors (furfural, HMF, formic acid, and levulinic acid). The method for SY and vanillin used these settings: the mobile phase was a mixture of 84% of 0.01 N sulfuric acid and 16% of acetonitrile; the flow rate was 0.35 ml/min and the temperature was set at 55 ^{o}C; the wavelength of the UV detector was set at 220 nm and 254 nm. The data was analyzed using Tukey's method in SAS (described in Chapter 2).

4.3 Results and discussion

4.3.1 The effect of syringaldehyde on the activities of C. tyrobutyricum

4.3.1.1 Cell growth

Fig. 4.1 shows the inhibitory effect of syringaldehyde on cell growth of *C. tyrobutyricum*. When the concentration of SY increased, the lag phase became longer. In the medium with 0.6 g/L of SY, the lag phase matched the control (36h). The lag phase was 48h for the third group, 84h for the fourth, and 96 h for the fifth. The prolong lag phase was also observed by Siqueira et al., (2015) that 1 g/L of SY increased the lag phase of H_2-production bacteria by 11h. Also, it took a longer time for cells to achieve their maximum cell-concentration when the SY concentration was increased. Fig. 4.2 provides more statistical information about cell growth. Maximum cell-concentration gradually decreased with the concentration of SY increased. For the group with 4.8 g/L of formic acid, the growth curve was diauxic. The first log phase was

96h to 120h. Then the cells suddenly went into stationary phase. The second log phase started from 132h to 192h. The diauxic growth-curve was probably related to the consumption of 2.4 g/L of syringaldehyde by cells (Fig. 4.3). One possible explanation is that, at the first 96h, cells were accommodating to the environment. From 96h, the lag phase ended, and cells started to consume syringaldehyde. The metabolites from consumed syringaldehyde accumulated from 96h. At 120h, the metabolites accumulated to a large amount and they caused synergistic effect with left syringaldehyde on cell growth, so cells went into stationary phase at 120h. With the accommodating of cells to the new environment and more syringaldehyde was consumed (less synergistic effect), cells went into the second log phase from 132h.

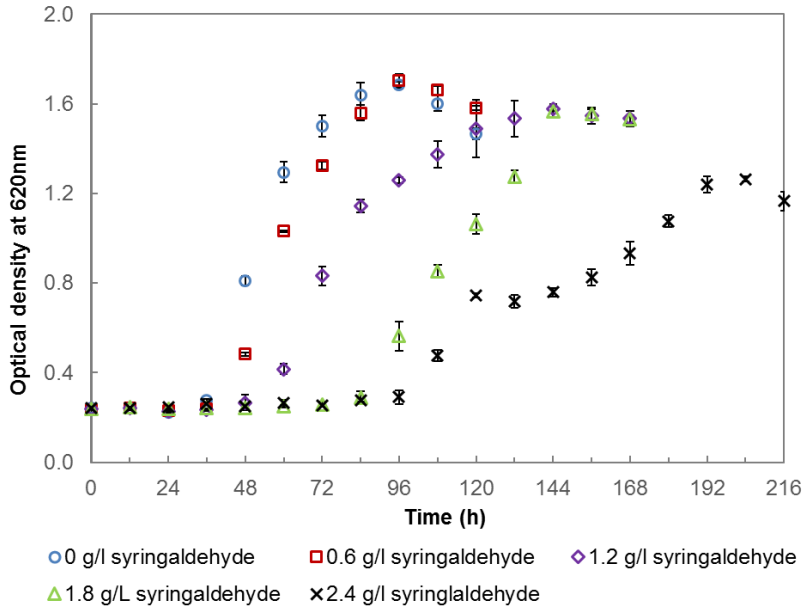

Figure 4.1 The cell growth curves of *C. tyrobutyricum* treated by five concentrations of syringaldehyde

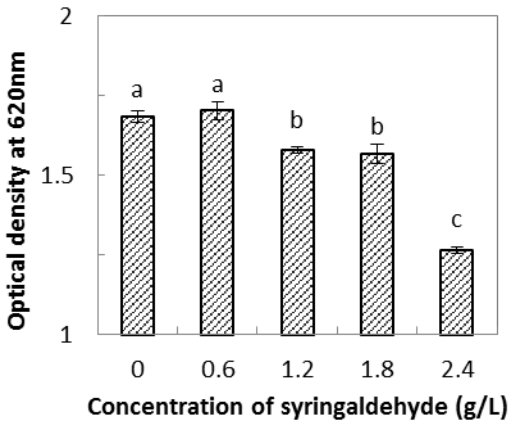

Figure 4.2 The maximum cell concentration at each concentration of syringaldehyde

4.3.1.2 Cell metabolism

The Fig. 4.3 presents the concentration of syringaldehyde during fermentation. SY was consumed by cells in each group. But the higher the concentration of SY, the longer the lag phase. The lag phases of consumption for this inhibitor were always same or 12h later when compared to those of cell growth. That is, the rate that cells metabolized this inhibitor was related to the growth of cells. SY can be assimilated to its alcohol form by *S. cerevisiae* (Kelly et al., 2008). *C. guilliermondii* was also able to convert aromatic aldehydes into their respective acids (syringyl acid) or alcohols (Syringyl alcohol) (Cortez et al., 2010). The assimilation metabolites from converting syringaldehyde by *C. tyrobutyricum* were not clear, but the metabolites could be syringyl alcohol or syringic acid. Aldehydes are generally more toxic for *S. cerevisiae* and *E. coli* than are alcohols and acids (Almeida et al., 2007). Thus, *C. tyrobutyricum* might be more tolerant of the metabolites of syringaldehyde than of syringaldehyde itself.

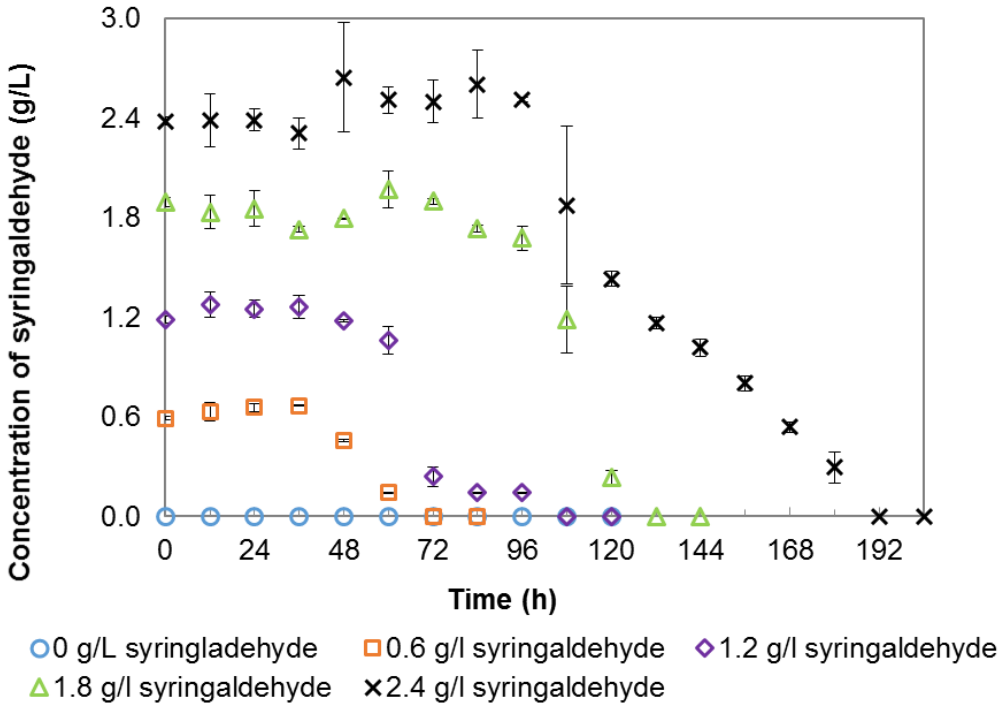

Figure 4.3 The concentration of syringaldehyde changing overtime in each group

In Figure 4.4, xylose in five groups was completely consumed, though the lag phase was prolonged by the increased concentration of SY. The lag phases were 48h, 48h, 60h, 84h, and 108h for 0, 0.6, 1.2, 1.8, and 2.4 g/L of SY respectively. In Fig, 4.5, the butyric acid in the first four groups was produced when cells started to consume xylose. In the fifth group, xylose was consumed from 96h, but butyric-acid production started from 144h. That is to say, from 96 to 144h, xylose was converted into other products instead of into butyric acid. After 144h, butyric acid might have been produced from the remaining xylose or converted by those products that were produced before 144h. As shown in Fig 4.6 a) and 4.6 b), the yield of butyric acid decreased severely with the increasing SY concentration. 0.6 g/L of SY had no effect on butyric-acid yield. 1.2 g/L of SY decreased the yield by 15%, 1.8 g/L by 43%, and 2.4 g/L by 80%. The influence of different concentrations of SY on butyric-acid productivity had the same

