

BUTYRIC AND DOCOSAHEXAENOIC ACIDS PRODUCTION FROM HEMICELLULOSE

by

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## Abstract

Many of the current industrial fermentation processes cannot use pentose as the carbon source. However, complete substrate utilization of sugars in lignocellulose is one of the prerequisites to render economic development of biofuels or bioproducts from biomass. In this study we proposed a new process for DHA production from renewable carbon sources by first using anaerobic bacteria, *Clostridium tyrobutyricum* to convert pentose into organic acids with butyric acid as the main product, and then using the organic acids to feed microalgae, *Cryptocodinium cohnii* to produce DHA.

The effect of glucose and xylose on the yield of butyric acid produced by *C. tyrobutyricum* was investigated, separately. Cell growth of *C. tyrobutyricum* increased with increasing initial glucose or xylose concentration, but was not affected significantly when the concentration was above 55g/l for glucose or 35g/l for xylose. Butyric acid yield increased as the initial sugar concentration increased in both xylose and glucose, but the conversion rate from xylose or glucose to butyric acid decreased as the sugar concentration increased. The xylose to glucose ratio in their mixture did not significantly affect cell growth or butyric acid yield.

The effect of arabinose on the yield of butyric acid produced by *C. tyrobutyricum* was also studied. As for butyric acid production, compared with glucose or xylose, the arabinose was in a low efficiency, with butyric acid output of 2.25g/l in 10g/l arabinose and a long lag period of about 3-4 d. However, a low concentration of arabinose could be used as a nutritional supplement to improve the fermentability of a mixture of xylose and glucose. The conversion rate of sugar to butyric acid increased as the supplement arabinose increased.

In order to obtain low cost xylose, corncobs were hydrolyzed and this xylose-rich product was used to culture *C. tyrobutyricum*. The results showed that at end of the 9 d fermentation, the concentration of butyric acid from corncob hydrolysate reached 10.56 g/l, and the mimic medium reached 11.3 g/l. This suggests that corncob hydrolysate can be used as a carbon source for butyric acid production by *C. tyrobutyricum*, although some inhibitory effects were found on cell growth with corncob hydrolysate.

The effect of butyric acid, lactic acid and acetic acid on the yield of DHA produced by *C. cohnii* was also investigated, separately. The DHA yield was highly related to both biomass and DHA content in the cell, whereas lower growth rate could bring higher DHA content. The best

concentration for DHA yield seemed to be 1.2g/l in three single organic acid media. In two organic acids mixture media, acetic acid tended to be beneficial for biomass accumulation, regardless whether butyric acid or lactic acid was mixed with acetic acid, the OD could reach 1.3 or above. When butyric acid was mixed with lactic acid, the highest DHA yield was achieved, due to increased DHA content from mutual influence between butyric acid and lactic acid.

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## ● Chapter 1-Introduction

### ■ 1.1 Plant biomass

Among all kinds of plant biomass, lignocellulose is the most abundant renewable biomass; its annual production has been estimated in  $1 \times 10^{10}$  MT worldwide (Sanchez, 2008). Plant biomass is mainly composed of cellulose, hemicellulose, and lignin, with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash (Jørgensen, Kristensen, & Felby, 2007). The composition of these constituents can vary from one plant species to another (Table 1.1) (P. Kumar, 2009). For example, hardwood has greater amounts of cellulose, whereas wheat straw and leaves have more hemicelluloses (Sun, 2002). In addition, the ratios between various constituents within a single plant vary with age, stage of growth, and other conditions (Pérez & J., 2002).

**Table 1.1 Cellulos, hemicellulose and lignin contents in common agricultural residues and wastes (P. Kumar, 2009)**

lignocellulosic material	cellulose (%)	hemicellulose (%)	lignin (%)
hardwood stems	40-55	24-40	18-25
softwood stems	45-50	25-35	25-35
nut shells	25-30	25-30	30-40
corn cobs	45	35	15
grasses	25-40	35-50	10-30
paper	85-99	0	0-15
wheat straw	30	50	15
sorted refuse	60	20	20
leaves	15-20	80-85	0
cotton seed hairs	80-95	5-20	0
newspaper	40-55	25-40	18-30
waste papers from chemical pulps	60-70	10-20	5-10
primary wastewater solids	8-15		
solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
coastal bermudagrass	25	35.7	6.4
switchgrass	45	31.4	12
swine waste	6	28	na

Lignocellulose is a cohesive composite material constructed around a framework of cellulose microfibrils, endowing the material with tensile strength and stiffness. Cellulose microfibrils provide the structural framework of the wall and these are associated with a coating

of hemicellulosic polysaccharides that hydrogen-bond to the microfibril surface and span the distance between fibrils, effectively tethering them to one another. This polysaccharide complex is effectively interpenetrated and encased by lignin, a polyphenolic polymer. The lignin is artificially thinned towards the left hand side of the image, simply to enable the polysaccharide components to be seen; in lignocellulosic biomass, the polysaccharides are mostly sealed up in the lignin matrix.

### ◆ *1.1.1 Cellulose*

Cellulose comprises about two-thirds of the total carbohydrate content for most woody plants and about half of the total carbohydrate content for herbaceous plants (Lynd, 1996). Cellulose microfibrils are macromolecular structures, composed of semicrystalline arrays of  $\beta$ -1, 4-glucan chains. The chains associate with one another through extensive hydrogen bonding. Each glucose residue is inversely relative to its neighbors, resulting in a linear chain of sugar residues. This linearity allows close associations to form with neighboring chains over long distances, resulting in a semicrystalline structure. In turn, the crystallinity of cellulose microfibrils makes them resistant to hydrolysis, because the absence of water from the structure and the strong associations between glucan chains impede the access of hydrolases to the individual  $\beta$ -1, 4-glucan chains. The  $\beta$ -glucan chains of cellulose are highly insoluble and the ordered nature of cellulose microfibrils arises, because the individual polymers in the fibril are synthesized simultaneously by the close association of numerous catalytic units (Turner, 2007). The result is a highly ordered, crystalline material that is recalcitrant to rapid reaction under many conditions.

### ◆ *1.1.2 Hemicellulose*

Hemicellulose is a polymer that occurs in association with cellulose and generally comprises 20–35% of the dry mass of biomass, representing the second most abundant sugar-based polymer in plant biomass (Lynd, 1996). Hemicellulose is composed primarily of several sugars, the identity and proportion of which depend on the type of plant material. These monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (e.g., 4-omethylglucuronic, D-glucuronic, and D-galactouronic acids). As the anhydrides, the xylan and arabinan contents of hemicelluloses vary with the plant species. The xylan content of hardwoods is generally much higher than that

of softwoods, ranging between 11% and 25% in the former and between 3% and 8% in the latter. Hemicelluloses in hardwoods contain appreciable amounts of D-xylose, D-mannose, acetyl, and uronic acid. The acetyl content ranges between 3% and 4.5% in hardwoods and between 1% and 1.5% in softwoods; uronic acid (as the anhydride) ranges between 3% and 5% in both hardwoods and softwoods. In conifers, the predominant hemicellulosic sugar is D-mannose, which, as mannan, averages about 11% of the total dry weight. Whereas the xylan content of softwoods is lower than in hardwoods, the lignin content is higher. The predominant hemicellulosic sugar of agricultural residues is D-xylose. The xylan content of corn residues varies from about 17% in the leaves and stalks to 31% in the cobs, but, on the average, it comprises about 24% of the total dry weight of corn stover (T. Jeffries, 1983).

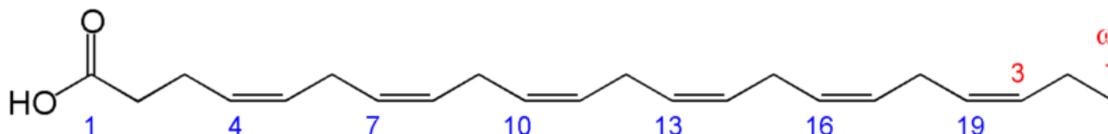
The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by  $\beta$ -(1, 4)-glycosidic bonds and occasionally  $\beta$ -(1, 3)-glycosidic bonds (Kuhad, 1997). Characterized by a branched structure, hemicellulose is essentially noncrystalline and generally more reactive than cellulose. Comparing with cellulose, hemicellulose can be readily hydrolyzed by dilute acids under moderate conditions, for hemicellulose has a relatively open structure, which facilitates diffusion of acid into the polymer and speeds hydrolysis. Meanwhile, hemicellulose can execute own hydrolysis: acetyl groups are readily hydrolyzed off, and the resulting acetic acid catalyzes the partial depolymerization of the hemicellulose (Jeffries, 1983).

### ◆ *1.1.3 Challenges in the utilization of hemicellulose*

In the biochemical conversion of lignocellulose to biofuels (e.g., ethanol and butanol), hemicellulose is usually separated before cellulose hydrolysis. Conversion of cellulose to glucose and biofuels is relatively easier compared to hemicellulose use. Hemicellulose, a heteropolymer of hexose and pentose sugars, comprises one-fifth to half of the total carbohydrates (Aristidou, 2000). Xylose, released from hemicellulose and the main portion of the sugar monomers, creates a roadblock to lignocellulosic fermentation because the yeast and bacterial species commonly used for cellulose fermentation do not metabolize pentose sugars such as xylose and arabinose efficiently because the sugars inhibit their activity (Chang, 2007; Gomez, 2008). To make products from plant biomass economical in industry, all the sugars derived from plant biomass must be converted into useful products. Therefore, effective utilization of xylose and arabinose in plant biomass is critical to the bioconversion of plant biomass to useful chemicals.

## ■ 1.2 DHA production from microbes

Lots of microalgae possess very high lipid contents. Consequently, some species are a good source of polyunsaturated fatty acid (M. De Swaaf, 2003; Pyle, 2008) and a potential source of biodiesel (Cheng et al., 2009; Gao, Zhai, Ding, & Wu, 2010). Among them, the docosahexaenoic acid (DHA) is the one of particular interest. DHA (molecular structure shown in Figure 1.1) is a polyunsaturated fatty acid (PUFA) that belongs to the  $\omega$ -3 group. DHA is a very high value product. It is reported that the DHA, a product like Neuromin is currently sold for about € 2,000 - 3,000/kg (M. De Swaaf, 2004). Recently, DHA draws much attention, due to its various beneficial contributions to human health. A partial list of diseases that may be prevented or ameliorated with n-3 fatty acids include coronary heart disease and stroke; essential fatty acid deficiency in infancy (retinal and brain development); autoimmune disorders (eg, lupus and nephropathy); Crohn disease; cancers of the breast, colon, and prostate; mild hypertension; and rheumatoid arthritis (Connor, 2000). Although the optimal intake of PUFAs has not yet been established, there is some consensus that the PUFA intake should be at least 3% and preferably 8-23% of the total lipid intake (Gill & GILL, 1997). The British Nutrition Foundation recommended a  $\omega$ -6 to  $\omega$ -3 PUFA ratio between 5:1 and 3:1 (British Nutrition Foundation 1992).



**Figure 1.1 Docosahexaenoic Acid Molecular Structure (M. De Swaaf, 2003)**

Currently, fish oil is the major source of DHA, however, it suffers several problems (Vázquez, 2011). As a food additive, fish oil has an unpleasant taste, and test. More importantly it has poor oxidative stability. The supply of fish oil is not stable, due to declining fish stocks in many oceans, and also the seasonal availability. The undesirable existence of EPA in the DHA will result in neonate growth retardation, when using in the infant food. The contamination of fish oil with metals, radiation and other pollutants will give rise to several unpredictable problems.

Some microorganisms with a high content of DHA could become promising alternatives. Among the heterotrophic marine dinoflagellates, *Cryptecodinium cohnii* has been identified as a prolific producer of DHA, for this microalga is extraordinary in that it produces no other PUFAs than DHA in its cell lipid in any significant amount, which makes the DHA purification process

very attractive.

### ■ 1.3 Objectives

In order to utilize hemicellulose in biomass and develop high value products from it, we developed a unique two-step fermentation to produce DHA. The two core parts of the fermentation are (1) xylose/arabinose fermentation by *Clostridium tyrobutyricum* to produce organic acid mixture with butyric acid as the main product, and (2) organic acid mixture fermentation by *C. cohnii* to produce DHA. The specific objectives of the research project were to:

- Understand the effect of varying the initial concentration and mixing ratios of glucose and xylose on butyric acid yield by *C. tyrobutyricum*
- Understand the fermentation of arabinose and the effect of adding arabinose to glucose/xylose mixture on butyric acid yield by *C. tyrobutyricum*, and demonstrate the feasibility of using corn cob hydrolysate as the fermentation substrate for *C. tyrobutyricum* to produce butyric acid
- Understand the effect of varying the initial concentration and mixing ratio of butyric acid, acetic acid, and lactic acid on DHA yield by *C. cohnii*

## ● Chapter 2 - Literature review

As previously described, efficient use of hemicellulose is of great importance to economic development of biofuels and bioproducts from lignocellulosic biomass. This chapter focuses on the literature of hemicellulose utilization and DHA generation by microbes.

### ■ 2.1 Utilization of xylose by microbes

#### ◆ 2.1.1 Filamentous fungi

Some filamentous fungi can ferment xylose. For example, the anaerobic *Chytridiomycete fungi* are important symbionts in the gastrointestinal tract of many herbivorous mammals (Trinci, 1994). They contribute substantially to the degradation of plant polymers that form a major constituent of the diets of both ruminants and hindgut fermenters (Boxma, 2004). These anaerobic eukaryotic microorganisms lack mitochondria; instead, they possess ATP-generating organelles called “hydrogenosomes” (Müller & Muller, 1993). Besides ATP, these fungal hydrogenosomes also produce hydrogen, CO<sub>2</sub>, acetate and formate (Akhmanova, 1999; Marvin Sikkema, 1990; Marvin Sikkema, 1993; Marvin Sikkema, 1994).

Aerobic filamentous fungi could tolerate industrial substrates well and ferment pentose sugars (Skoog, 1988). However, while concentrations of ethanol produced by filamentous fungi are surprisingly high for organisms traditionally considered non-fermentative, they are still too low for industrial production. Additionally, the rates of ethanol productivity are slow when compared to those of *S. cerevisiae* or *Zymomollas mobilis* (Skory, 1997). But filamentous fungi also have a proven record in the industry where they are used in large scale for production of antibiotics and acids (Atkinson and Mavituna 1991). Example are numerous in enzyme production (amylase, cellulase, xylanase, lipase, protease), food production (sake, soy sauce, rice beer, tempe), and production of heterologous proteins for food or pharmaceutical uses. It is probable that only a question of time before engineering of filamentous fungi for xylose fermentation will be attempted if the problem of low productivity could be overcome (Hahn Hågerdal, 2007).

#### ◆ 2.1.2 Yeast

Whereas a large number of yeast species metabolize xylose and arabinose and display

fermentative capacity (Barnett 2000), only a few have the potentials for fermentation in the industry. In the early 1980s, following the discovery that *S. cerevisiae*, *Schizosaccharomyces pombe* and other yeasts can ferment D-xylulose to ethanol (Wang, 1980a; Wang, 1980b), intensive screening efforts rapidly revealed that some can convert xylose to ethanol directly under aerobic or oxygen-limiting conditions (Schneider, 1981; Slininger, 1982). Attention was focused on *Pachysolen tannophilus*, *C. shehatae* (Du Preez & Preez, 1983) and *Pichia stipitis*, which are the best native xylose-fermenting yeasts known (Slininger, 1982). Right now, *saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae* are four major excessively researched yeasts. In the initial steps in the conversion of xylose to ethanol, xylose is transported by proton symport (Kilian, 1988) into the cell where xylose is reduced to xylitol via xylose reductase (XR) and thereafter is oxidized to xylulose with xylitol dehydrogenase (XDH). The xylulose is phosphorylated, channeled into the pentose phosphate shunt, and converted to C6 and C3 compounds. These compounds then enter glycolysis.

Oxygen plays an important role in the conversion of xylose to ethanol by yeasts, and there is a critical level of oxygenation at which the ethanol yield and productivity are high and the cell yield is low (Ligthelm, 1988; Rizzi, 1989; Watson, 1984). For example, *C. shehatae* requires oxygen for growth on xylose (Delgenes, 1986; Preez, 1984), but it is capable of anaerobic xylose fermentation. Fermentation rates are, however, higher with moderate aeration (semi-aerobic condition) (T. W. Jeffries, 1985; Sreenath, 1986). *C. shehatae* displays two distinct metabolic patterns in continuous culture depending on the level of aeration. Therefore, levels of oxygen are required carefully controlled. However, such precise oxygenation is technically impossible to maintain in large-scale industrial conditions.