trend. More time was needed to achieve a stable concentration of butyric acid in the presence of higher concentrations of SY. The groups control and 0.6 g/L of SY achieved similar highest productivity, whereas the productivity in groups 1.2, 1.8, and 2.4 g/L of SY were 61%, 37%, and 11% of control, respectively. One conclusion is that with a relatively higher concentration of SY, butyric acid was not the major product. The low yield of butyric acid indicated that high concentrations of SY interfered with the reactions along the glycolytic pathway to butyryl-CoA which led to a variety of products besides butyric acid (Cho et al., 2009; Ezeji et al., 2007). The effect of SY on cell growth turned out to be less than the effect on butyric-acid production. This result was in agreement with Cho et al. that 1g/L of SY inhibited cell growth and butanol production by C. *beijerinckii* by 3% and 26% (Cho et al., 2009). A similar phenomenon was observed by Ezeji, in that 0.3 g/L of SY was potent to butanol production but with little effect on cell growth (Ezeji et al, 2007).

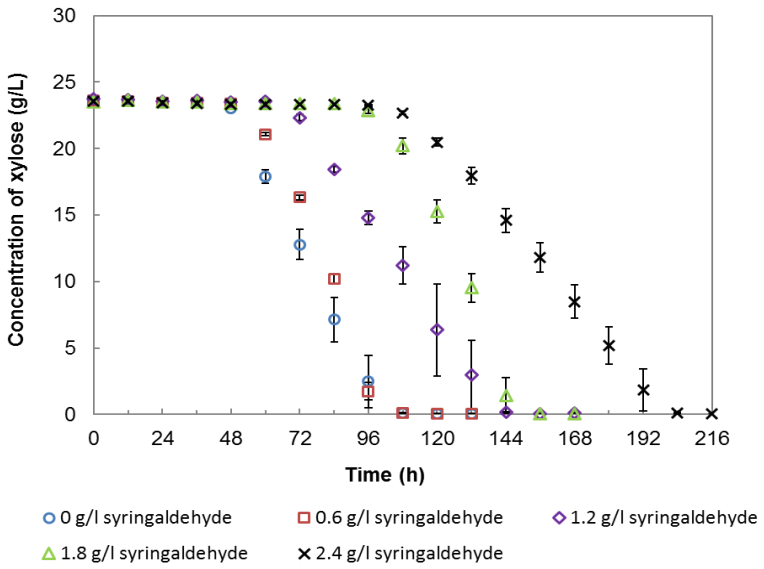

Figure 4.4 The consumption of xylose by *C.tyrobutyricum* during fermentation treated by five concentrations of syringaldehyde

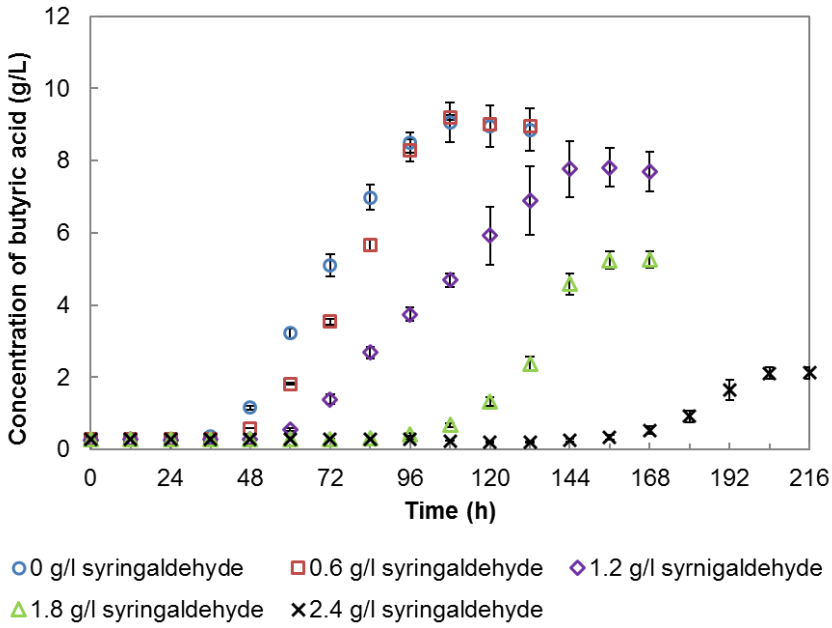

Figure 4.5 The production of butyric acid during fermentation treated by five concentrations of syringaldehyde

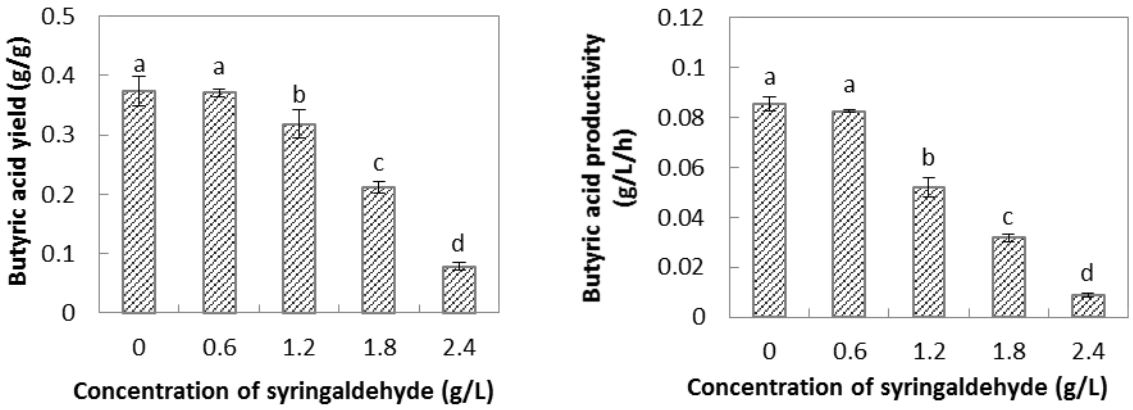

Figure 4.6 a) The yield of butyric acid at each concentration of syringaldehyde; b) The productivity of butyric acid at each concentration of syringaldehyde

4.3.1.3 Relationship between optical density and its corresponding butyric-acid concentration treated by syringaldehyde

Fig. 4.7 and Fig. 4.8 displays the relationship between optical density and its corresponding butyric-acid concentration (exponential and linear after transformed) in syringaldehyde groups of 0, 0.6, 1.2, 1.8, and 2.4g/L. The exponential regression shows that with the optical density increased, more butyric acid was produced per OD unit. The equation for the linear regression is y=2.330x-1.960. The equation in the original scale is $y=0.141e^{2.330x}$. The R-square for linear regression is 0.915, and some data points lie outside the 95% prediction limits (Fig. 4.8). These data points are in the 2.4 g/L of SY group. In Fig. 4.1 and Fig. 4.5, cell growth started from 96 hours, whereas butyric acid was produced from 144 hours. The asynchronicity of optical density and butyric-acid production caused a phenomenon such that when the optical density increased, the accumulation of butyric acid stayed the same. Thus, the exponential model under-fit outlying points.