### ◆ 2.1.3 Bacteria

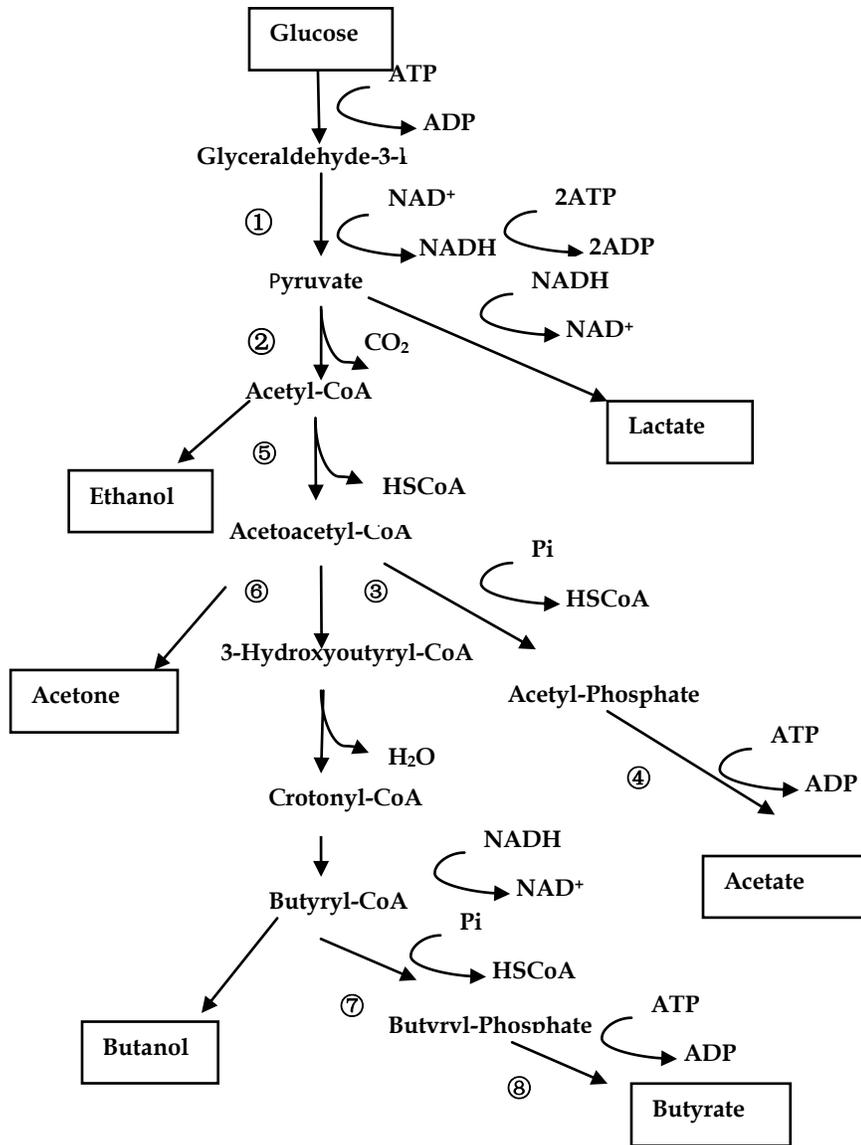
Some obligate anaerobic bacteria could ferment all lignocellulose-derived sugars, including their oligomers and polymers, to ethanol, other solvents, and acids (Wiegel, 1985). These bacteria are more severely inhibited than other bacteria by high sugar concentrations and end-products. Also, these bacteria generally display mixed acid product formation, where ethanol is a minor product. Furthermore, their optimal pH, which is around 6–7, makes bacterial fermentation susceptible to infection and their low tolerance to lignocellulose-derived inhibitors requires a detoxification step to be included in the fermentation process (Hahn H ägerdal, 1994).

Recently, one of the most efficient microorganism for fermentation of detoxified lignocellulose hydrolysates is recombinant strains of *Escherichia coli* (Dien, 2003; Hespell, 1996; Ingram, 1987). In contrast to other bacteria, *Zymomonas mobilis* produces ethanol with stoichiometric yields (Swings, 1977). It also displays high specific ethanol productivity (Joon Lee, 1979). Despite intensive efforts over the past 20 years, the industrial exploitation of *Z. mobilis* has so far not materialized. In relation to the variety of sugars present in lignocellulosic raw materials, the substrate range of *Z. mobilis* is limited (Hahn Hågerdal, 2007). Recombinant xylose- and arabinose-fermenting strains, capable of fermenting these sugars in detoxified lignocellulose hydrolysates, have been constructed (Mohagheghi, 2002). However, *Z. mobilis* would also need pathways for the metabolism of mannose, galactose and arabinose, which constitute a considerable fraction of some lignocellulosic raw materials (Hayn et al. 1993). Another promising bacterium capable of using xylose is *C. tyrobutyricum*. *C. tyrobutyricum* can produce butyric acid, acetic acid, lactic acid, hydrogen and carbon dioxide from various carbohydrates including glucose and xylose.

## ■ 2.2 Fermentation of *C. tyrobutyricum*

### ◆ 2.2.1 Fermentation of glucose by *C. tyrobutyricum*

The strain *Clostridium* has two parallel metabolic pathways by using glucose shown in Figure 2.1. Products of the first pathway (acidogenesis) are acids (butyrate and acetate), and products of the second pathway (solventogenesis) are solvents (butanol and acetone) (Zigová 2000). The main products are butyrate and butanol, by-products are acetate and acetone; lactate and ethanol can also be produced in small amounts (Girbal, 1995).

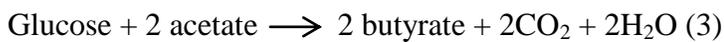
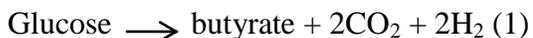


**Figure 2.1 Fermentative metabolic glucose by *C. tyrobutyricum* (Thauer, 1977)**

Approximately the same conditions are required for the production of butyric acid and acetic acid. During acetic acid production, 4 mols of ATP are formed, whereas only 3 mols of ATP are formed during butyric acid production. The ratio of butyric acid to acetic acid is strongly influenced by the growth rate of the bacteria, which is decided by the concentration of substrate, because the production of butyric acid and acetic acid is essentially interpreted as aimed at fulfilling the energy requirements of the cell (Thauer, 1977). The availability of the carbon source could possibly influence the ratio of butyric acid to acetic acid.

At high growth rates, cells are more likely to produce more acetic acid, because more ATP will be produced during this process, which can compensate for the ATP consumed to

maintain a functional pH gradient. In all cases, however, the production of acetic acid remained lower than that of butyrate, probably because it entails the thermodynamically unfavorable electron flow from reduced nicotinic adenine dinucleotide (NADH) produced by glycolysis to protons via ferredoxin (Thauer, 1977). At a lower growth rate, cells tend to produce more butyric acid. Sometimes in glucose-limited, fed-batch cultures, initially produced acetic acid was re-utilized, resulting in exclusive production of butyrate (Michel Savin, 1990), which shows in the following reaction. The gain for the cell is not obvious because acetic acid re-utilization also involves the energetic cost of acylation of acetic acid to acetyl-CoA. One possible explanation could be that maintaining the external concentration of acetic acid is also energy-consuming, thus leading to its re-utilization. Noting that acetic acid conversion to butyric acid involves the utilization of protons, and that pH has been found to affect the selectivity of butyric acid production, which may hint at a relationship with the proton balance of metabolism (Michel Savin, 1990).



Butanol and acetone are two by-products. Butyric acid will only be produced subsequently if there are sufficiently high levels of the enzymes involved in the pathway from butyryl-CoA to butyrate present (Hartmanis, 1984). These enzymes are influenced by the ATP concentration and the NADH: NAD ratio. A minimal intracellular ATP concentration and a high NADH: NAD ratio stimulate solventogenesis (Grobben, 1993). So a shift from acidogenesis to solventogenesis may be influenced indirectly with the change of oxidative and reductive relations in cells or directly by inhibitory compounds (Zigov 2000).

### ◆ 2.2.2 Fermentation of xylose by *C. tyrobutyricum*

This strain of bacteria *C. tyrobutyricum* could also use xylose. Liu et al. (2006) reported that by using a fibrous-bed bioreactor (FBB), the final butyric acid concentrations in fed-batch fermentations could reach 51.6 g/L from xylose. The metabolic pathway of xylose is almost the same with glucose as Figure 6. Acetyl-CoA produced from pyruvate is a branch-point intermediate located at the node dividing two analogous pathways leading to the formation of acetic acid and butyric acid. In these pathways, acetyl-CoA and butyryl-CoA are first converted

to acetyl phosphate and butyryl phosphate by phosphotransacetylase (PTA) and phosphotransbutyrylase (PTB), respectively. These acyl phosphates are then converted to acetic acid and butyric acid by acetate kinase (AK) and butyrate kinase (BK), respectively. The activities of these four enzymes, thus, play critical roles in affecting the production of acetic and butyric acids in *C. tyrobutyricum*. Also, there are two key enzymes involved in lactate production—lactate dehydrogenase (LDH), which is the enzyme catalyzing the formation of lactate from pyruvate with the regeneration of  $\text{NAD}^+$  and  $\text{NAD}^+$ -independent LDH (iLDH), which is responsible for the production of pyruvate from lactate (Zhu, 2004).

### ◆ 2.2.3 pH shift of the fermentation of *C. tyrobutyricum*

In general, the medium pH not only affects cell growth and fermentation rate, but also changes final product yield and purity (Zhu, 2004). Changing the medium pH also may induce a metabolic shift. Culturing *C. tyrobutyricum* with different pH, a shift of productivity of different organic acid carried out as pH changed. Butyric acid production increased and acetic acid decreased with increasing the pH. A maximum butyric acid concentration was produced at pH 6.3, with only a little of acetic acid and negligible amount of lactic acid as byproducts. In contrast, acetic acid and lactic acid production increased as the pH decreased to below 6.0. When pH dropped to 5.0, acetic acid and lactic acid became the major products and only a small amount of butyric acid was produced in the fermentation. It appeared that at pH 6 and higher, acetic acid production was inhibited by butyric acid and reduced to almost a halt. The probable reason for this situation is the pH drastically affects the activities of various enzymes during this process.

## ■ 2.3 The fermentation of *C. cohnii* with different carbon sources for DHA production

De Swaaf studied the DHA production by *C. cohnii* ATCC 30772, using glucose as the carbon source (M. E. de Swaaf & DESWAAF, 1999). In these experiments, the biomass increased from 1.5 g/l to 27.7 g/l in 74 h; and the total amounts of lipid and DHA after 91 h were 3.7 g/l and 1.6 g/l, respectively. Compared with glucose, in the case of glucose and glycerol as carbon sources, DHA contents of 32.5% and 43.1%, respectively, were obtained. The maximum DHA yields of 4.20 g/l and 4.15 g/l were therefore obtained, using 9% glucose and 12% glycerol respectively (Yokochi, 1998). Although the usage of glycerol is bigger than glucose, taking into

the price of glycerol, the low price of \$0.05/lb, it is still a very promising alternative.

Acetic acid also can be used for DHA production. For *C. cohnii*, a feed consisting of acetic acid (50% w/w) resulted in a higher overall volumetric productivity of DHA (rDHA) than a feed consisting of 50% (w/v) glucose (38 and 14 mg/L/h, respectively)(M. E. De Swaaf, Sijtsma, & Pronk, 2003). Referring to biochemistry and subcellular location of acetyl-CoA metabolism in yeasts (Pronk & PRONK, 1996), this difference may be that during growth on glucose, export of acetyl-CoA from the mitochondrial matrix to the cytosol is required to make it available for lipid synthesis, while acetate can be directly activated to acetyl-CoA by the action of acetyl-Coenzyme A synthetase, and then this would curtail the need for translocation of acetyl-CoA from the mitochondrial matrix.

In another literature (Ratledge, 2001), when microalgae were cultivated in medium containing sodium acetate as principal carbon source; the pH was maintained at a constant value by addition of acetic acid, which also provided an additional carbon source in a controlled manner. Moreover, its addition to microbial cultures can be easily controlled by a pH-stat mechanism. The accumulation of lipid by *C. cohnii* in this pH-auxostat culture was significantly greater than previously reported for batch cultures using glucose as principal carbon source, with the cells reaching 20 to 30 g dry weight per liter after 98 to 144 h and containing in excess of 40% (w/w) total lipid.

Ethanol also can be used. The specific growth rate was optimal with 5 g/l ethanol, and higher ethanol concentrations inhibited growth of microalgae like glucose. As the ethanol concentration increased, the microalgae exhibited a longer lag phase. Moreover, growth did not occur above 15 g/l. Experiment showed that ethanol had a great advantage in DHA production. The maximum overall volumetric productivity of DHA (rDHA) in ethanol-grown fed-batch cultures of *C. cohnii* (53 mg/l/h) (M. De Swaaf, 2003) was much higher than the highest rDHA values reported for cultures grown on glucose (19 mg/l/h) and even higher than Ratledge reported a rDHA of 36 mg/l/h during cultivation of *C. cohnii* for 98 h on acetic acid (Ratledge, 2001; de Swaaf et al, 2003). Ethanol can beat acetic acid because far less ethanol is required than acetic acid in order to produce a similar amount of biomass. Also, ethanol is less corrosive than acetic acid (Lide, 2004), which may reduce capital investment and maintenance costs for fermentation hardware.

## ■ 2.4 summary

In summary, *C. tyrobutyricum* could utilize xylose and glucose to generate butyric acid, and the truth of this fermentation is the balance of ATP, proton yield, individual inhibitory and uncoupling effects etc. of these different organic acids, which is influenced by pH, substrate type, substrate concentration, and other fermentation factors. In the end, due to the multiple differences, the fermentation ends up with different cell biomass and acids. Although some experiments about initial concentration of xylose and glucose were done, we think how both glucose and xylose initial concentration between 5g/l to 75g/l in batch fermentation may affect the organic acid type and yield have not been figured out yet.

*C. cohnii* could utilize several carbon sources to produce DHA. Factors that determine DHA yield are biomass concentration, lipid content of the cells, DHA content of the lipid and cultivation time (Sijtsma, 2004). In order to make DHA by *C. cohnii* economic, high cell densities, high lipid content of the cells, high DHA content of the lipid and short cultivation time are required, which are affected by key medium components and environmental conditions, and a large number of studies have been published, aiming at DHA production optimization. Many substrates can be used to produce DHA by *C. cohnii*. Comparing DHA production by *C. cohnii* ATCC 30772 from glucose, acetate and ethanol, it is easily found that acetyl-CoA may be supplied in the cytosol in a more direct way by cultivation of the organism on C2 compounds, like acetate and ethanol. The conversion of acetate into acetyl-CoA involves a one-step enzymatic reaction catalyzed by the enzyme acetyl-CoA synthetase. The utilization of ethanol by *C. cohnii* could suggest the presence of an alcohol dehydrogenase, which converts ethanol to acetaldehyde, and an acetaldehyde dehydrogenase, which converts acetaldehyde to acetate (Sijtsma, 2004). Meanwhile, it was recently reported that the *C. cohnii* can use butyric acid and lactic acid, but no detail was discussed. In addition, it was also reported that this kind of microalgae is capable of using glycerol as a supply, which is a C3 carbon source as lactic acid. However, little attention has been paid to butyric acid and lactic acid as a potential carbon source.

- **Chapter 3 - Butyric acid production of *Clostridium tyrobutyricum* using glucose and xylose**

- **3.1 Abstract:**

*C. tyrobutyricum* was cultured in various media containing glucose, xylose or their mixtures. Maximal optical density (OD at 620nm) increased from 0.866 to 1.804 as the initial xylose concentration increased from 5g/l to 35g/l. Further increase of xylose concentration to 75g/l did not significantly increase cell growth. The maximal OD also increased as the initial glucose concentration increased from 5g/l to 55g/l, with higher cell densities than growing in xylose medium at the same sugar concentration. Butyric acid concentration increased as the initial sugar concentration increased in both xylose and glucose media. Butyric acid yield increased from 2.62g/l to 27.0g/l as glucose concentration increased from 5g/l to 75g/l, whereas butyric acid yield in xylose increased from 3.24g/l to 16.29g/l as xylose initial concentration increased from 5g/l to 75g/l, but the conversion rate from xylose or glucose to butyric acid decreased as the sugar concentration increased. In xylose/glucose mixture, the ratio did not significantly affect the maximal OD and butyric acid yield. Butyric acid yields from five media were more or less the same (14.24g/l for medium with 20% xylose; 14.27g/l for medium with 40% xylose; 14.52g/l for medium with 50% xylose; 15.97g/l for medium with 60% xylose and 15.2g/l for medium with 80% xylose). Also, glucose was likely to be utilized more quickly than xylose, so the medium with a high glucose ratio had shorter lag period and faster cell growth.