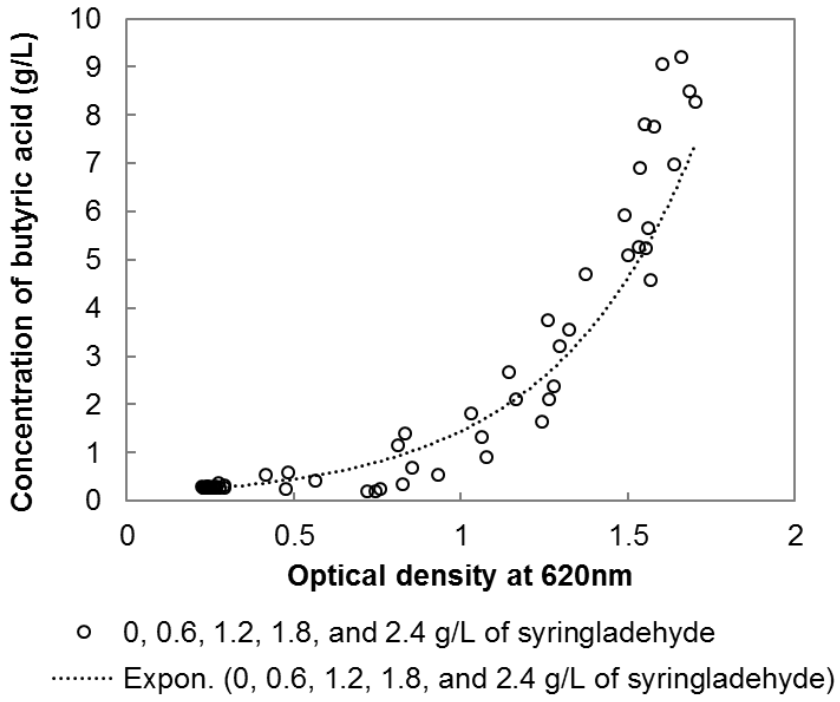

Figure 4.7 The relationship between optical density and its corresponding butyric-acid concentration treated by syringaldehyde

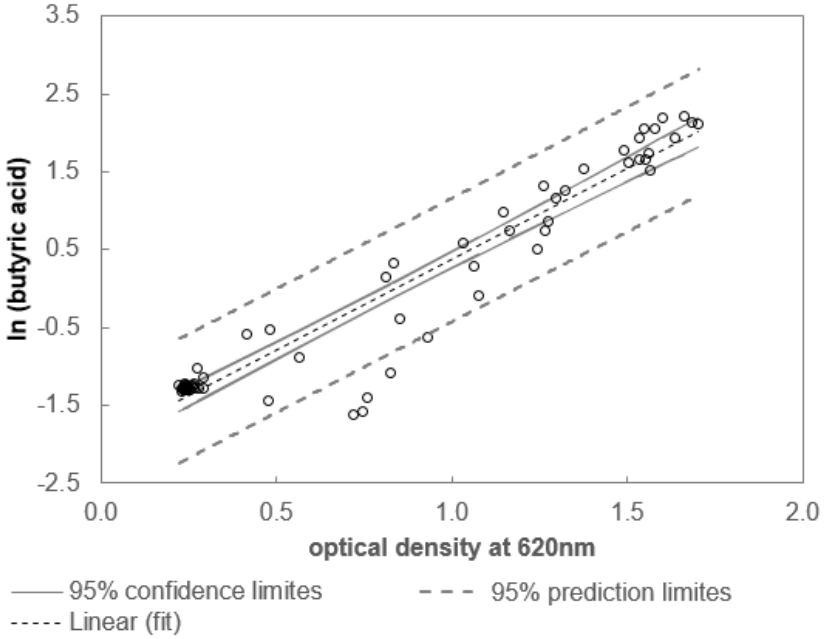

Figure 4.8 The relationship between optical density at 620nm and its corresponding butyric-acid concentration treated by syringaldehyde (after natural log response)

4.3.2 The effect of vanillin on the activities of C. tyrobutyricum

4.3.2.1 Cell growth

Fig. 4.9 presents cell growth curves for five groups with different concentrations of vanillin in the fermentation. No prolonged lag-phase was observed with the increasing vanillin concentrations (12h for each group). But, it took a longer time for cells to achieve their maximum cell-concentration with higher concentrations of vanillin. As shown in Fig 4.10, the maximum cell-concentration decreased with elevating inhibitor-concentration. Note that for 1.8 and 2.4 g/L vanillin groups, cells grew following a diauxic curve. That is, the cells went into a second stationary phase before they decayed.

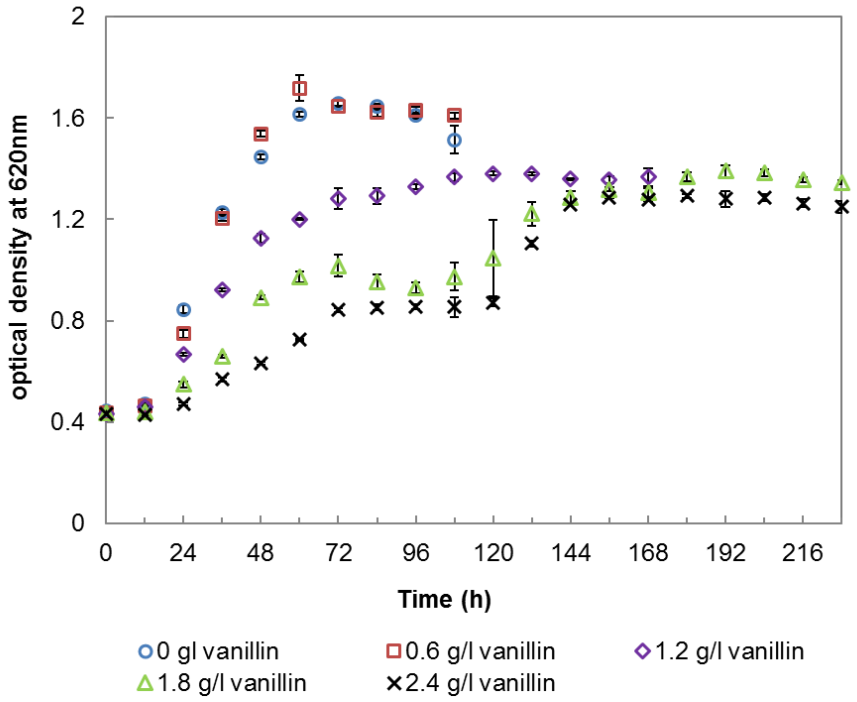

Figure 4.9 The cell growth curves of *C. tyrobutyricum* during fermentation at each concentration of vanillin

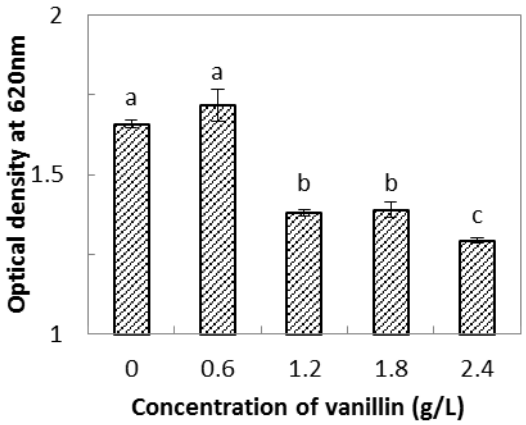

Figure 4.10 The maximum cell concentration at each concentration of vanillin

4.3.2.2 cell metabolism

Vanillin was consumed by cells after 12h for each group (Fig. 4.11). But, it took longer for vanillin with higher concentrations to be depleted. Vanillin was consumable to its less inhibitory alcohol form, vanillyl alcohol, by *S. cerevisiae* (Nguyen et al., 2014). *C. guilliermondii* was also able to convert vanillin into vanillic acid and vanillyl alcohol (Cortez et al., 2010). For *C. tyrobutyricum*, the products that were assimilated from vanillin might also be vanillic acid and vanillyl alcohol. In the 1.8 and 2.4 g/L vanillin groups, the accumulation of large amounts of assimilation products might cause a second lag-phase for cell growth. After adaptation to the new environment, cells went into a second growth-cycle.