**Keywords:** *Clostridium tyrobutyricum*, butyric acid, glucose, xylose, cell growth

- **3.2 Introduction**

As one of the most abundant renewable resources on earth, plant biomass is a promising source for future chemical products (R. Kumar, 2008; Rivas, Domínguez, Domínguez, & Parajó, 2002; Wyman, 1999). Plant biomass is mainly composed of cellulose, hemicellulose, and lignin, with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash (Jørgensen et al., 2007). Generally, lignin accounts for approximately 25% of total biomass, and carbohydrate polymers, including cellulose and hemicellulose, account for the rest. In addition, among sugar monose xylose is one of the most important components of hemicellulose, but hard to be utilized.

As a result, effective utilization of xylose becomes critical to the bioconversion of plant biomass.

For years, research has been carried out to study how to convert xylose into useful chemicals efficiently by different microorganisms. Some aerobic filamentous fungi (Skoog, 1988) can use xylose, but concentration of the products, such as ethanol, is well beyond the ability of filamentous fungi to withstand, so the process does not meet industry efficiency standards (Hahn H ägerdal, 2007). Some yeasts also can utilize xylose. *Saccharomyces cerevisiae* (K ätter, 1993), *Zymomonas mobilis* (Delgenes, 1996), *Pichia stipitis* (T. W. Jeffries, 2007), and *Candida shehatae* (Du Preez & Preez, 1983) are the four major yeasts that have been extensively researched. Oxygen plays an important role in their fermentation of xylose (Preez, 1984; Skoog, 1990); however, harsh oxygenation control is technically impossible to maintain in large-scale industrial conditions. Still, some obligate anaerobic bacteria such as *Escherichia coli* (Dien, 2003; Dien, 2003) and some recombinant xylose- and arabinose-fermenting strains (Mohagheghi, 2002) possess the ability to convert xylose to valuable produces. *C. tyrobutyricum* is one of the most promising bacteria in using xylose. *C. tyrobutyricum* is a gram-positive, spindle-form bacillus (Liu, 2006) that can be isolated from soil, waste water, animal digestive systems, or contaminated dairy products (Zigov á 2000). It is an anaerobic prokaryote that requires an absolute absence of oxygen to grow (Liu, 2006). Optimal growth pH ranges from 4.5 to 7.0, and optimal temperature is from 35°C to 37°C (Zigov á 2000). Butyric acid produced by *C. tyrobutyricum* is a valuable chemical that is used in the preparation of various butyrate esters and has several potential applications. Low-molecular-weight esters of butyric acid, such as methyl butyrate, have mostly pleasant aromas or tastes. As a result, they can be used as food and perfume additives (Zhu, 2002). In addition, butyric acid and its derivatives (in mixtures with other compounds; e.g., cellulose, acetic acid) play an important role in the plastic materials and textile fibers industries (Playne MJ, 1985) and are used in the pharmaceutical industry to treat colorectal cancer and hemoglobinopathies (Pouillart, 1998).

Literatures have been reported that microorganisms produce butyric acid, acetic acid, lactic acid, hydrogen, and carbon dioxide from various sugars, such as glucose and xylose (Wu & Yang, 2003). Some research has focused on the effect of initial sugar concentration on fermentation by *C. tyrobutyricum*. Michel Savin compared the initial glucose concentrations of 125g/l and 85g/l (Michel Savin, 1990) and found that 125g/l successfully increased final butyric acid concentrations but led to a longer initial lag period. Research carried out by Fayolle also

proved that initial glucose concentration of 130g/l could result in long initial lag time (Fayolle, 1990). Qiu et al (2009) reported that initial xylose concentration between 5 to 40g/l could affect the hydrogen production. As a result, we think that both glucose and xylose initial concentration from 5g/l to 75g/l in batch fermentation may affect the organic acid type and yield. In another project, Zhu (2002) studied the effect of fermentation of glucose/xylose mixture (1:1) and found that the acid production pattern from xylose did not change in the presence of glucose and observed no inhibition of xylose uptake; however, we think the mixture ratio may affect the fermentation of glucose/xylose mixture. Therefore, our experiment had two objectives: 1) to determine the effect of initial concentration of glucose and xylose on organic acid yield, and 2) to understand the effect of mixture ratio on the organic acid yield.

### ■ 3.3. Materials and methods

*C. tyrobutyricum* (ATCC 25755) was obtained from ATCC. Synthetic medium (Huang, 1998) with sugar supplements was used for inoculation and culture experiments. In the single sugar experiments, glucose (Fisher Chemicals, CAS 50-99-7) or xylose (Cascade Analytical Reagents & Biochemicals, CAS 5328-36-0) was added to the synthetic medium at different initial concentrations, including 5g/l, 15g/l, 35g/l, 55g/l, and 75g/l. In the sugar mixture experiments, bacteria were fed on the glucose/xylose mixtures. The total sugar of mixture was set at 30g/l. The ratio of xylose to glucose ranged from 1:4, 2:3, 1:1, 3:2, to 4:1. All media contained yeast extract (5g/l),  $(\text{NH}_4)_2\text{SO}_4$  (3 g/l),  $\text{K}_2\text{HPO}_4$  (1.5 g/l),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.6 g/l),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.03 g/l). All media were adjusted to pH 6.4 every day by adding  $\text{NH}_4\text{OH}$ . All media were sterilized at 121 °C for 25 min before inoculation. All the media were cultured in 500ml beakerflask with 300ml medium and cultured in 120r/min shaking table. The anaerobic condition was kept by airlock.

Cell density was analyzed by measuring the optical density (OD) of cell suspension at a wavelength of 620 nm ( $\text{OD}_{620}$ ) with a spectrophotometer (Sequoia-turner, model 340).

A high performance liquid chromatography (HPLC) system (Agilent Technologies 1200 Series) was used to analyze the organic compounds, including xylose, glucose, arabinose, lactate, butyrate, and acetate in the fermentation broth. The HPLC system consisted of an automatic injector (Agilent Technologies 1200 Series), a pump (Agilent Technologies 1200 Series), an organic acid analysis column (ROA organic acid rezex or phonomonex column, size:

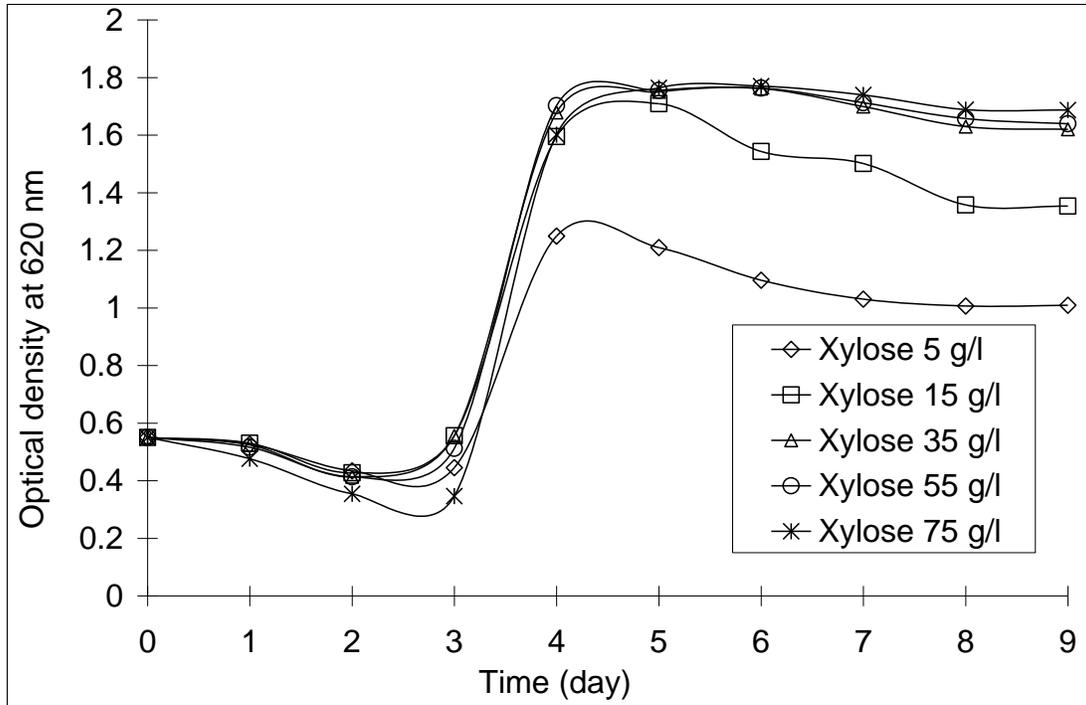
300×700mm), a column oven at 45 °C, and DAD (Agilent Technologies 1200 Series) and ELSD (Agilent Technologies 1200 Series) detectors. The mobile phase was 0.005mol/l H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 ml/min.

## ■ 3.4 Results and Discussion

### ◆ 3.4.1 *Fermentation of single sugars*

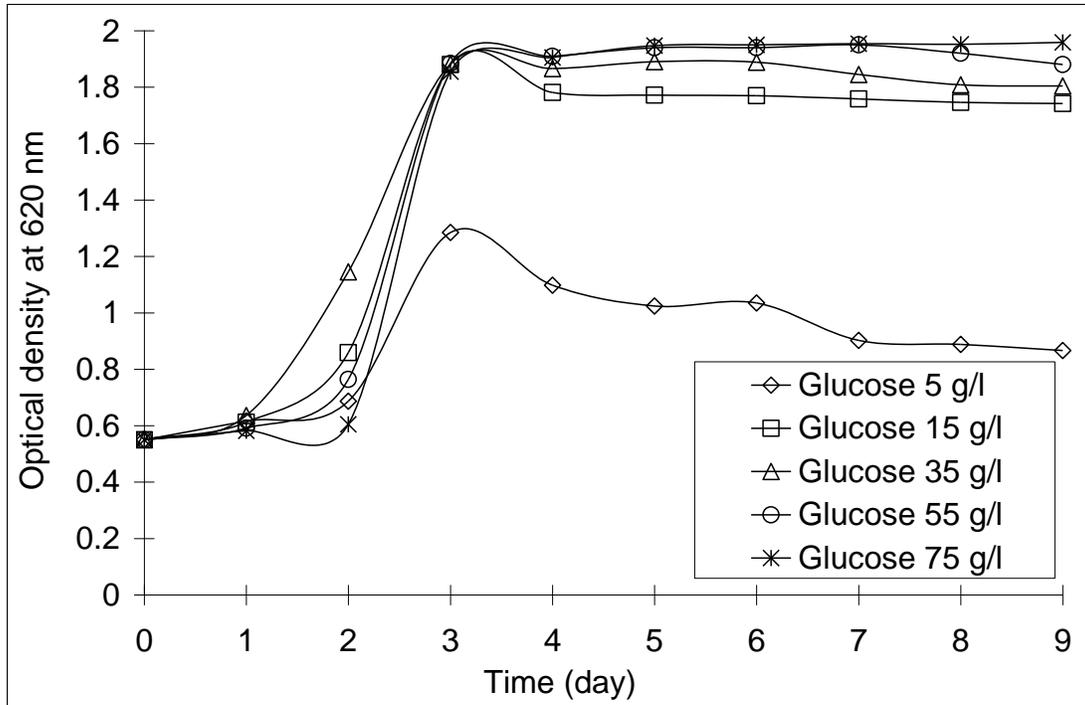
#### ● 3.4.1.1 *Cell growth*

In xylose, bacteria at all the initial concentrations had 2-3 d lag period, but bacteria in 75g/l xylose were inhibited more seriously than in all other initial concentrations, possessing a low OD of 0.605 in the lag period (Figure 3.1). When the initial sugar concentration was too high, the excess carbon source was often a reason for osmotic dehydration, which affected the formation of acids, their form (dissociated, undissociated), membrane transport and cell lysis (Edwards, 1970). The bacteria in xylose had a longer lag phase compared with those in glucose medium. One possible reason for this lag is that the seed was cultured in the glucose, so the bacteria may need a longer time to adjust to the xylose environment. Another reason could be that glucose is more easily metabolized than xylose by *C. tyrobutyricum*. Maximal OD increased as the initial xylose concentration increased from 5g/l to 35g/l. Maximal ODs of 35g/l to 75g/l were almost the same.



**Figure 3.1 Optical density of *C. tyrobutyricum* in xylose.**

In glucose, the bacteria exhibited a shorter lag period, which also had little to do with the initial sugar concentration (Figure 3.2). The maximal OD increased as the initial glucose concentration increased from 5g/l to 55g/l because higher glucose concentration provided more energy for bacteria to grow in the logarithmic phase. When the glucose concentration was from 55g/l to 75g/l, the biomass was more or less the same; for example, the highest OD from 75g/l was 1.959, whereas the highest OD from 55g/l was 1.95. This similarity occurred because cell growth took place mainly during the logarithmic phase, so energy-offering capacity in that phase was almost the same in both 55g/l and 75g/l.



**Figure 3.2 Optical density of *C. tyrobutyricum* in glucose.**

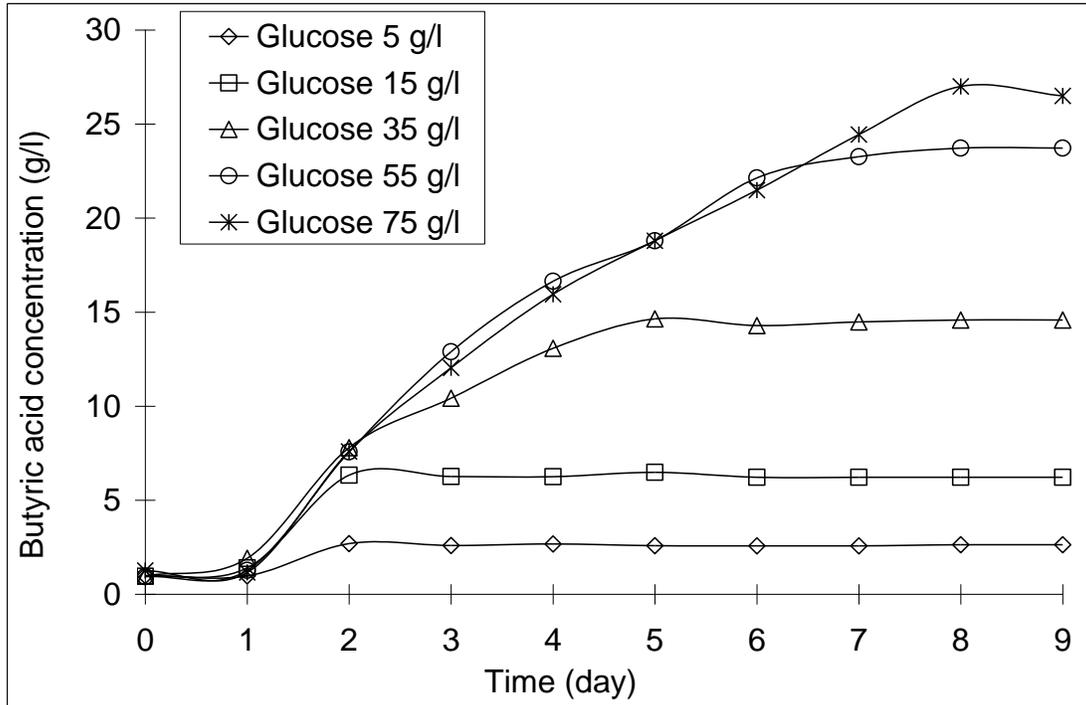
The cell growth in xylose was less than that in the glucose at the same concentration. The major reason was that when xylose enters the cell of bacteria, it needs more energy to go through the cell membrane, because one more mol ATP (as in the following formula) was needed to actively transport of D-xylose across the cell membrane (Heyndrickx, 1991). Unlike glucose transportation by means of a phosphoenolpyruvate-dependent phosphotransferase system, the xylose transportation was energized by a proton motive force and/or directly with a high-energy phosphate compound. Therefore, compared with glucose, less net ATP was produced by xylose, which led to lower cell growth rate and productivity (Zhu, 2002).



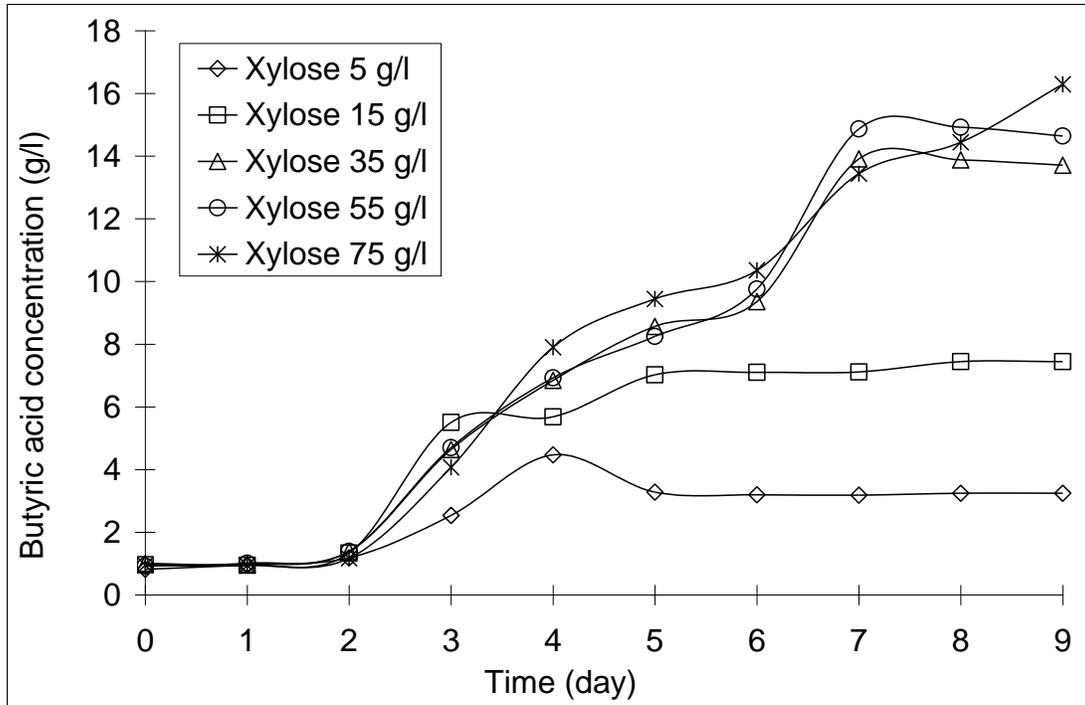
#### ● 3.4.1.2 Butyric acid production

The major acid produced by xylose and glucose was butyric acid because of the pH, as pointed out by research showing that the maximum butyrate concentration was produced at pH 6.3, with only a little acetate and a negligible amount of lactate as the acid by-products (Zhu, 2004). In our experiment, the lactic acid and acetic acid concentrations were detected, but because the concentrations were all very low (1-2g/l), thus they were not shown in the results.

The butyric acid yield increased as the initial sugar concentration increased in both xylose and glucose. Butyric acid yield increased from 2.628g/l to 27g/l when glucose concentration increased from 5g/l to 75g/l (Figure 3.3), whereas butyric acid yield in xylose increased from 3.24g/l to 16.29024g/l as xylose initial concentration increased from 5g/l to 75g/l (Figure 3.4).

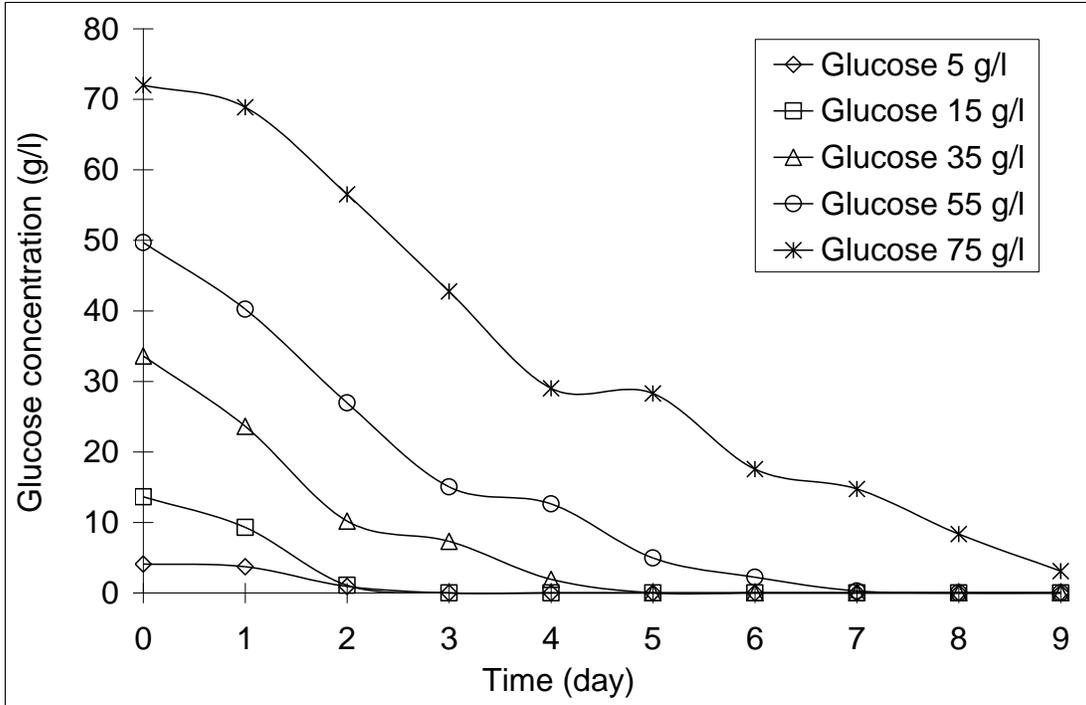


**Figure 3.3 Butyric acid production in glucose.**

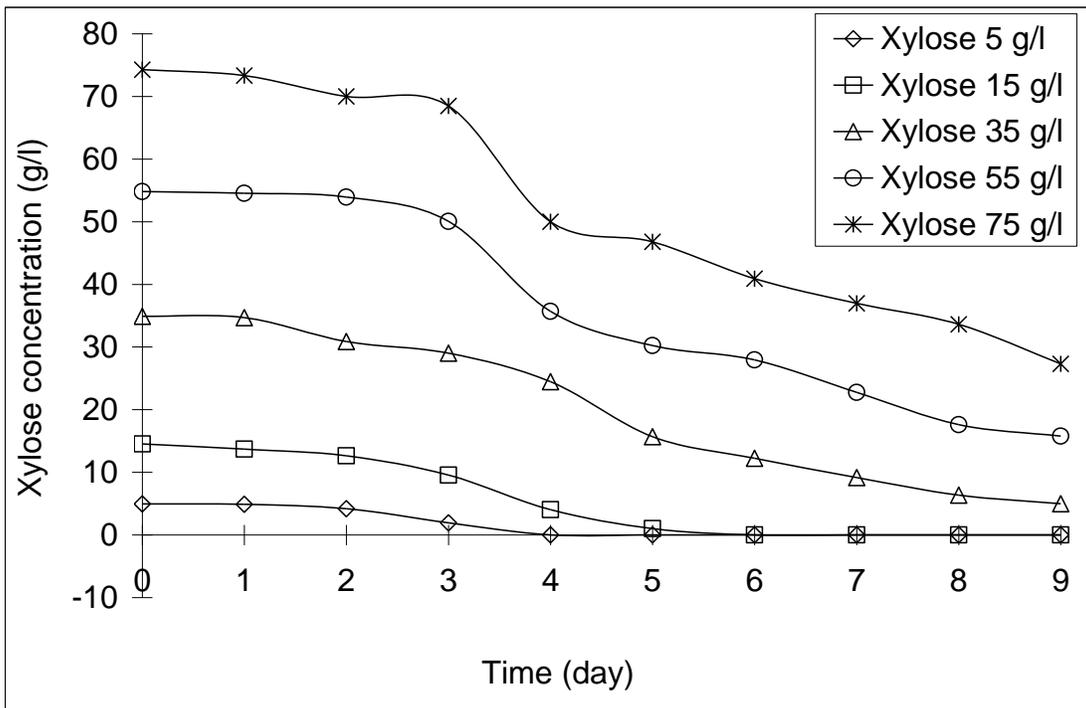


**Figure 3.4 Butyric acid production in xylose.**

In a higher sugar concentration, bacteria in glucose produced more butyric acid than the bacteria in xylose. The first reason for this is that when the experiment is done within 9 days, all the glucose was utilized but not all the xylose was used (Figure 3.5 and Figure 3.6). Xylose from 35g/l to 75g/l could not be completely used within 9 days (Figure 3.6). Because the bacteria in xylose grow at a very low speed near the end, the experiment stops before the xylose was completely used. Still, compared Figure 3.5 with Figure 3.6, the glucose was utilized more quickly than xylose. A second explanation is that compared Figure 3.5 with Figure 3.6 glucose utilization enjoyed a higher efficiency than xylose, because part of the xylose was used to offer energy when the sugar enters the cell.



**Figure 3.5** Glucose concentrations in fermentation of glucose by *C. tyrobutyricum*



**Figure 3.6** Xylose concentrations in fermentation of xylose by *C. tyrobutyricum*

Table 3.1 summarizes the conversion efficiency from sugar to butyric acid. In can be seen that the conversion rate from xylose or glucose to butyric acid decreased as the sugar

concentration increased; that is, low concentration sugar can utilize the sugar to produce butyric acid more efficiently. At low sugar concentrations (5g/l and 15g/l), xylose concentration is higher than glucose concentration, whereas the reverse is true at high sugar concentrations (35g/l to 75g/l).

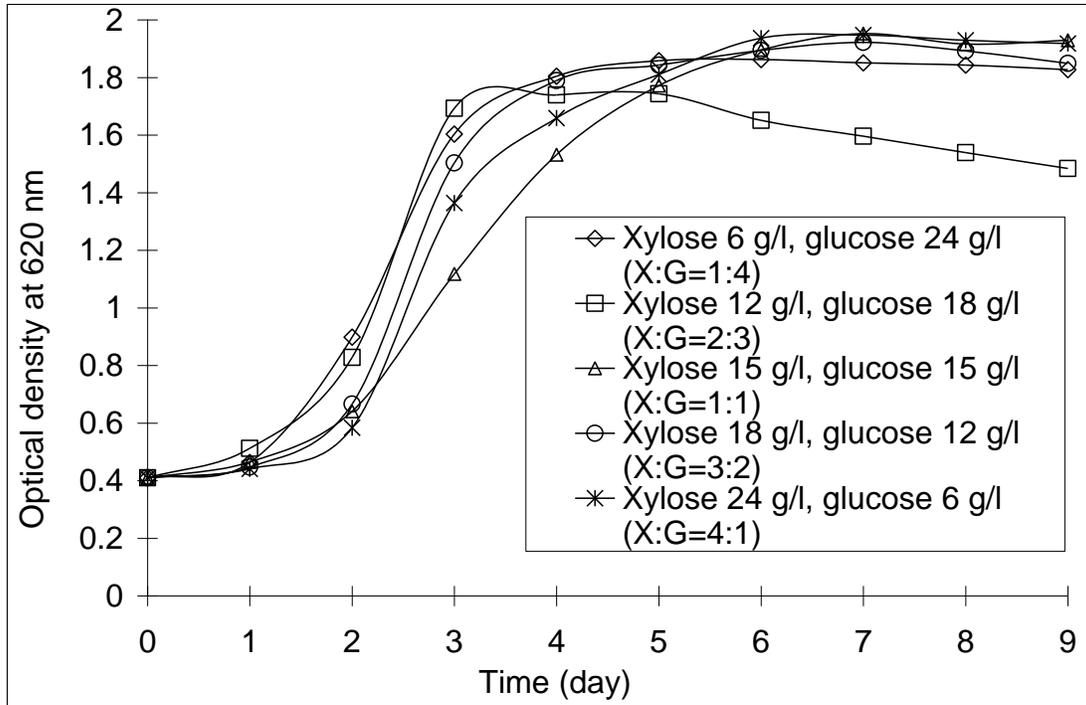
**Table 3.1 Conversion rate and butyric acid yield in glucose and xylose.**

Sugar concentration g/l	Butyric acid yield from xylose g/l	Conversion rate from glucose to butyric acid	Butyric acid yield from xylose g/l	Conversion rate from xylose to butyric acid
5	2.628	52.56%	3.24	64.80%
15	6.222	41.48%	7.44	49.60%
35	14.58	41.66%	13.7	39.17%
55	23.72	43.13%	14.6	26.62%
75	27	36%	16.29	21.72%

### ◆ 3.4.2 Fermentation of glucose/xylose mixture

#### ● 3.4.2.1 The cell growth in glucose/xylose mixture

In this part of our research, bacteria were cultured in five different media with a mixture of xylose and glucose, and the total sugar concentration in every medium was 30g/l. But the media varied in xylose concentration and glucose concentration. Media with more glucose clearly exhibited more cell growth more quickly at the beginning, but later the media with more xylose caught up and reached higher OD. Also, glucose was likely to be utilized more quickly than xylose, so media with more glucose had a shorter lag period. Figure 3.8 and Figure 3.9 clearly showed that glucose was likely to be utilized more quickly than xylose, so in the beginning of fermentation, the medium with more glucose could offer more energy to support the cell growth. As the glucose was exhausted, xylose became the major energy source to further support cell growth, so the medium with more xylose could reach a slightly higher maximal OD, but the ratio of the mixture could not significantly affect the maximal OD.



**Figure 3.7 Optical density of *C. tyrobutyricum* in xylose/glucose mixture.**

From Figure 3.8 and Figure 3.9, we could conclude that glucose was likely to be used more quickly than xylose. Zhu (2002) pointed out that in fed-batch fermentation of xylose and glucose mixtures 1:1, the bacteria metabolized glucose and xylose simultaneously without preference for either, but glucose consumption was faster initially and the consumption of xylose became faster after the first batch. Therefore, the bacteria might not tend to utilize glucose just because it needs more time to activate the enzyme in xylose metabolic pathways.

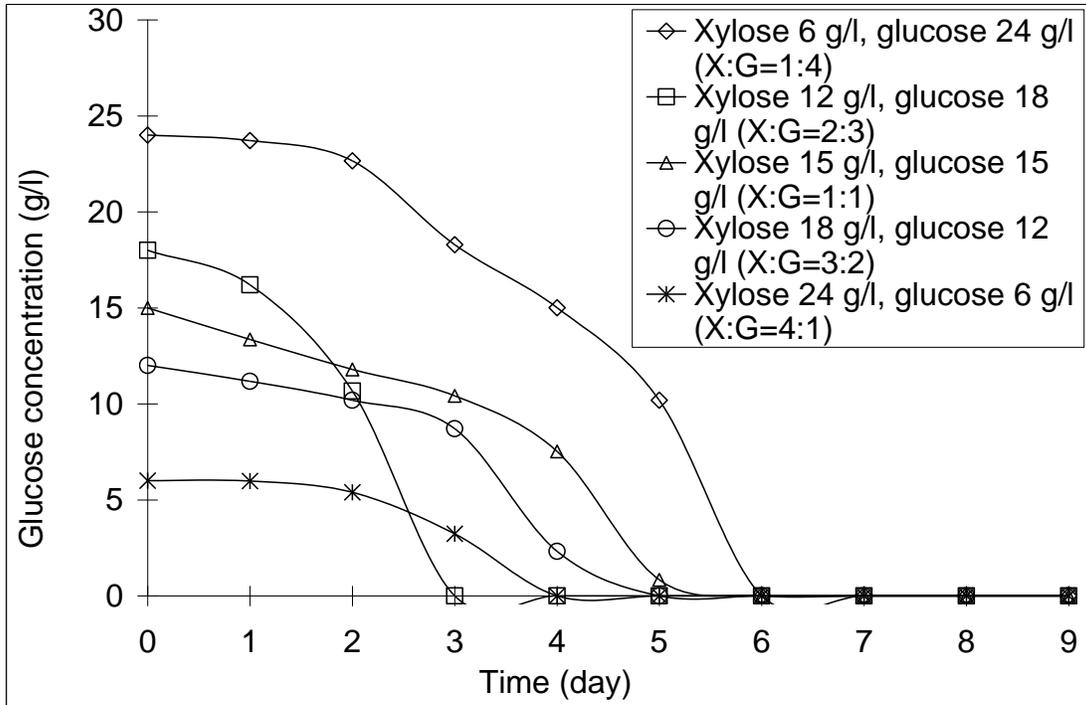


Figure 3.8 Glucose concentrations in fermentation of glucose/xylose by *C. tyrobutyricum*