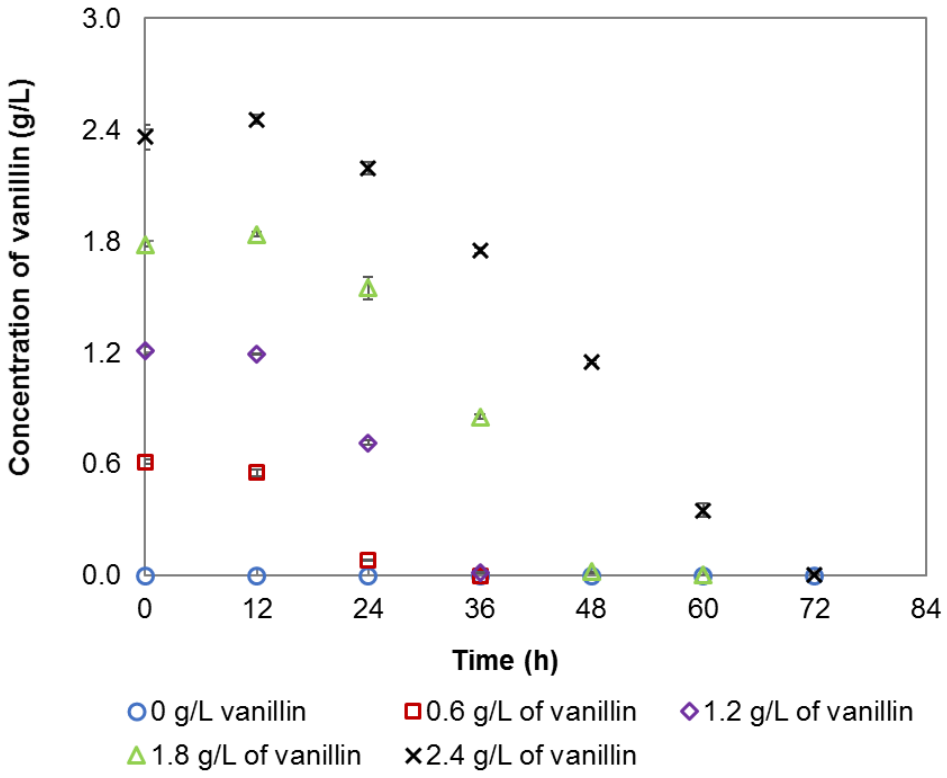

Figure 4.11 The concentration of vanillin during fermentation in each group

Xylose consumption and butyric-acid production during fermentation with different concentrations of vanillin are displayed in Fig 4.12 and 4.13. Longer lag phases of both xylose consumption and butyric-acid production were observed for higher concentrations of vanillin. Furthermore, cells took more time to deplete xylose and to achieve a stable concentration of butyric acid. Fig. 4.14 a) and Fig. 4.14 b) show that in the medium with 0.6 g/L of vanillin, the yield and productivity of butyric acid were at same level as the control. 1.2 g/L of vanillin severely prolonged cell metabolism, resulting in the low butyric-acid productivity, but the vanillin had no effect on butyric-acid yield. With 1.8 g/L of vanillin, xylose consumption continued until 228h, but butyric acid achieved a stable concentration from 72h to 156h.

Butyric acid then increased again up to its maximum concentration (5.04 g/L). In the medium that was treated by 2.4 g/L of vanillin, the concentration of butyric acid was stable after 84h, whereas xylose was still consumed after that time. In the SAS analysis charts, the yield and productivity of butyric acid decreased gradually with the increasing of concentration of vanillin.

In a summary, a low concentration of vanillin (0.6 g/L) had no effect on cell activities, other than growth rate. When the concentration of vanillin increased to 1.2 g/L, both cell growth and productivity were inhibited, whereas the yield of final product was not affected. When vanillin was increased to a high concentration (1.8 and 2.4 g/L), both cell growth and activities were influenced, and butyric-acid production was affected more than cell growth. Cho et al. demonstrated a similar conclusion with SY that vanillin affected butanol production (completely inhibited) more than cell growth (a 22% decrease) (Cho et al., 2009). Moreover, the low yield of butyric-acid production was due to the interference of vanillin on glycolytic pathway to butyryl- CoA (Cho et al., 2009).

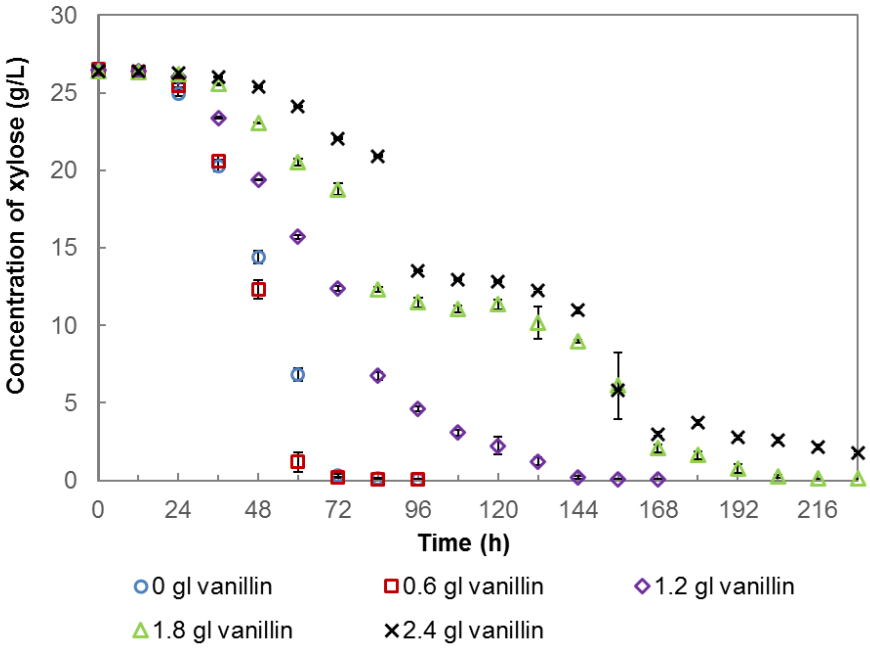

Figure 4.12 The consumption of xylose treated by five concentrations of vanillin

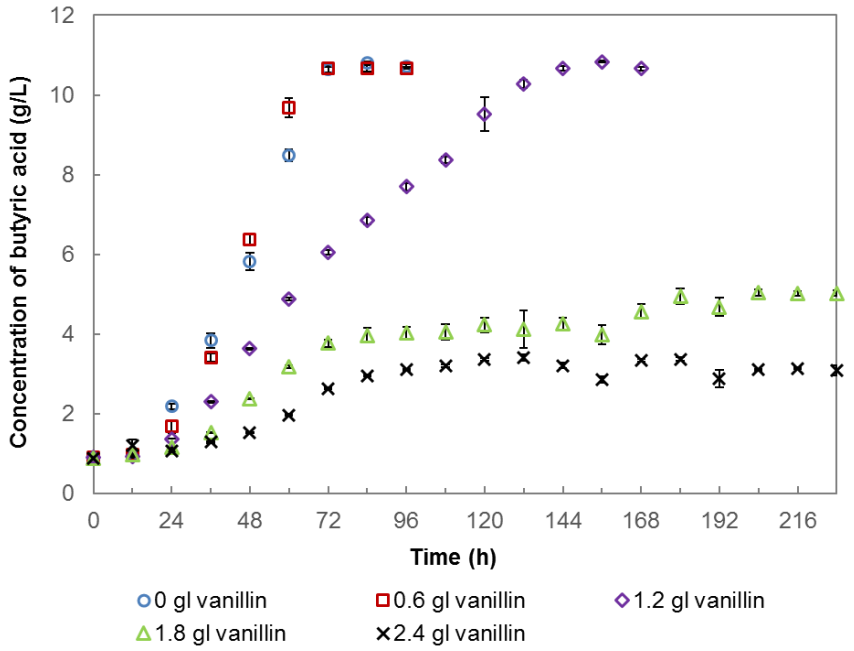

Figure 4.13 The production of butyric acid during fermentation treated by five concentrations of vanillin

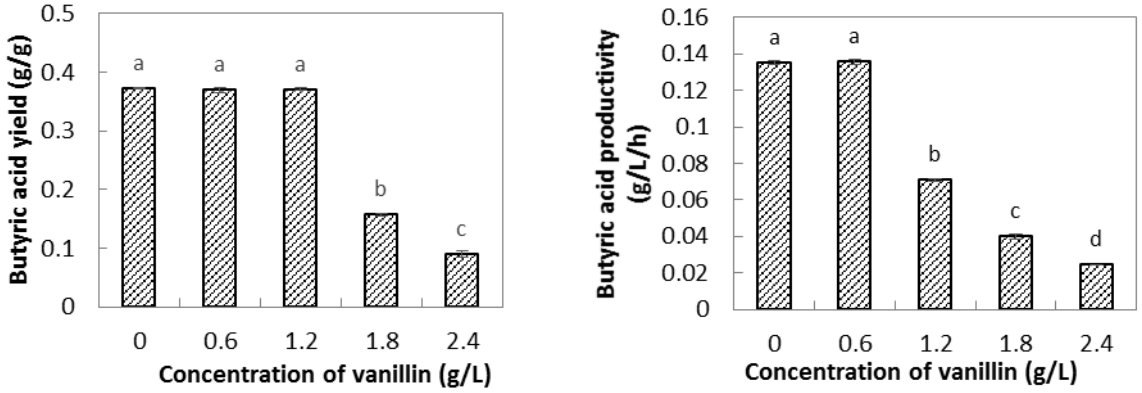

Figure 4.14 a) The yield of butyric acid at each concentrationof vanillin; b) The productivity of butyric acid at each concentration of vanillin

5.4.2.3 Relationship between optical density and its corresponding butyric-acid concentration treated by vanillin

Fig 4.15 displays the relationship between optical density and its corresponding butyric-acid concentration in the vanillin groups 0, 0.6, 1.2, 1.8, and 2.4g/L. The data in groups 0 and 0.6 g/L followed one exponential curve, whereas the data in 1.2 g/L group followed a second exponential curve. The exponential regression shows that with the optical density increased, more butyric acid was produced per OD unit. The linear regressions are shown in Fig. 4.16 and Fig. 4.17 (after a natural-log transformation). The high R-square for these two linear regressions (R^2=0.988 for group 0 and 0.6 g/L, R^2=0.972 for 1.2 g/L) indicates that the linear regressions fit the data well. For the group of 0 and 0.6 g/L vanillin, the equations for linear and exponential regression are y=1.922x-0.908 and $y=0.380e^{1.922x}$, respectively. For the group of 1.2 g/L vanillin, the equations for linear and exponential regression are y=2.583x-1.367 and $y=0.255e^{2.583x}$.