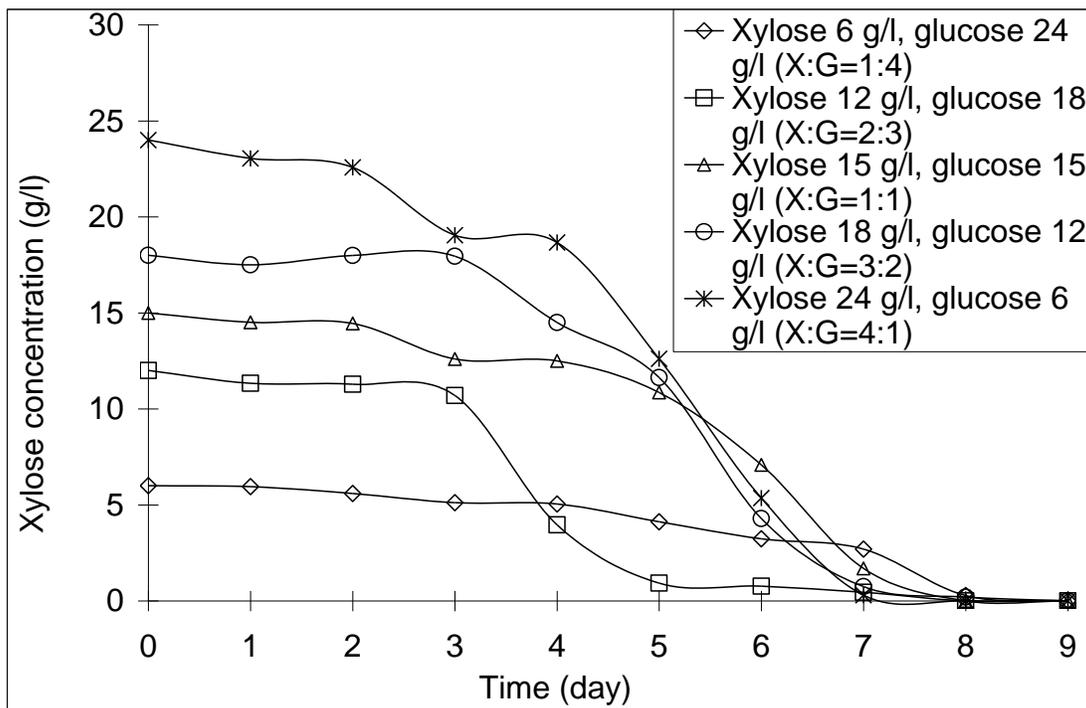
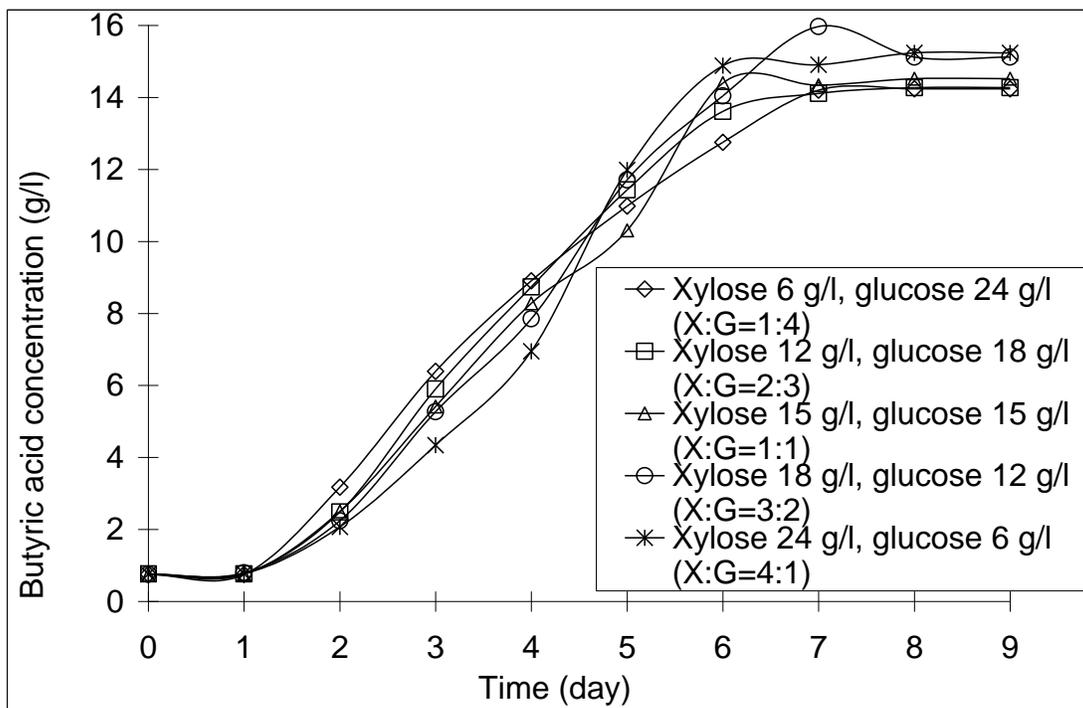


Figure 3.9 Xylose concentrations in fermentation of glucose/xylose by *C. tyrobutyricum*

● 3.4.2.2 The butyric acid production from glucose/xylose mixtures

Butyric acid was the major acid product (Figure 3.10). The butyric acid concentration

from five media was more or less the same (14.24g/l for medium with 20% xylose; 14.27g/l for medium with 40% xylose; 14.52g/l for medium with 50% xylose; 15.97g/l for medium with 60% xylose and 15.2g/l for medium with 80% xylose)), but butyric acid output from the medium with more xylose was slightly higher than from the medium with more glucose.



**Figure 3.10 Butyric acid productions in glucose/xylose medium.**

### ■ 3.5 Conclusions

Cell growth of *C. tyrobutyricum* increased with increasing initial glucose or xylose concentration, but was not affected significantly when the concentration was above 55g/l for glucose or 35g/l for xylose. The cell growth in xylose was less than that in the glucose at the same concentration, due to extra energy needed when D-xylose entered the cell membrane. Butyric acid yield increased as the initial sugar concentration increased in both xylose and glucose, but the conversion rate from xylose or glucose to butyric acid decreased as the sugar concentration increased. In addition, the xylose to glucose ratio in mixture did not significantly affect cell growth or butyric acid yield. In summary, *C. tyrobutyricum* could ferment glucose and xylose efficiently to produce butyric acid, which laid a good foundation for the fermentation of agricultural residue hydrolysates by this bacterium.

- **Chapter 4 - Butyric acid production of *Clostridium tyrobutyricum* using sugar mixtures and corncob hydrolysate**

- **4.1 Abstract:**

*C. tyrobutyricum* was cultured in different media, including arabinose alone, three-sugar mixture, corncob hydrolysate, and mimic corncob hydrolysate, to study the metabolic features of *C. tyrobutyricum* using three different sugars and prove the feasibility of butyric acid production from an inexpensive source. As for butyric acid production, compared with glucose or xylose, the arabinose was in a low efficiency, with butyric acid output of 2.25g/l in 10g/l arabinose and a long lag period of about 3-4 d. However, we found that a low concentration of arabinose could be used as a nutritional supplement to improve the fermentability of a mixture of xylose and glucose. The conversion rate of sugar to butyric acid increased as the supplement arabinose increased: 48.4% for medium without supplement, 49.375% for medium supplement with 2g/l arabinose, and 57.94% for medium with 5g/l arabinose. In three-sugar mixture, the result showed that 9.46g/l butyric acid was produced, which was from 14.52g/l (from 15g/l glucose and 15g/l xylose) to 8.3115g/l (from 20g/l glucose) or 9.007g/l (from 20g/l xylose). That is, arabinose could promote butyric acid productivity, but was less effective than the 30g/l glucose/xylose mixture. Also, in three-sugar mixture, it is easy to conclude that glucose was the favored substrate, followed by xylose. In addition, we demonstrated the feasibility of fermenting xylose-rich corncob hydrolysis to butyric acid. At end of the 9 d fermentation, the concentration of butyric acid from corncob hydrolysis reached 10.56 g/l, and the mimic medium reached 11.3 g/l.

**Key words:** *Clostridium tyrobutyricum*, corncob, butyric acid, cell growth

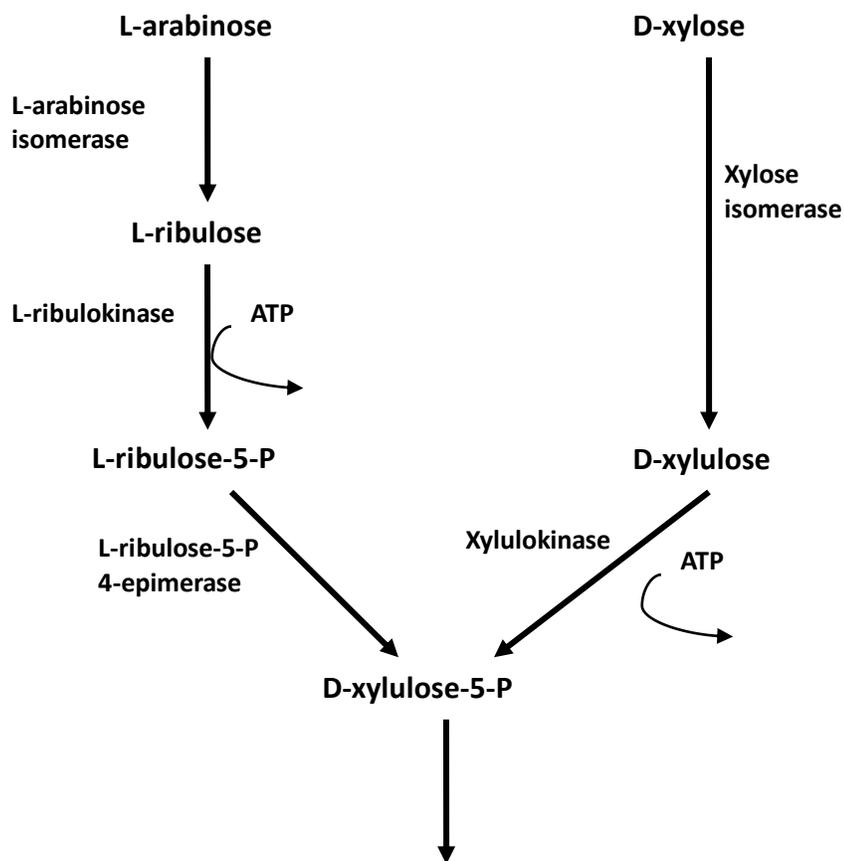
- **4.2 Introduction**

Corn is one of the most abundant agricultural materials worldwide. The United States produces 40% of the world's harvest, followed by China, Brazil, Mexico, Indonesia, India, and France (<http://faostat.org>). In 2009, over 159 million hectares of maize were planted worldwide, with a yield of over 5 tonnes/hectare, and its total production reached 817 million tonnes, which is more than rice (678 million tonnes) or wheat (682 million tonnes) ("Iowa corn crop poised to set record," 2009). Due to the huge quantity of corn production, its by-product productivity is

also very high; however, not enough attention has been paid to using agriculture and agro-industry residues, the non-product outputs from the growing and processing of raw agricultural products, such as corncobs. In fact, such residues may contain valuable materials, but their apparent cost of collection, transportation and processing for beneficial use are more than their current economic values (Tsai, 2001).

Due to their lignocellulosic nature, corncobs are a promising source for value-added chemical products. Researchers are exploring various methods of utilizing this by-product. Corncobs can be used as fertilizers and soil amendments, as animal and poultry hovel (Tsai, 2001), as fiber and fodder in animal feed (Bagby and Widstrom, 1987), and as an energy source (Lin, 1995; Mullen, 2010). Corncobs also can play a role in bioconversion as a biological substrate for the production of forage protein (Perotti & Molina, 1988), carbon adsorbents (Tsai, 2001), ethanol (de Carvalho Lima, 2002), xylitol (Dominguez, 1997; Latif, 2001), biochar (Mullen, 2010), and furfural (Mckillip and Sherman, 1980).

As one important component of corn cob, arabinose is also a notable pentose, but bacteria utilize arabinose in a metabolic pathway (Figure 4.1) differently from xylose, and the content of arabinose in plant biomass is much less than xylose, so less attention has been paid to fermentation of arabinose. Although some studies have begun to focus arabinose fermentation, such as a project on recombinant *Saccharomyces cerevisiae* (Richard & RICHARD, 2003; Wiedemann, 2008), research exploring arabinose's effects on fermentation media by *C. tyrobutyricum* has not been reported.



**Figure 4.1 Fermentative metabolic xylose and arabinose by bacteria (Hahn Hägerdal, 2007; Patrick, 1968).**

As a result, this study had two objectives: 1) to ferment the low-value corncob efficiently with *C. tyrobutyricum* to obtain valuable butyric acid, and 2) to study the effect of arabinose in fermentation media, especially in low concentration, by *C. tyrobutyricum*.

### ■ 4.3 Materials and methods

Corncoobs were collected from Kaytee Products Inc. (Chilton, WI) and ground using a Retsch SM2000 rotary cutting mill (Retsch Inc., Newtown, PA) with a 1.0 mm screen to mill into a particle size smaller than 1 mm. The hydrolysate was prepared by dilute acid treatment: 50 g of corncob was immersed in 50 ml of 5% sulphuric acid (vol/vol) and autoclaved at 121 °C for 30 min. The solid and liquid phases were separated by filtration (Fisherbrand, Coarse cellulose fiber papers remove particles >25µm). Hydrolysate was stored at 4 °C until use.

*C. tyrobutyricum* ATCC 25755 seed was cultured in a synthetic medium (Huang et al., 1998) as follows: glucose 30g/l, yeast extract 5g/l, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 3 g/l, K<sub>2</sub>HPO<sub>4</sub> 1.5 g/l, MgSO<sub>4</sub> ·7H<sub>2</sub>O 0.6 g/l, FeSO<sub>4</sub> ·7H<sub>2</sub>O 0.03 g/l and pH 6.4.

The fermentation media composition varied according to different parts of the experiment; we substituted glucose with different sugars or hydrolysate as the carbon source, but all media contained yeast extract 5g/l, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 3 g/l, K<sub>2</sub>HPO<sub>4</sub> 1.5 g/l, MgSO<sub>4</sub> ·7H<sub>2</sub>O 0.6 g/l, FeSO<sub>4</sub> ·7H<sub>2</sub>O and 0.03 g/l. In the first part of the experiment, media with 5g/l arabinose or 10g/l arabinose were prepared for the test. The medium for testing the mixture of three sugars contained 30g/l of total sugar, including xylose (10g/l), arabinose (10g/l), and glucose (10g/l). The arabinose-supplement experiment media contained 2 g/l or 5 g/l arabinose with 15 g/l glucose and 15 g/l xylose. The corncob mimic medium was prepared due to HPLC analysis result of corncob hydrolyses and contained xylose (19.4g/l), arabinose (2.1g/l), and glucose (1.6g/l). All media were adjusted to pH 6.4 three times a day by adding NH<sub>4</sub>OH or HCl. Anaerobic environments were maintained by aerating high-purity nitrogen gas. All media were autoclaved at 121 °C for 25 min before use. All the media were cultured in 500ml beakerflask with 300ml medium and cultured in 120r/min shaking table. The anaerobic condition was kept by airlock.

Cell density was analyzed by measuring the optical density of the cell suspension at a wavelength of 620 nm (OD<sub>620</sub>) with a spectrophotometer (Sequoia-turner, model 340).

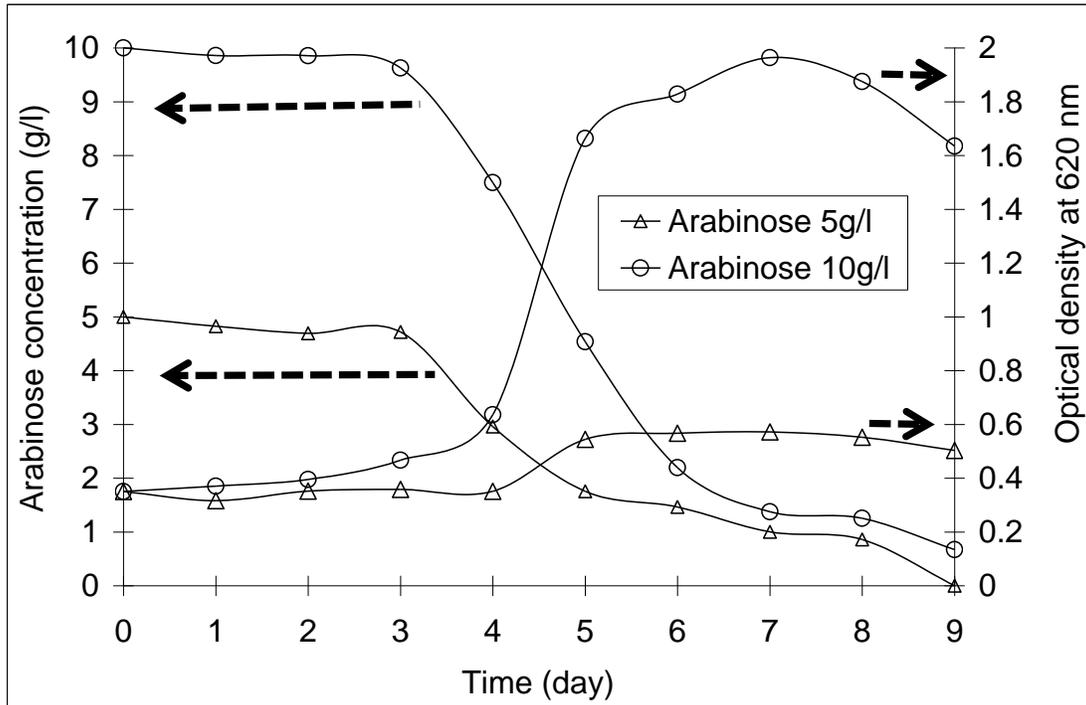
A high performance liquid chromatography (HPLC) system (Agilent Technologies 1200 Series) was used to analyze the organic compounds, including xylose, glucose, arabinose, lactate, butyrate, and acetate in the fermentation broth. The HPLC system consisted of an automatic injector (Agilent Technologies 1200 Series), a pump (Agilent Technologies 1200 Series), an organic acid analysis column (ROA organic acid rezex or phonomonex column, size: 300×700mm), a column oven at 45 °C, and a DAD (Agilent Technologies 1200 Series) and ELSD (Agilent Technologies 1200 Series) detector. The mobile phase was 0.005mol/l H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 ml/min.

## ■ 4.4 Results and discussion

### ◆ 4.4.1 Fermentation of arabinose

#### ● 4.1.1.1 Cell growth

When culturing the bacteria with low concentration of arabinose (5g/l and 10g/l), it grew very slowly and had a long lag period of about 3-4 d. After 3 d, the arabinose began to be used and on the fourth day, and the growth of bacteria was still in lag period with OD 0.451 in 5g/l and 0.636 in 10g/l (Figure 4.2). The bacteria reached maximal OD 0.644 in 5g/l arabinose after 6 d, and OD 1.875 in 5g/l arabinose after 7 d. It is easy to see that cell growth increased with increased arabinose concentration, because the maximal OD in 10g/l arabinose was much higher than that in the 5g/l arabinose.

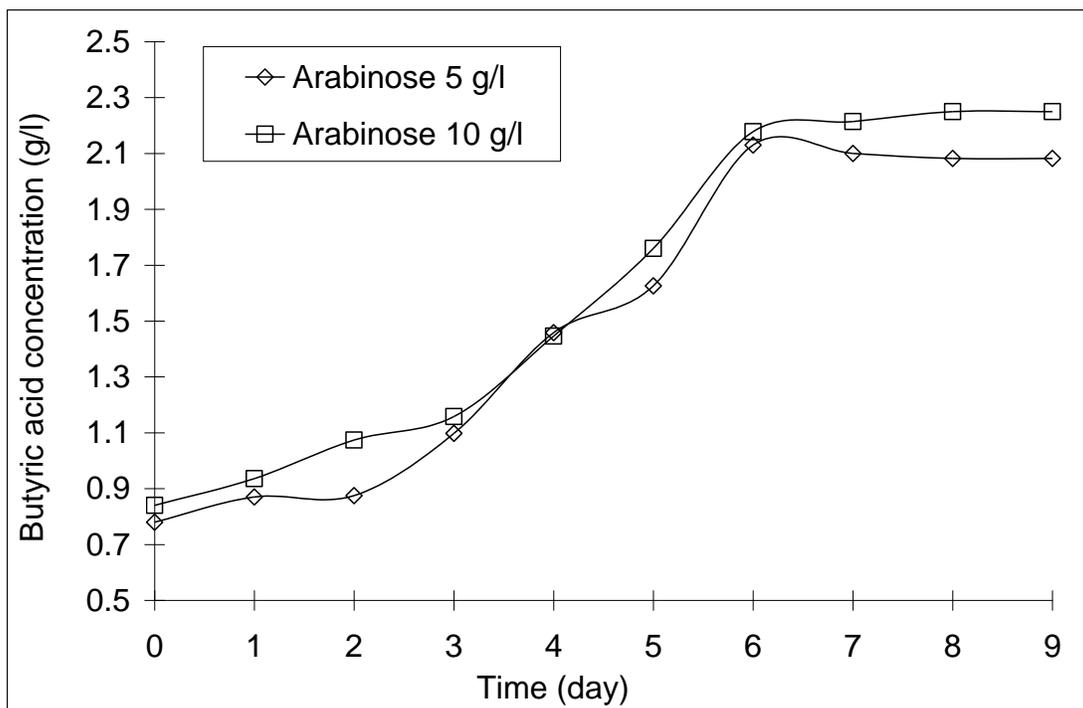


**Figure 4.2 Optical density of *C. tyrobutyricum* and arabinose concentration in the fermentation of 5g/l and 10g/l arabinose.**

#### ● 4.1.1.2 Butyric acid production

As for butyric acid production, compared with glucose or xylose, the arabinose was in a low efficiency. Arabinose was not used up after 9 days cultivation (Figure 4.2), and 2.082g/l butyric acid was produced in 5g/l arabinose and 2.25g/l butyric acid was produced from 10g/l

arabinose (Figure 4.3). Although the difference in concentration of butyric acid between two arabinose concentrations was not huge, 10g/l arabinose brought out higher maximal OD than 5g/l arabinose. That is, compared with 5g/l arabinose, more arabinose was used to support cell growth. Although the 10g/l arabinose resulted in almost the same maximal OD as glucose or xylose, the butyric acid output of 2.25g/l in 10g/l arabinose was less than 4.61g/l in 10g/l glucose. The butyric acid production in 10g/l glucose was calculated by a linear interpolation method from 5g/l and 15g/l glucose data. The lower efficiency in arabinose may be due to the extra energy needed to enter the cell membrane as xylose (Heyndrickx, 1991) and because compared with xylose, more enzymes are needed to be inspired in the metabolic pathway (Figure 4.1). On the other hand, the bacteria were cultured in a higher concentration of arabinose but showed an extremely low growth rate (data not shown) due to substrate inhibition.



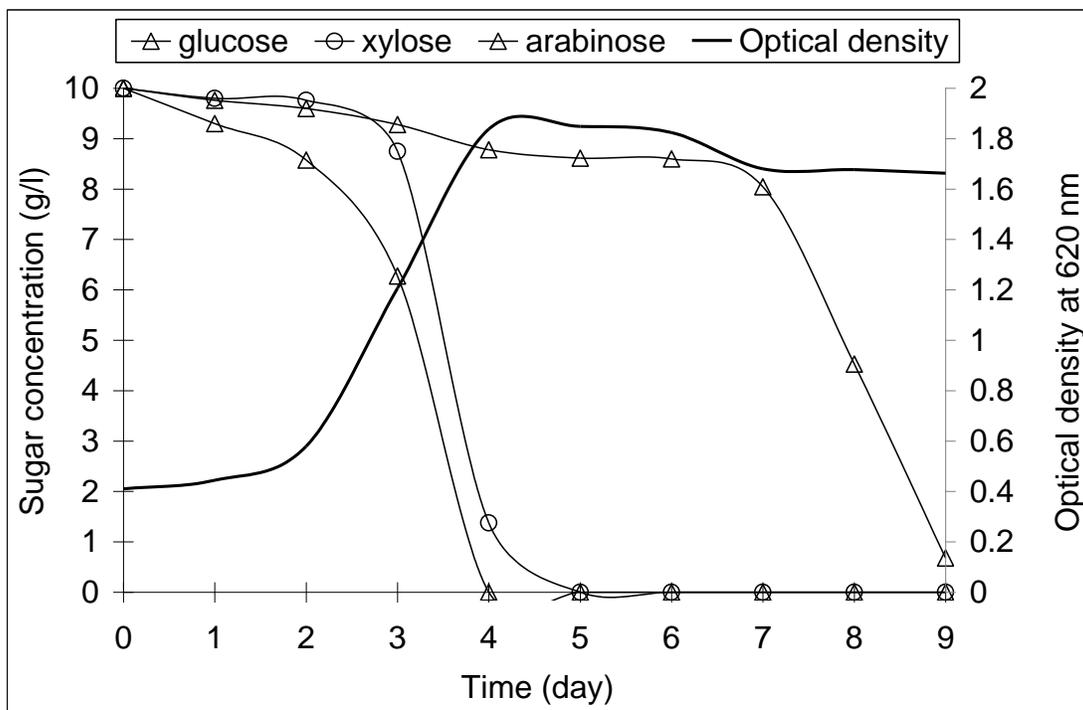
**Figure 4.3 Butyric acid production in 5g/l and 10g/l arabinose by *C. tyrobutyricum*.**

#### ◆ 4.4.2 Fermentation of glucose/xylose/arabinose mixture

##### ● 4.4.2.1 Cell growth

When culturing this bacterium with a three-sugar mixture of 10g/l glucose, 10g/l xylose, and 10g/l arabinose, it is easy to see that the glucose was the favored substrate, followed by xylose. Meanwhile, arabinose was not easily utilized; on the 7th d, arabinose just began to be

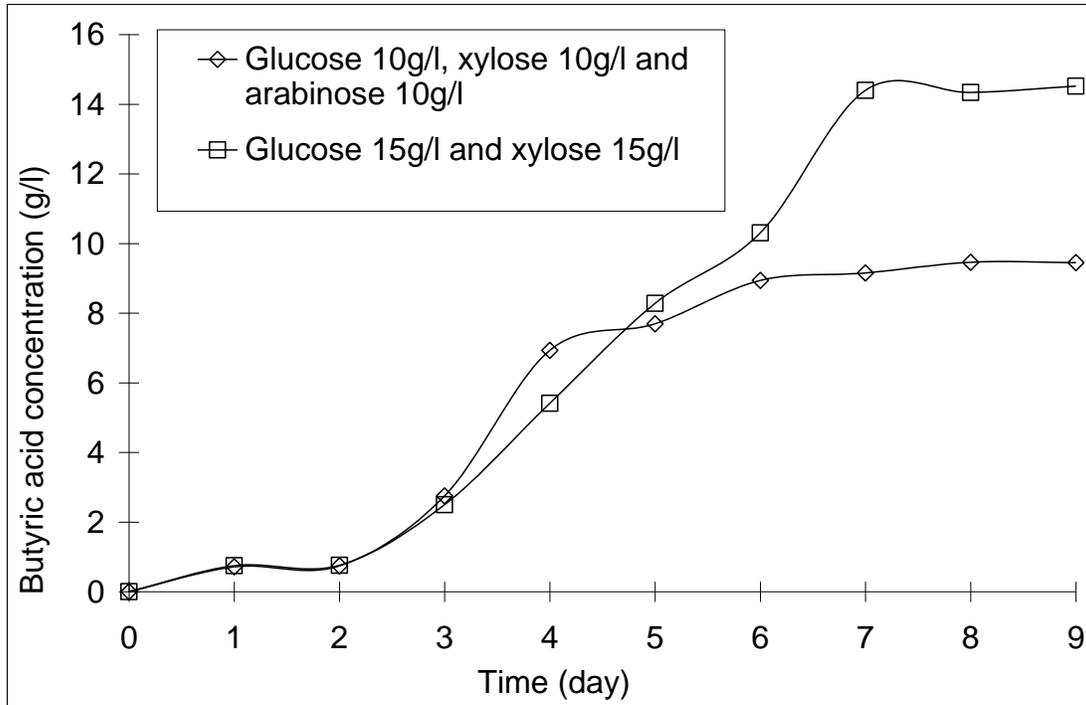
used (Figure 4.4). This result indicates that arabinose could not be used efficiently as xylose and glucose, which was consistent with the previous experiment that cultured the bacteria with arabinose alone.



**Figure 4.4 Optical density of *C. tyrobutyricum* and sugar concentration in glucose/xylose/arabinose mixture.**

● **4.4.2.2 Butyric acid production**

The bacteria in the 30g/l three-sugar mixture medium could produce 9.46 g/l butyric acid after 9 days (Figure 4.5), whereas bacteria in the medium with 15g/l glucose and 15g/l xylose could produce 14.52g/l within 9 days. We calculated by linear interpolation that 20g/l glucose could produce 8.3115g/l butyric acid and 20g/l xylose could produce 9.007g/l butyric acid. Compared with 8.3115g/l or 9.007g/l butyric acid from 20g/l glucose or xylose and 14.52g/l in 30g/l xylose/glucose mixture, we could find that 9.46g/l butyric acid is from 14.52g/l to 8.3115g/l or 9.007g/l. That is, arabinose can promote butyric acid productivity, but is less effective than the 30g/l glucose/xylose mixture.

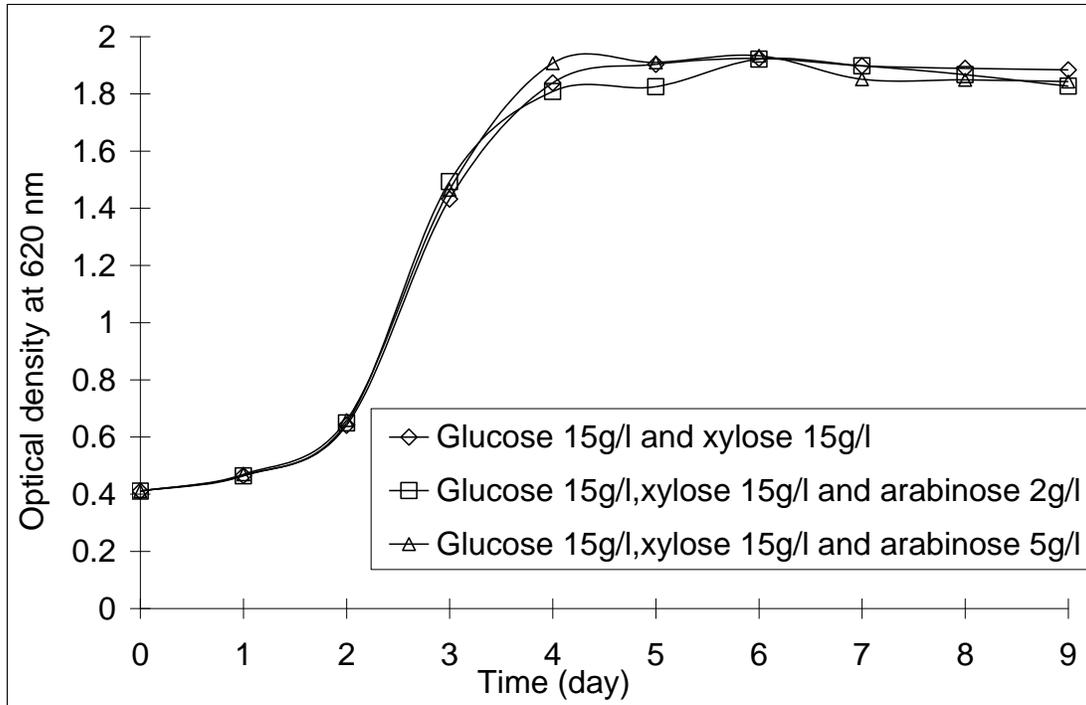


**Figure 4.5 Butyric acid production from three-sugar mixture and medium with 15g/l glucose and 15g/l xylose.**

◆ **4.4.3 Fermentation of arabinose-supplement medium**

● **4.4.3.1 Cell growth**

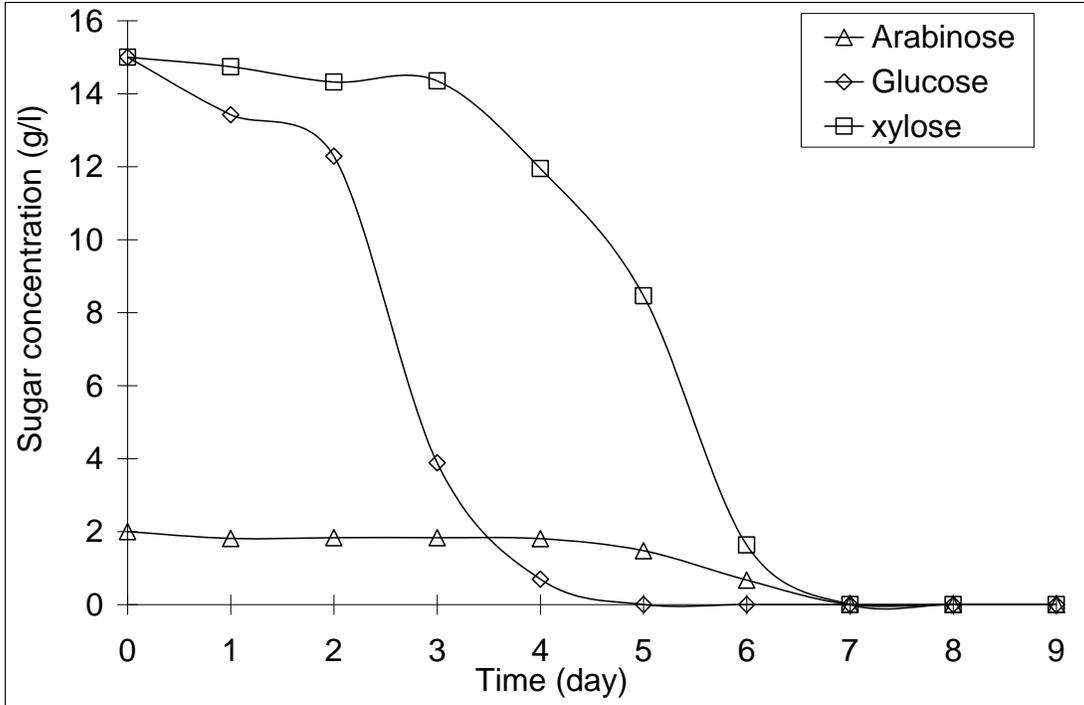
Regardless of the medium, with 15g/l glucose and 15g/l xylose or the media supplemented with arabinose, *C. tyrobutyricum* could reach maximal OD around 1.8 (Figure 4.6), which means that the supplement with 2g/l or 5g/l arabinose could not significantly affect OD because cell growth continued in the logarithmic phase and the energy supply capacity was the same at that time.