Fig 4.15 indicates that 1.2 g/L of vanillin had negative effects on cell growth without affecting butyric-acid production. A similar phenomenon was observed by Cortez et al., (2010), that 2 g/L of vanillin decreased the cell growth about 20% whereas xylitol production was not affected. The interaction between vanillin and membrane-embedded proteins caused the loss of integrity of the membrane. However, a significant proportion of the cell populations had functional membrane, which could normally produce butyric acid (Fitzgerald et al., 1997). Although the rate of cell growth in log phase was decreased in the group of 1.2 g/L, the stationary phase increased from about 12h (in the control group) to 60h. Thus, actually more biomass was formed during the fermentation in the medium that was treated by 1.2 g/L of vanillin, though the maximum cell-concentration was 83% of control. Hence, since the membranes of a large proportion of cell populations were still functional, the yield of butyric acid was not significantly different from the control.

Data in the group of 1.8 and 2.4 g/L of vanillin formed two clouds, which makes the data not follow a single exponential curve. The clouds formed because the diauxic growth curves of cells in mediums with 1.8 and 2.4 g/L of vanillin (shown in Fig. 4.9). The two stationary phases in the diauxic curves resulted because one optical-density value had several response butyric-acid concentrations. Thus, in the scatterplot, these data formed two clouds. The cells acted differently when they were treated by 1.8 and 2.4 g/L of vanillin, compared to other concentration of vanillin. The difference might be due to the higher toxicity of metabolites when 1.8 and 2.4 g/L of vanillin were consumed.

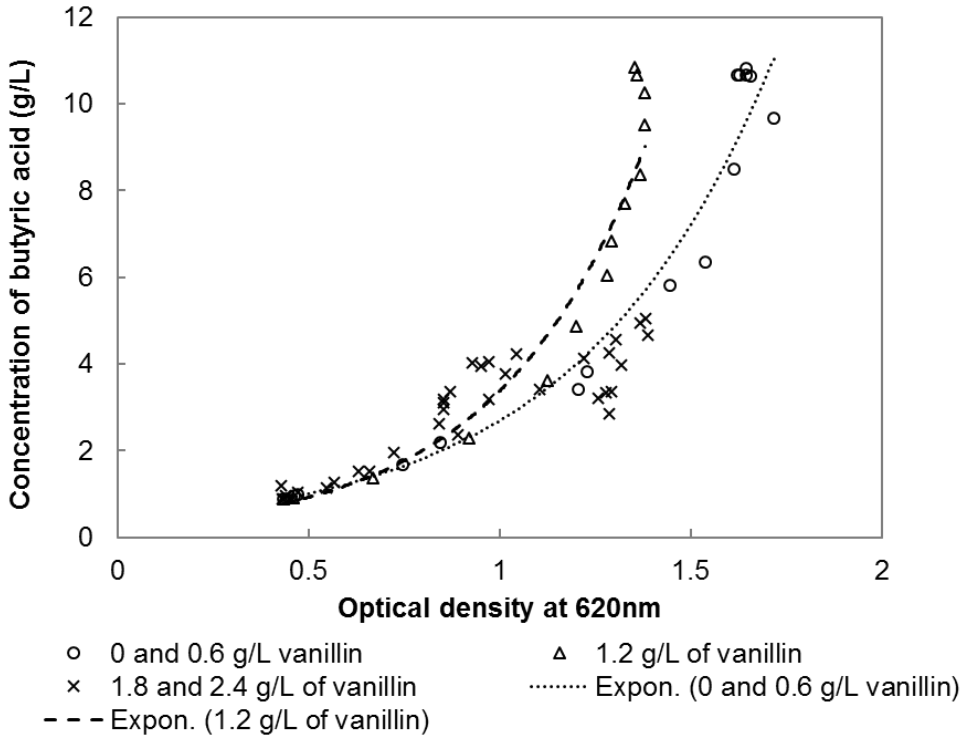

Figure 4.15 The relationship between optical density and its corresponding butyric-acid concentration treated by vanillin

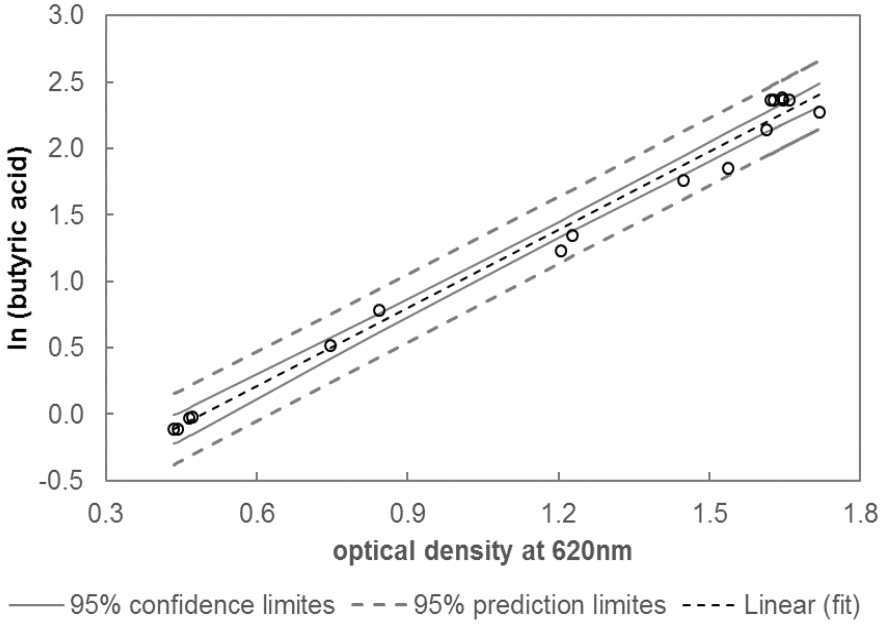

Figure 4.16 The relationship between optical density at 620nm and its corresponding butyric-acid concentration treated by 0 and 0.6 g/L of vanillin (after natural log response)

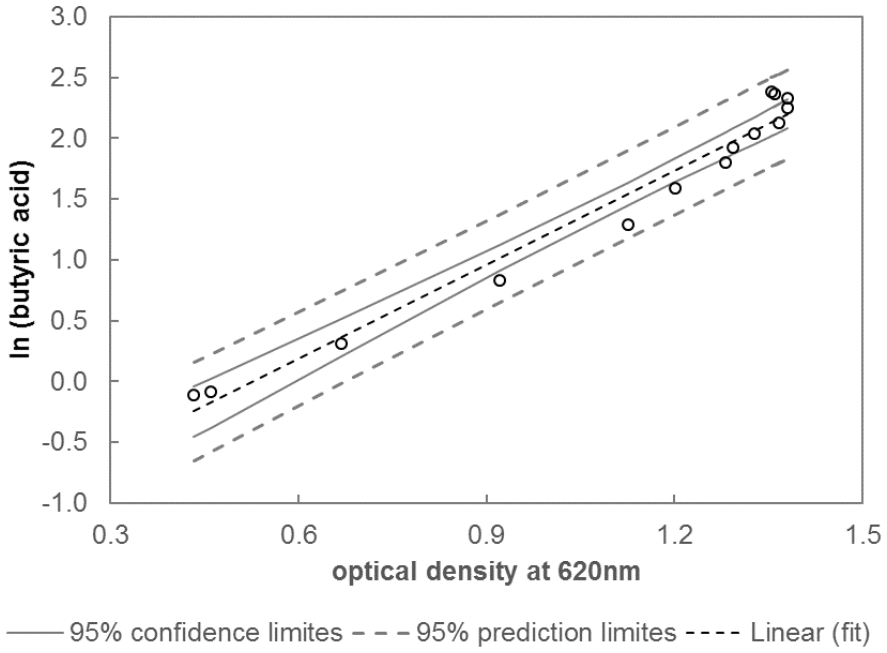

Figure 4.17 The relationship between optical density at 620nm and its corresponding butyric-acid concentration treated by 1.2 g/L of vanillin (after natural log response)

4.4.3 Comparison between syringaldehyde and vanillin

Generally speaking, vanillin is more toxic than syringaldehyde. In the research of Cumin et al., 12 mM of syringaldehyde decreased biomass and lipid content to 84.3% and 94.8% of control. However, vanillin almost completely inhibited cell activities at the same concentration (Hu et al., 2009). One explanation is that the inhibiting ability of aldehydes depends on their hydrophobicities. A more hydrophobilic aldehyde has a stronger inhibitory effect (Hu et al., 2009). However, in our study, the difference of inhibitory severity between these two inhibitors was not obvious. The cell concentrations for 1.8 and 2.4 g/L of SY were 93% and 75% respectively, which were 84% and 78% of those of vanillin. The yields of butyric acid for 1.8

and 2.4 g/L of SY were 57% and 21% of the control, while for vanillin, the yields were 42% and 24%.

4.4 Conclusion

In this chapter, the effects of syringaldehyde and vanillin at different concentrations (0, 0.6, 1.2, 1.8, and 2.4 g/L) on *C. tyrobutyricum* activities were studied. Some conclusions were obtained according to above results.

1. The lag phases for cell growth, xylose consumption, and butyric-acid production were all prolonged with increasing concentrations of both inhibitors, except that the lag phases of cell growth for all vanillin treatment groups were the same.
2. Longer times were required for cells to achieve their maximum cell concentration and maximum butyric-acid production with higher concentrations of each inhibitor.
3. With no more than 0.6 g/L of SY and vanillin, maximum cell-concentration and butyric-acid production were not affected. The solubility of these inhibitors is limited (Palmqvist et al., 2000) and the concentrations of phenolic compounds that were used in model studies were usually far higher than are actually present in the hydrolysate. Thus, these two compounds would likely have little impact on butyric-acid production by *C. tyrobutyricum* when actual lignocellulosic hydrolysate was applied (Hu et al., 2009).
4. When the concentration of SY and vanillin increased from 0.6 g/L to 2.4 g/L, maximum cell-concentration, yield, and production of butyric-acid decreased gradually.
5. Both SY and vanillin could be assimilated by *C. tyrobutyricum*.

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CHAPTER 5 Summaries and Contributions

5.1 Summaries

In order to better utilize lignocellulosic biomass in butyric-acid fermentation by *Clostridium tyrobutyricum*, the inhibitory effects of six potential inhibitors (furfural, HMF, formic acid, levulinic acid, syringaldehyde, and vanillin) that are usually released during pretreatment and hydrolysis of lignocellulosic biomass were studied in this project. The effects of inhibitors on biomass accumulation, yield, and productivity of butyric acid, are summarized in Tables 1, 2, and 3 (Shown in the Appendix). Some conclusions could be drawn as follows:

1. Furfural was the most toxic among the six inhibitors because 1.2 g/L of it could inhibit cell activities completely. But when the concentration of furfural fell to 0.9 g/L or lower, only the lag phase was prolonged. The biomass and butyric-acid production were not affected.

   A low concentration of HMF (less than 0.6 g/L) in the growth medium had no effect on cell activities. With the increase in HMF concentration, biomass accumulation was only reduced to 73.15%, whereas butyric-acid yield and productivity both decreased greatly. In the medium with high concentrations of HMF (1.8 and 2.4 g/L), butyric acid was not generated. Furfural and HMF were both metabolized by *C. tyrobutyricum*.
2. Within certain range of concentrations, weak acids had mild effects on cell growth. When the concentration of formic acid increased from 0 to 4.8 g/L, the biomass and the yield of butyric acid only decreased to 85.78% and 89.12%, respectively. The production of butyric acid decreased because of longer lag and log phases, which were

caused by higher concentrations of formic acid.

With 2.4 and 4.8 g/L of levulinic acid in the growth medium, biomass and butyric-acid production were not influenced. Neither formic acid nor levulinic acid were consumed by *C. tyrobutyricum*.

3. As phenolic compounds, low concentrations of syringaldehyde and vanillin (0.6 g/L) had no inhibitory effects on cell growth. When the concentration increased from 0.6 to 2.4 g/L, both of these two inhibitors gradually reduced the maximum cell-concentration, yield, and productivity of butyric acid. Syringldehyde and vanillin were assimilated by *C. tyrobutyricum*.

5.2 Contributions

Much previous research explored the inhibitory effects of substances that are released in biomass hydrolysates. These studies focused mainly on biofuels production and on strains that are capable of producing biofuels. But the effects of inhibitors that work on valuable bio-products, such as butyric acid, were not fully investigated. This project provided significant information about the effects of three groups of inhibitors, furan derivatives (furfural and HMF), organic acids (formic acid and lenulinic acid), and phenolic compounds (syringaldehyde and vanillin) on *C. tyrobutyricum* biomass-accumulation and butyric-acid production. First, in general, the results of this project supported previous research, in that different types of inhibitors with different concentrations had varying inhibitory effects on cell growth and butyric-acid production. Second, the information that was obtained from experiments could be applied to guide practical production of butyric acid when using

lignocellulosic biomass as the carbon source. For example, if the concentration of levulinic acid in hydrolysate is less than 4.8 g/L, its impact could be neglected, thereby saving time and energy when producing butyric acid. Third, performance information about the specific ranges of inhibitors in this project provides a way to manage and control the fermentation by adjusting the concentration of inhibitors to achieve certain biomass accumulation and butyric-acid production. Fourth, this project is also a fundamental study for other researchers to utilize in their future research.

5.3 Future work

To complete the study of inhibitors that affect *C. tyrobutyricum*, more work should be conducted.

1. Due to limited time and conditions, six kinds of potential inhibitors that are classified in three groups were selected as typical inhibitors in this project. Other inhibitors, for example, hydroxybenzaldehyde and ferulic acid, might also be worthwhile for study by similar experimental processes.
2. Presently the inhibitory mechanisms of some inhibitors, such as phenolic compounds, are still unknown. One solution is to label the key enzymes in *C. tyrobutyricum* cells (such as KB, PTA, and so on), and measure the enzymatic activities during fermentation. The inhibitory mechanisms of the chemicals on the processes of cell replication and metabolism can be investigated.
3. It was known that synergistic effects would occur when more than one kind of inhibitor was present at significant concentrations in hydrolysates. Study about interaction

effects among six inhibitors is necessary after single-inhibitor investigation. A response-surface methodology (RSM) could be designed for this purpose to analyze the synergistic effects comprehensively.

4. This research was based on laboratory-scale experiments. These experiments could be extended to a pilot scale to verify the obtained results, and to apply to industrial production.

APPENDIX

Appendix A- Summarization of effects by inhibitors on *C. tyrobutyricum*

Table 1 Summarization of effects on maximum cell concentration for each inhibitor at different concentration

Concentration (g/L)	Furfural	HMF	Formic acid	Levulinic acid	Syringaldehyde	Vanillin
Control (0)	100% a	100% d	100% g	100% k	100% l	100% p
0.3	97.67%±1.32% a	-	-	-	-	-
0.6	96.50%±2.78% a	98.87%±1.39% d	-	-	101.16%±2.13% l	103.60%±3.03% p
0.9	97.46%±1.18% a	-	-	-	-	-
1.2	16.63%±0.87% b	99.88%±5.69% d	93.19%±5.01% gh	-	93.81%±1.34% m	83.26%±1.07% q
1.8	-	73.15%±4.02% e	-	-	93.08%±1.14% m	83.81%±2.06% q
2.4	-	79.29%±2.33% e	93.59%±4.26% gh	101.44%±2.38% k	75.14%±1.33% n	77.95%±0.98% r
3.6	-	-	88.76%±7.16% gh	-	-	-
4.8	-	-	85.78%±1.96% h	97.93%±1.07% k	-	-

*****1) The control values at each inhibitor group were set as 100%. The percentage was obtained by using values at different concentrations of each inhibitor, divided by the control values of the same group. 2) Different groups of letters: ab, de, gh, k, lmn, and pqr were used for six inhibitors to indicate significant differences between each concentration respectively, in the order a>b, d>e, g>h, l>m>n, and p>q>r.