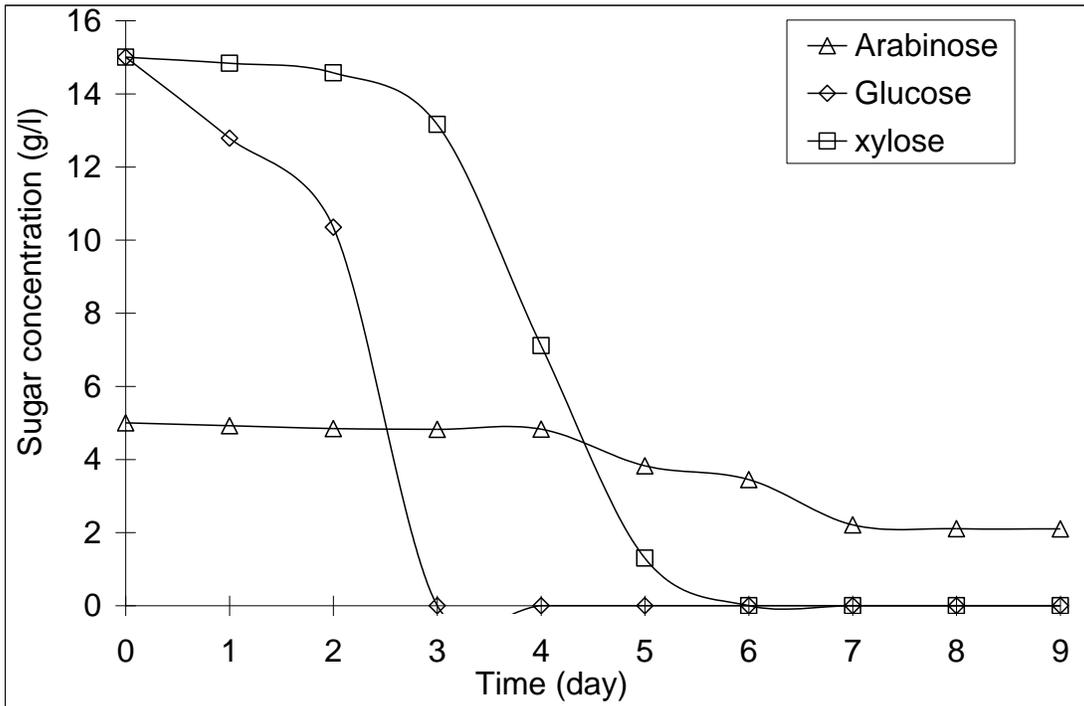
**Figure 4.6 Optical density of *C. tyrobutyricum* in arabinose-supplement medium.**

● **4.4.3.2 Butyric acid production**

Bacteria were cultured in three different media. From Figure 4.7 and Figure 4.8, it is easy to see that the glucose was the favored substrate, followed by xylose, which was the same trend like previous glucose/xylose/arabinose mixture experiment. The 5g/l supplement arabinose was not completely used within 9 d (Figure 4.8).



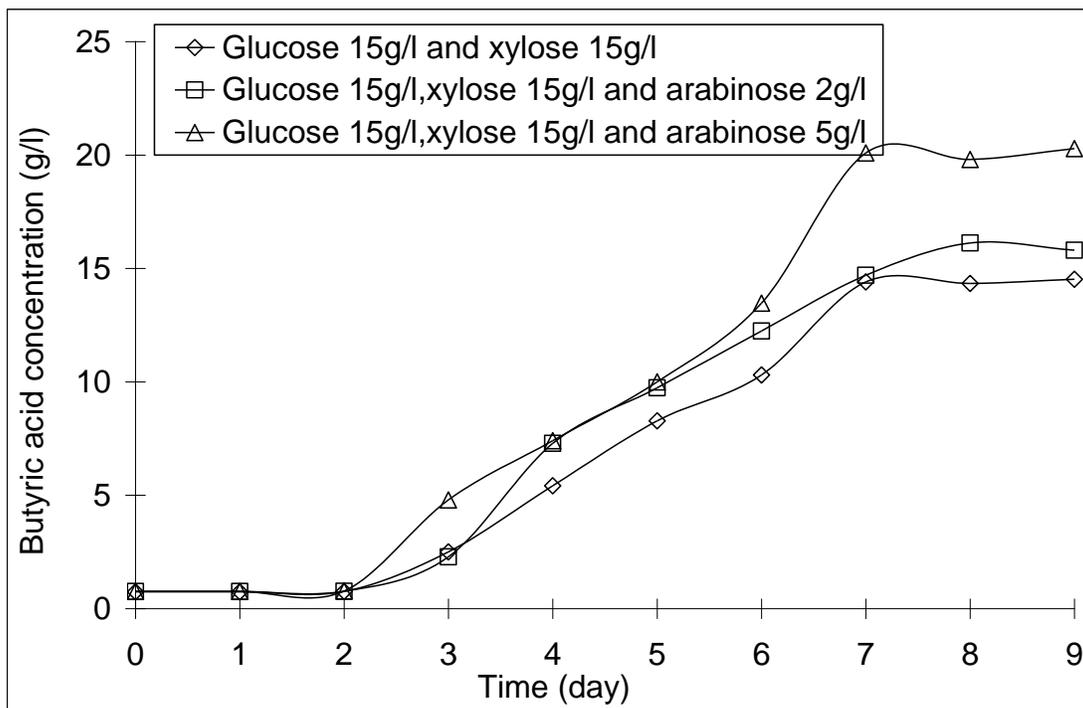
**Figure 4.7 Sugar concentrations in medium supplement with 2g/l arabinose.**



**Figure 4.8 Sugar concentrations in medium supplemented with 5g/l arabinose.**

We found that when the medium was supplemented with 5g/l arabinose, the productivity of butyric acid increased greatly. The medium without supplement that produced 14.52g/l butyric

acid, but the medium with 5g/l arabinose produced 20.28g/l butyric acid (Figure 4.9). The conversion rate of sugar to butyric acid increased as the supplement arabinose increased: 48.4% for medium without supplement, 49.375% for medium supplement with 2g/l arabinose, and 57.94% for medium with 5g/l arabinose. Two reasons could explain this result. One is the utilization of the arabinose from the fourth day, which means more sugar was used to support the production. But the increasing total sugar concentration was not the main reason. Because from the xylose and glucose cultivation, it was concluded that the conversion rate should decreased as the sugar concentration increased. If the increasing butyric acid concentration was just because the increasing total sugar used, the conversion rate should decrease. If we presumed that the supplement did not increased effectiveness, we could use direct proportion function to predict that when 35g/l sugar involved in the fermentation, 16.94g/l butyric acid should be produced. However, the actual productivity was higher than that. As a result, another probable reason was proposed that arabinose may be beneficial for cultivation with the mixture, and it might reinforce the enzyme activity of xylose or glucose, because in the third day the butyric acid concentration from the medium with 5g/l supplement (4.794g/l) was already higher than in the medium without supplement (2.4978g/l). Further proof should be obtained after the molecular mechanism and metabolic pathways of mixture sugar cultivation are elucidated.



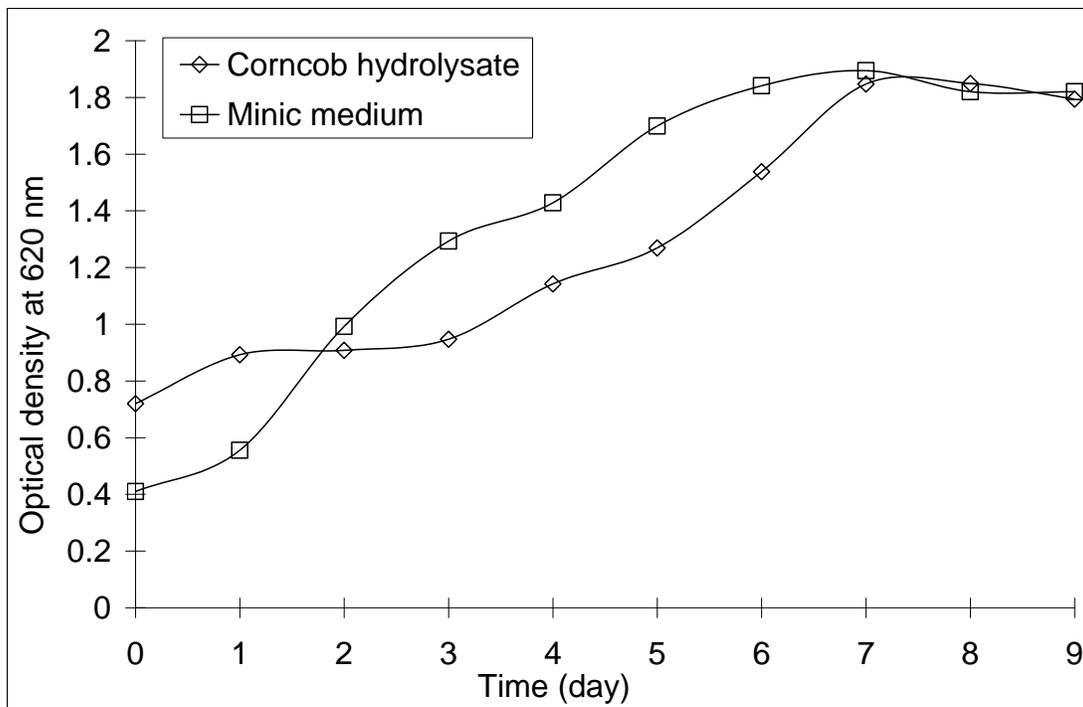
**Figure 4.9 Butyric acid productions from arabinose-supplement medium.**

#### ◆ 4.4.4 Fermentation of Corncob hydrolysate and mimic medium

##### ● 4.4.4.1 Cell growth

The bacteria was cultured in corncob hydrolysate with yeast extract as the nitrogen source and was also cultured in the medium with sugar concentrations mimicked from hydrolysate. The major component of corncob was xylose, followed by arabinose, glucose, and galactose. In our experiment, total sugars from corn fibre were released by acid hydrolysis using sulfuric acid. The acid hydrolysis through HPLC analysis showed that the mixture of sugar in corncob was composed of xylose (19.4g/l), arabinose (2.1g/l) and glucose (1.6g/l). That is, xylose accounts for 83.6% of total sugar. Ehrental et al. reported the hydrolysis products of methylated corncob hemicellulose to have a xylan portion containing 83% xylose with 5.9% L-arabinose and 1.7% D-glucose (Ehrental, 1954). Another experiment reported that through sequential steps of autohydrolysis (in aqueous media) and posthydrolysis (in the presence of sulphuric acid), the composition of the liquors after posthydrolysis was 28.2 g xylose, 2.1 g glucose and 3.2 g arabinose (Rivas, 2002). One researcher reported that two hemicelluloses, A and B, can be isolated from corncobs and that the qualities of sugars varied in the two hemicelluloses. There were consistently higher amounts of xylose and less arabinose in hemicellulose A than in B; meanwhile, the content of hemicelluloses A and B in one kind of corncob is decided by different synthetic genes (Donnelly, 1973). Therefore, although our result is not exactly the same as the report from Ehrental et al and B. Rivas, it is still within a reasonable range.

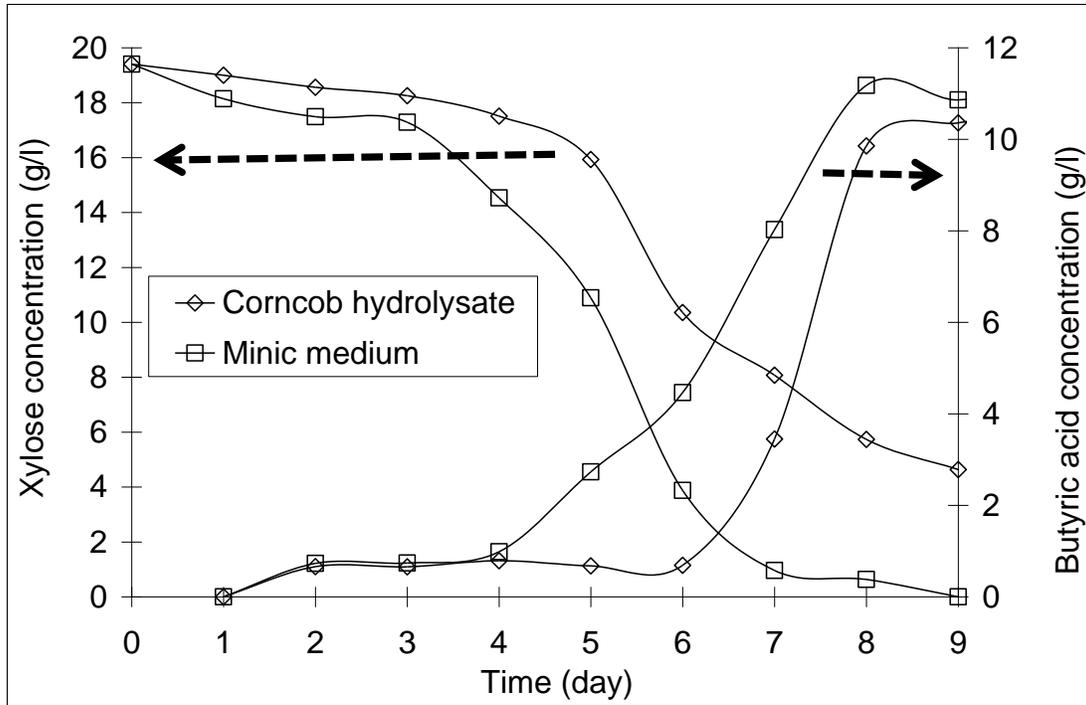
As for cell growth, compared with the mimic medium, the corncob hydrolysis had a longer lag phase. After 3 d cultivation, the bacteria in the mimic medium reached logarithmic phase, but bacteria in corncob hydrolysis remained in lag phase (Figure 4.10). This happened because acid pretreatment efficiently releases sugar monomers from hemicellulose, but it sometimes can generate inhibitory substances, including phenolic compounds, furan derivatives, and aliphatic acids (Larsson, 2000; Nilvebrant, 2003; Ranatunga, 1997; Ranatunga, 2000).