Table 2 Summarization of effects on yield of butyric acid for each inhibitor at different concentration

Concentration (g/L)	Furfural	HMF	Formic acid	Levulinic acid	Syringaldehyde	Vanillin
Control (0)	100% a	100% d	100% g	100% k	100% l	100% p
0.3	98.76%±1.44% a	-	-	-	-	-
0.6	100.21%±2.94% a	98.11%±3.91% d	-	-	99.45%±5.92% l	99.43%±0.87% p
0.9	97.82%±1.30% a	-	-	-	-	-
1.2	0.00%±0.00% b	100.40%±6.06% d	97.08%±3.54% gh	-	85.44%±7.44% m	99.82%±0.35% p
1.8	-	11.22%±13.99% e	-	-	56.94%±1.20% n	42.32%±0.73% q
2.4	-	0.00%±0.00% e	89.12%±1.89% i	99.85%±1.29% k	20.94%±2.11% o	24.16%±1.48% r
3.6	-	-	93.83%±3.81% ghi	-	-	-
4.8	-	-	92.35% ±3.09% hi	104.37%±4.67% k	-	-

*****1) The values for control at each inhibitor group were set as 100%. The percentage was obtained by using values at different concentrations of each inhibitor, divided by the control values of the same group. 2) Different groups of letters: ab, de, ghi, k, lmno, and pqr were used for six inhibitors to indicate significant difference between each concentration respectively, in the order a>b, d>e, g>h>i, l>m>n>o, and p>q>r.

Table 3 Summarization of effects on productivity of butyric acid for each inhibitor at different concentration

Concentration (g/L)	Furfural	HMF	Formic acid	Levulinic acid	Syringaldehyde	Vanillin
Control (0)	100% a	100% d	100% g	100% k	100% l	100% p
0.3	99.64%±0.38% a	-	-	-	-	-
0.6	79.19%±1.30% b	100.47%±4.34% d	-	-	96.65%±2.58% l	100.26%±0.39% p
0.9	78.53%±1.04% b	-	-	-	-	-
1.2	0.00%±0.00% c	74.44%±5.38% e	75.56%±2.58% h	-	60.98%±6.44% m	52.51%±0.90% q
1.8	-	6.12%±7.72% f	-	-	37.29%±1.80% n	29.49%±1.01% r
2.4	-	5.18%±2.19% f	71.71%±2.51% h	100.18%±1.15% k	10.54%±1.19% o	17.99%±0.31% s
3.6	-	-	52.26%±0.65% i	-	-	-
4.8	-	-	44.36%±2.26% j	101.04%±6.75% k	-	-

*****1) The values for control at each inhibitor group were set as 100%. The percentage was obtained by using values at different concentrations of each inhibitor, divided by the control values of the same group. 2) Different groups of letters: abc, def, ghij, k, lmno, and pqrs were used for six inhibitors to indicate significant differences between each concentration respectively, in the order a>b>c, d>e>f, g>h>i>j, l>m>n>o, and p>q>r>s.

Appendix B- The data used for presenting the relationships between optical density (at 620 nm) and butyric acid production in each group

Table 4 The data used for presenting the relationships between optical density and butyric acid production in the groups of furan derivatives

furfural			**HMF**				
0, 0.3, 0.6, 0.9, and 1.2 g/L of furfural			**0, 0.6, and 1.2 g/L of HMF**			**1.8 and 2.4 g/L of HMF**	
optical density at 620nm	**butyric acid (g/L)**	**ln (butyric acid)**	**optical density at 620nm**	**butyric acid (g/L)**	**ln (butyric acid)**	**optical density at 620nm**	**butyric acid (g/L)**
0.221	0.301	-1.202	0.282	0.362	-1.015	0.266	0.357
0.238	0.307	-1.180	0.258	0.363	-1.013	0.267	0.364
0.258	0.336	-1.091	0.276	0.369	-0.996	0.256	0.375
0.276	0.341	-1.076	0.273	0.372	-0.988	0.260	0.379
0.242	0.341	-1.075	0.260	0.375	-0.980	0.250	0.379
0.248	0.346	-1.060	0.260	0.376	-0.979	0.254	0.378
0.275	0.352	-1.043	0.269	0.380	-0.968	0.237	0.381
0.276	0.357	-1.031	0.266	0.384	-0.958	0.265	0.398
0.270	0.358	-1.028	0.269	0.400	-0.918	0.349	0.463
0.262	0.359	-1.024	0.256	0.407	-0.898	0.891	0.767
0.268	0.362	-1.015	0.277	0.425	-0.856	1.101	0.833
0.266	0.366	-1.006	0.551	0.771	-0.260	1.225	0.675
0.267	0.372	-0.990	0.648	0.935	-0.067	1.213	0.696
0.291	0.396	-0.927	0.352	0.995	-0.005	1.103	1.030
0.444	0.532	-0.631	0.528	1.721	0.543	1.050	1.304
0.676	0.743	-0.298	1.013	2.114	0.749	1.197	1.476
0.675	0.839	-0.175	0.814	2.413	0.881	0.286	0.358
0.774	1.123	0.116	1.116	2.448	0.895	0.286	0.362
0.903	1.718	0.541	1.122	3.352	1.209	0.274	0.364
1.099	2.286	0.827	1.358	3.988	1.383	0.279	0.366

1.167	2.540	0.932	1.380	4.347	1.469	0.272	0.364
1.258	3.197	1.162	1.542	5.584	1.720	0.272	0.367
1.242	3.517	1.258	1.534	6.162	1.818	0.311	0.366
1.339	4.533	1.511	1.528	6.439	1.862	0.298	0.388
1.458	5.031	1.616	1.648	7.203	1.974	0.620	0.615
1.466	5.245	1.657	1.632	8.240	2.109	0.447	0.624
1.440	5.426	1.691	1.648	8.317	2.118	0.510	0.852
1.520	6.450	1.864	1.607	9.808	2.283	0.848	1.036
1.602	7.571	2.024	1.676	9.823	2.285	1.275	0.595
1.582	7.584	2.026	1.657	9.849	2.287	1.330	0.378
1.576	7.638	2.033	1.622	9.989	2.301	-	-
1.598	8.360	2.123	1.673	10.019	2.304	-	-
1.666	9.354	2.236	-	-	-	-	-
1.663	9.435	2.244	-	-	-	-	-
1.646	9.783	2.281	-	-	-	-	-
1.706	9.880	2.291	-	-	-	-	-
1.652	10.306	2.333	-	-	-	-	-
1.615	10.321	2.334	-	-	-	-	-
1.535	10.328	2.335	-	-	-	-	-

* The column name “butyric acid” refers to the concentration of butyric acid in the medium

Table 5 The data used for presenting the relationships between optical density and butyric acid production in the groups of organic acids

formic acid			levulinic acid		
0, 1.2, 2.4, 3.6, and 4.8 g/L of formic acid			0, 2.4, and 4.8 g/L of levulnic acid		
optical density at 620nm	**butyric acid (g/L)**	**ln (butyric acid)**	**optical density at 620nm**	**butyric acid (g/L)**	**ln (butyric acid)**
0.239	0.319	-1.144	0.255	0.317	-1.149
0.243	0.321	-1.135	0.233	0.321	-1.137
0.253	0.325	-1.125	0.265	0.337	-1.088
0.257	0.325	-1.123	0.258	0.338	-1.086
0.263	0.326	-1.120	0.257	0.340	-1.078
0.254	0.332	-1.102	0.256	0.341	-1.075
0.261	0.333	-1.100	0.239	0.342	-1.073
0.243	0.333	-1.100	0.262	0.343	-1.071
0.241	0.363	-1.012	0.264	0.345	-1.065
0.238	0.365	-1.008	0.304	0.429	-0.846
0.241	0.365	-1.008	0.313	0.431	-0.842
0.241	0.366	-1.005	0.317	0.434	-0.835
0.259	0.366	-1.004	0.890	1.452	0.373
0.226	0.377	-0.976	0.863	1.487	0.397
0.264	0.383	-0.961	0.866	1.567	0.449
0.274	0.386	-0.951	1.265	3.462	1.242
0.244	0.403	-0.909	1.301	3.627	1.288
0.298	0.435	-0.832	1.354	3.765	1.326
0.287	0.449	-0.800	1.457	5.246	1.657
0.273	0.472	-0.751	1.515	5.506	1.706
0.395	0.634	-0.456	1.541	5.681	1.737
0.503	0.897	-0.109	1.551	6.780	1.914

0.799	1.181	0.166	1.609	7.279	1.985
0.635	1.213	0.193	1.610	7.302	1.988
0.674	1.259	0.230	1.600	8.123	2.095
0.852	1.801	0.588	1.633	8.806	2.175
0.763	1.896	0.640	1.575	8.819	2.177
0.808	1.903	0.644	1.657	8.963	2.193
0.815	2.056	0.721	1.564	9.159	2.215
0.900	2.518	0.924	-	-	-
1.023	2.665	0.980	-	-	-
0.991	2.754	1.013	-	-	-
1.277	3.245	1.177	-	-	-
1.185	3.257	1.181	-	-	-
1.050	3.515	1.257	-	-	-
1.295	3.804	1.336	-	-	-
1.163	3.829	1.343	-	-	-
1.155	3.919	1.366	-	-	-
1.250	4.521	1.509	-	-	-
1.358	4.874	1.584	-	-	-
1.280	4.919	1.593	-	-	-
1.409	4.990	1.607	-	-	-
1.516	5.558	1.715	-	-	-
1.528	5.760	1.751	-	-	-
1.278	6.321	1.844	-	-	-
1.376	6.497	1.871	-	-	-
1.495	6.639	1.893	-	-	-
1.433	7.379	1.999	-	-	-
1.527	7.765	2.050	-	-	-
1.646	7.856	2.061	-	-	-
1.611	8.080	2.089	-	-	-

1.545	8.483	2.138	-	-	-
1.449	8.537	2.144	-	-	-
1.604	8.698	2.163	-	-	-
1.394	9.100	2.208	-	-	-
1.477	9.370	2.238	-	-	-
1.484	9.578	2.259	-	-	-
1.382	9.622	2.264	-	-	-
1.721	9.891	2.292	-	-	-
1.682	10.158	2.318	-	-	-

* The column name “butyric acid” refers to the concentration of butyric acid in the medium

Table 6 The data used for presenting the relationships between optical density and butyric acid production in the groups of phenolic compounds

syringaldehyde			vanillin							
0, 0.6, 1.2, 1.8, and 2.4 g/L			0 and 0.6 g/L			1.2 g/L			1.8 and 2.4 g/L	
optical density at 620nm	butyric acid (g/L)	ln (butyric acid)	optical density at 620nm	butyric acid (g/L)	ln (butyric acid)	optical density at 620nm	butyric acid (g/L)	ln (butyric acid)	optical density at 620nm	butyric acid (g/L)
0.718	0.198	-1.618	0.436	0.894	-0.112	0.433	0.891	-0.115	0.434	0.890
0.745	0.205	-1.586	0.442	0.897	-0.109	0.459	0.920	-0.083	0.440	0.970
0.476	0.240	-1.429	0.465	0.971	-0.030	0.668	1.371	0.316	0.548	1.145
0.759	0.249	-1.390	0.471	0.979	-0.021	0.921	2.298	0.832	0.659	1.523
0.228	0.270	-1.311	0.747	1.679	0.518	1.126	3.630	1.289	0.892	2.366
0.239	0.271	-1.306	0.844	2.180	0.779	1.201	4.879	1.585	0.972	3.185
0.244	0.272	-1.303	1.205	3.409	1.226	1.281	6.050	1.800	1.017	3.763
0.233	0.274	-1.296	1.228	3.834	1.344	1.293	6.848	1.924	0.953	3.965
0.238	0.274	-1.295	1.448	5.817	1.761	1.328	7.696	2.041	0.930	4.026
0.240	0.274	-1.295	1.538	6.361	1.850	1.367	8.377	2.125	0.974	4.052
0.238	0.274	-1.293	1.614	8.490	2.139	1.381	9.512	2.253	1.045	4.223
0.241	0.275	-1.291	1.718	9.680	2.270	1.380	10.269	2.329	1.221	4.119
0.250	0.275	-1.291	1.658	10.639	2.364	1.359	10.668	2.367	1.286	4.256
0.242	0.276	-1.289	1.646	10.661	2.367	1.355	10.838	2.383	1.318	3.976
0.277	0.278	-1.280	1.622	10.666	2.367	-	-	-	1.306	4.561
0.244	0.278	-1.279	1.629	10.672	2.368	-	-	-	1.369	4.947
0.290	0.278	-1.279	1.645	10.810	2.381	-	-	-	1.390	4.680
0.241	0.279	-1.278	-	-	-	-	-	-	1.384	5.044
0.231	0.279	-1.278	-	-	-	-	-	-	0.433	0.881
0.246	0.280	-1.274	-	-	-	-	-	-	0.428	1.195
0.250	0.280	-1.273	-	-	-	-	-	-	0.472	1.052

0.266	0.280	-1.273	-	-	-	-	-	-	0.568	1.281
0.252	0.280	-1.273	-	-	-	-	-	-	0.632	1.525
0.243	0.282	-1.267	-	-	-	-	-	-	0.724	1.970
0.261	0.282	-1.266	-	-	-	-	-	-	0.843	2.634
0.235	0.283	-1.263	-	-	-	-	-	-	0.853	2.951
0.259	0.288	-1.246	-	-	-	-	-	-	0.854	3.103
0.242	0.290	-1.238	-	-	-	-	-	-	0.854	3.196
0.241	0.292	-1.231	-	-	-	-	-	-	0.871	3.362
0.223	0.292	-1.231	-	-	-	-	-	-	1.104	3.404
0.267	0.297	-1.214	-	-	-	-	-	-	1.258	3.208
0.239	0.298	-1.211	-	-	-	-	-	-	1.287	2.853
0.290	0.318	-1.147	-	-	-	-	-	-	1.279	3.332
0.825	0.338	-1.086	-	-	-	-	-	-	1.293	3.367
0.275	0.358	-1.026	-	-	-	-	-	-	-	-
0.564	0.417	-0.874	-	-	-	-	-	-	-	-
0.932	0.534	-0.628	-	-	-	-	-	-	-	-
0.416	0.553	-0.592	-	-	-	-	-	-	-	-
0.482	0.587	-0.533	-	-	-	-	-	-	-	-
0.853	0.681	-0.384	-	-	-	-	-	-	-	-
1.076	0.914	-0.090	-	-	-	-	-	-	-	-
0.811	1.159	0.147	-	-	-	-	-	-	-	-
1.063	1.329	0.284	-	-	-	-	-	-	-	-
0.833	1.394	0.332	-	-	-	-	-	-	-	-
1.241	1.648	0.499	-	-	-	-	-	-	-	-
1.032	1.815	0.596	-	-	-	-	-	-	-	-
1.265	2.117	0.750	-	-	-	-	-	-	-	-
1.166	2.118	0.751	-	-	-	-	-	-	-	-
1.276	2.368	0.862	-	-	-	-	-	-	-	-
1.145	2.680	0.986	-	-	-	-	-	-	-	-

1.295	3.214	1.168	-	-	-	-	-	-	-	-
1.324	3.541	1.265	-	-	-	-	-	-	-	-
1.259	3.740	1.319	-	-	-	-	-	-	-	-
1.567	4.583	1.522	-	-	-	-	-	-	-	-
1.375	4.695	1.547	-	-	-	-	-	-	-	-
1.501	5.093	1.628	-	-	-	-	-	-	-	-
1.554	5.247	1.658	-	-	-	-	-	-	-	-
1.533	5.266	1.661	-	-	-	-	-	-	-	-
1.559	5.662	1.734	-	-	-	-	-	-	-	-
1.489	5.925	1.779	-	-	-	-	-	-	-	-
1.534	6.898	1.931	-	-	-	-	-	-	-	-
1.637	6.980	1.943	-	-	-	-	-	-	-	-
1.579	7.763	2.049	-	-	-	-	-	-	-	-
1.548	7.811	2.056	-	-	-	-	-	-	-	-
1.703	8.274	2.113	-	-	-	-	-	-	-	-
1.683	8.501	2.140	-	-	-	-	-	-	-	-
1.602	9.065	2.204	-	-	-	-	-	-	-	-
1.661	9.203	2.220	-	-	-	-	-	-	-	-

* The column name “butyric acid” refers to the concentration of butyric acid in the medium