**Figure 4.10 Optical density of *C. tyrobutyricum* in corn cob hydrolysate and its mimic medium.**

● **4.4.4.2 Butyric acid production**

The main product of both media was butyric acid. After 9 d, all the xylose in the mimic medium was used, but 4.633g/l xylose remained in corn cob hydrolysate. At the end of the cultivation, the concentration of butyric acid from the corn cob hydrolyses reached 10.56 g/l, whereas the mimic medium reached 11.3 g/l (Figure 4.11). These results show that the corn cob hydrolysate could reach a 45.71% conversion rate from sugar to butyric acid and 48.92% for the mimic medium. Although the conversion rate from hydrolysate is lower than the mimic medium due to the influence of inhibitory substances from acid pretreatment, compared with the conversion rate from glucose to butyric acid of 41.48% in 15g/l glucose, it was high enough. Therefore, corn cob hydrolysate could feasibly be used as the substrate for *C. tyrobutyricum* fermentation. In addition, the corn cob hydrolysate reached 54.4% conversion rate from xylose to butyric acid and 58.2% for mimic medium. Compared with the 49.6% conversion rate from xylose to butyric acid in 15g/l xylose, it also could suggest that low concentration of glucose or arabinose enhanced the conversion rate of xylose.



**Figure 4.11 Butyric acid productions and xylose concentration in corncob hydrolysate and mimic medium.**

#### ■ 4.5 Conclusions

When supplemented with yeast extract, corn cob hydrolysate was found to be a good feedstock for butyric acid production by *C. tyrobutyricum*. At end of the 9 d fermentation, compared with the concentration of butyric acid from mimic corncob hydrolysate (11.3g/l), corncob hydrolysate reached 10.56g/l. Thus, they can be used as a low valued substrate to significantly reduce production costs and improve the economics of value-added butyric acid fermentation. This fermentation process also would benefit the agriculture development by providing a reasonable method of better residue utilization. Also, we found that a low concentration of arabinose can be used as a nutritional supplement to improve the fermentability of a mixture of xylose and glucose by enhancing the conversion rate of sugar to butyric acid. In three-sugar mixture, the results showed that glucose was the favored substrate, followed by xylose and arabinose could promote butyric acid productivity, but was less effective than the 30g/l glucose/xylose mixture. Arabinose alone was in a low efficiency in butyric acid fermentation. However, a low concentration of arabinose could be used as a nutritional supplement to improve the fermentability of a mixture of xylose and glucose.

- **Chapter-5 Docosahexaenoic acid production by *Cryptocodinium cohnii* using organic acids**

- **5.1 Abstract**

Cell growth of *C. cohnii* increased with increasing initial butyric acid concentration from 0.4g/l to 0.8g/l, but *C. cohnii* in 1.2g/l butyric acid gave out a low cell growth due to substrate inhibition. The initial acetic acid concentration did not significantly affect the cell growth. Cell growth of *C. cohnii* increased with increasing initial lactic acid concentration, but was not affected significantly when the concentration was above 1.2g/l. The DHA yield was highly related to both biomass and DHA content in the cell, whereas lower growth rate gave higher DHA content. The best concentration for DHA yield seemed to be 1.2g/l in three single organic acid media. In two organic acids mixture media, acetic acid tended to be beneficial for biomass accumulation, regardless whether butyric acid or lactic acid was mixed with acetic acid, the OD reached 1.3 or above. When lactic acid was mixed with butyric acid, the OD was always around 0.72 and as the butyric acid content increased, the growth rate decreased. When lactic acid was mixed with butyric acid, they could produce highest DHA yield (20.738 mg/l) due to low growth rate. As a result, it could be concluded that lactic acid and butyric acid could greatly influence each other in metabolic pathway and promote DHA production.

- **5.2 Introduction**

Currently, DHA has received much attention, due to its versatile applications in medicine and health care. The main sources of DHA are fatty fish species, such as herring, mackerel, sardine and salmon (Gunstone, 1996). However, the quality of fish oil depends on fish species, season and location of catching sites. Because some microalgae species possess a very high content of lipid (M. De Swaaf, 2003; Pyle, 2008), microalgae have been considered an alternatives to oils.

Among the heterotrophic marine dinoflagellates, *Cryptocodinium cohnii* has been identified as a prolific producer of DHA. This microalga is extraordinary in that it produces no other PUFAs in any significant amount, which makes the DHA purification process very attractive. This heterotrophic marine microalga, formerly known as *Gyrodinium cohnii*, has been studied since the end of the nineteenth century (BHAUD, 1991). Two forms of *C. cohnii* exist;

swarming cells and cysts. Both forms can vary strongly in size (10-50  $\mu\text{m}$  and beyond). Swarming or swimming cells are motile due to two flagella; a transverse and a longitudinal one. A normal speed of swimming cells is about 1 m/h. The cysts are either in a resting/survival or dividing stage (M. E. de Swaaf & DESWAAF, 1999). Out of one cyst, 1, 2, 4 or 8 swarming daughter cells can originate. In nature, *C. cohnii* can be found on rotting seaweed. On a medium containing yeast extract, sodium acetate and peptone in sea water, *C. cohnii* can readily be propagated in the laboratory (Pringsheim & PRINGSHEIM, 1956).

Many organic compounds can serve as the carbon sources for microalgae. Acetate is a promising feedstock for several microalgae. *Cryptocodinium cohnii* (Ratledge, 2001) and *Chlamydomonas reinhardtii* (M. E. De Swaaf, 2003) could grow heterotrophically on acetate (1 g/L or less). However this group is very sensitive to pH. For *Cryptocodinium cohnii*, a feed consisting of acetic acid (50% w/w) resulted in a higher overall volumetric productivity of DHA (rDHA) than a feed consisting of 50% (w/v) glucose (38 and 14 mg/L/h, respectively) (M. E. De Swaaf, Sijtsma, & Pronk, 2003). Meanwhile, it was recently reported that *C. cohnii* can use butyric acid and lactic acid, but no detail was discussed. In addition, it was also reported that this kind of microalgae is capable of using glycerol and lactic acid as a C3 carbon source. However, little attention has been paid to butyric acid and lactic acid as a potential carbon source.

In previous studies, we have demonstrated that *C. tyrobutyricum* can convert low value corn cob hydrolysate into organic acids mixture, including acetic acid, butyric acid and lactic acid. Our goal is to use the mixture of organic acids directly. The goal of this current study is to determine the effect of varying the initial concentration of butyric acid, acetic acid and lactic acid on DHA yield by *C. cohnii* and to investigate the effect of two organic acid combination ratios on the DHA yield by *C. cohnii*.

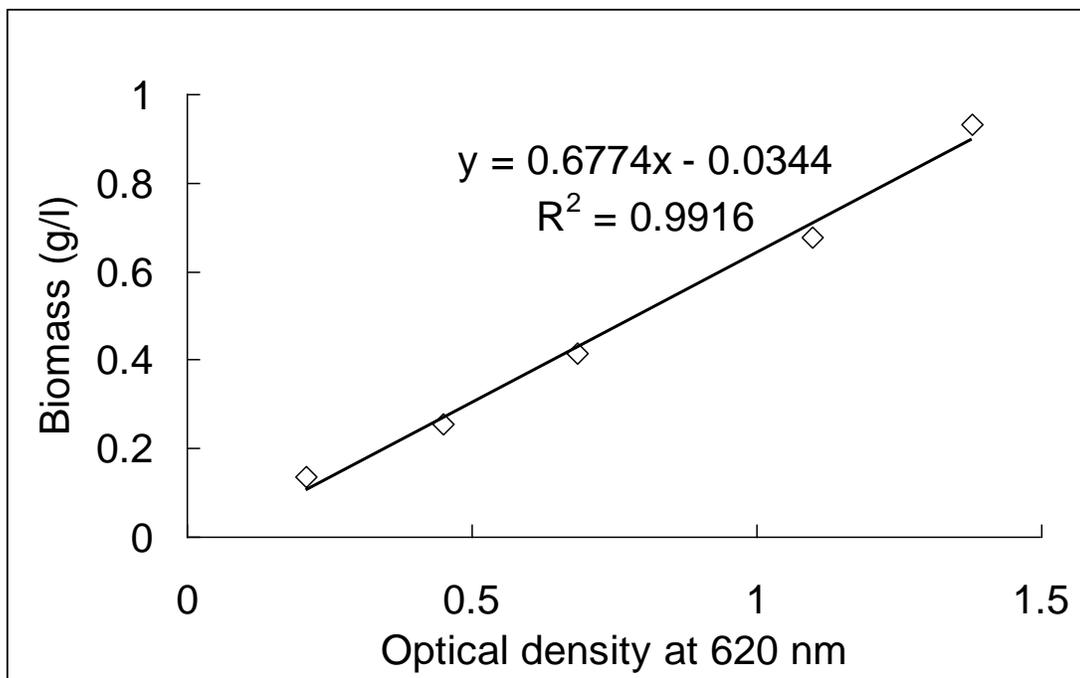
### ■ 5.3 Materials and methods

*C. cohnii* (ATCC 30772) was obtained from American Type Culture Collection and cultured in a synthetic medium as follows: NaCl 23.48 g,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  10.63 g,  $\text{Na}_2\text{SO}_4$  3.92 g,  $\text{CaCl}_2$  (anhydrous) 1.11 g, KCl 0.66 g,  $\text{NaHCO}_3$  0.19 g, KBr 0.1 g,  $\text{H}_3\text{BO}_3$  0.03 g,  $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$  0.04 g, Metal Mixture (see below) 3.0 ml,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  0.01 g, Sodium glycerophosphate 0.15 g,  $(\text{NH}_4)_2\text{SO}_4$  0.05 g, Tris Buffer 3.0 g, Vitamin Solution (see below) 1.0 ml,  $\text{K}_2\text{HPO}_4$  0.01 g, Glucose 3.0 g, Glutamic acid 1.5 g, Distilled water 1.0 L; the Metal Mixture includes EDTA 1.0

g,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  0.05 g,  $\text{H}_3\text{BO}_3$  1.0 g,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.15 g,  $\text{ZnCl}_2$  0.01 g,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.005 g, Distilled water 100.0 ml; Vitamin Solution includes Biotin 0.003 g, Thiamine 1.0 g, Distilled water 1.0 L. The medium was adjusted to pH 6.4 - 6.6. After culturing in above medium for three weeks, the seed was transferred to a medium including 2 g/l yeast extract (Oxoid), 9 g/l glucose and 27.8 g/l sea salt (Sigma) and was maintained as the seed.

The fermentation media composition was varied by substituting glucose with different organic acids as the carbon source, but all contained 2 g/l yeast extract (Oxoid), and 27.8 g/l sea salt (Sigma) (M. E. de Swaaf & DESWAAF, 1999). In single organic acid fermentation experiments, media with 0.4g/l, 0.8g/l or 1.2g/l butyric acid were prepared for the test. Also media with 0.4g/l, 0.8g/l, 1.2g/l and 1.6g/l acetic acid and lactic acid were prepared. In the second part of experiment, microalgae cultured in mixture of two organic acids, the total acid used in culture was 1.2g/l and the ratio of the two organic acids included 1:4, 2:3, 3:2 to 4:1. All media were sterilized at 121 °C for 30 min before inoculation. Also, all media were prepared with at stirrer speed (100 rpm) in the dark. The pH was adjusted to 6.4 every 8h by addition of 2 M HCl.

Cell density was analyzed by measuring the optical density of the cell suspension at a wavelength of 620 nm ( $\text{OD}_{620}$ ) with a spectrophotometer (Sequoia-turner, model 340). OD can be converted into biomass according to the following formula ( $\text{Dry weight} = 0.6774 \times \text{OD}_{620} - 0.0344$ ).



**Figure 5.1 Relationship between biomass and Optical density at 620nm**

Dry microalgae samples containing 5-25 mg fatty acids were added to 15 ml screw cap tubes. Two ml of benzene containing internal standard (methyl-C13, 400  $\mu\text{g/ml}$ ) and 3 ml of methanolic-HCl were added and tubes were capped, vortexed, and incubated at 70°C for 2 hours. After cooling, 5 ml of 6%  $\text{K}_2\text{CO}_3$  was added and tubes were vortexed. The benzene layer was separated by centrifuging at 1000  $\times g$  for 10 minutes and transferred to vials for analysis of fatty acid methyl esters. The DHA content was tested by GC Analysis. Samples were analyzed for fatty acid methyl esters using a HP 5890 GC with a SP-2560 capillary column (100m  $\times$  0.25mm  $\times$  0.2 film, Supelco, Inc., Bellefonte, PA). Injection port and detector temperatures were 250 °C with a flow rate of 1 ml/min helium, split ratio of 70:1, and 1  $\mu\text{l}$  injection volume. Oven temperature began at 140 °C and increased at 2 °C /min to 200 °C then at 4 °C /min to 245 °C and held for 17 minutes.

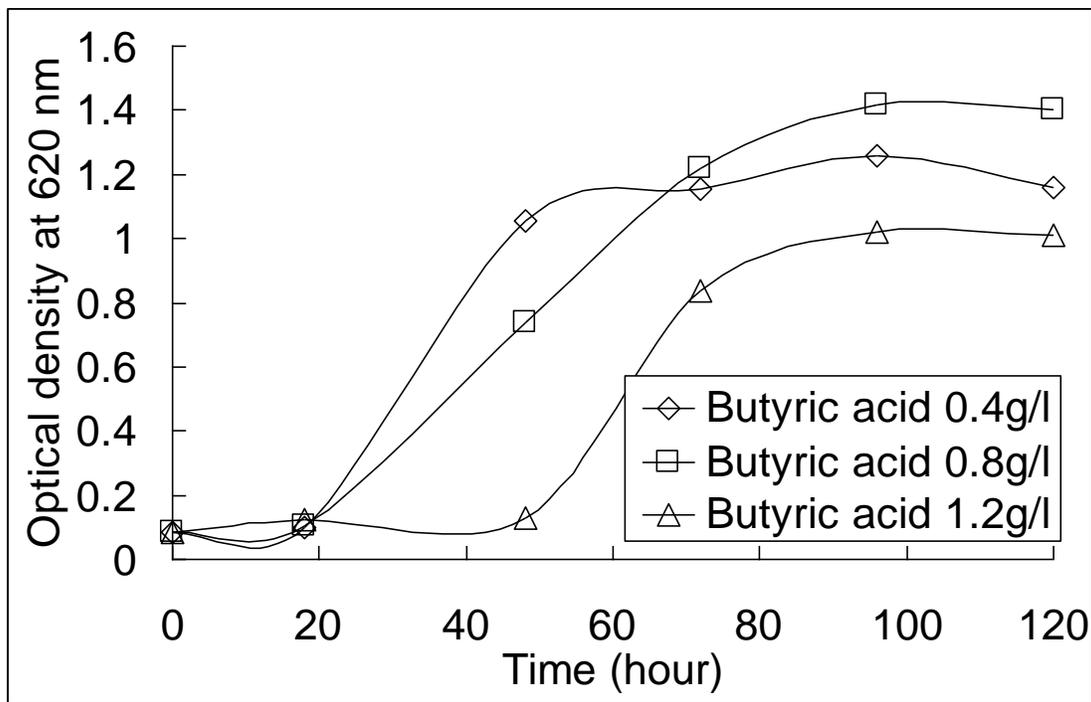
## ■ 5.4 Results and discussion

### 5.4.1 Fermentation *C. cohnii* in single organic acid

#### 5.4.1.1 Cell growth

A significant growth lag period of *C. cohnii* was observed in 1.2 g/l butyric acid,

indicating that higher butyric acid concentrations inhibit growth of *C. cohnii* (Figure 5.2). This was a kind of substrate inhibition. Severe inhibition might even result in negative values when a physiological rate is measured, indicating a progressive loss in that particular function and sometimes a concurrent loss in viability by the cells in the population. Possible reasons for this include the following: The higher butyric acid concentrations could modify chemical potential of substrates, intermediates, or products; alter cell's permeability; change activity of one or more enzymes; dissociate one or more enzyme or metabolic aggregates; affect enzyme synthesis by interaction with genome or transcription process; and/or influence functional activity of the cell (Edwards, 1970). The 0.8g/l butyric acid was the best concentration for cell growth.



**Figure 5.2 Optical density of *C. cohnii* by butyric acid**

In the beginning, the microalgae in the 1.6g/l acetic acid grew slower than others due to a slight substrate inhibition. Later on all concentrations reached almost the same maximal OD and there was no observable difference in growth pattern in the various concentrations of acetic acid (Figure 5.3). The initial concentration did not affect the biomass significantly. Considering 1.2g/l did not have substrate inhibition and reached the maximal OD more quickly, 1.2g/l was considered the best concentration for the biomass accumulation. As for lactic acid, from concentration 0.4 to 1.2g/l, the growth rate increased as the initial concentration increased, while 1.2g/l and 1.6g/l reached almost the same maximal OD in different time (Figure 5.4).

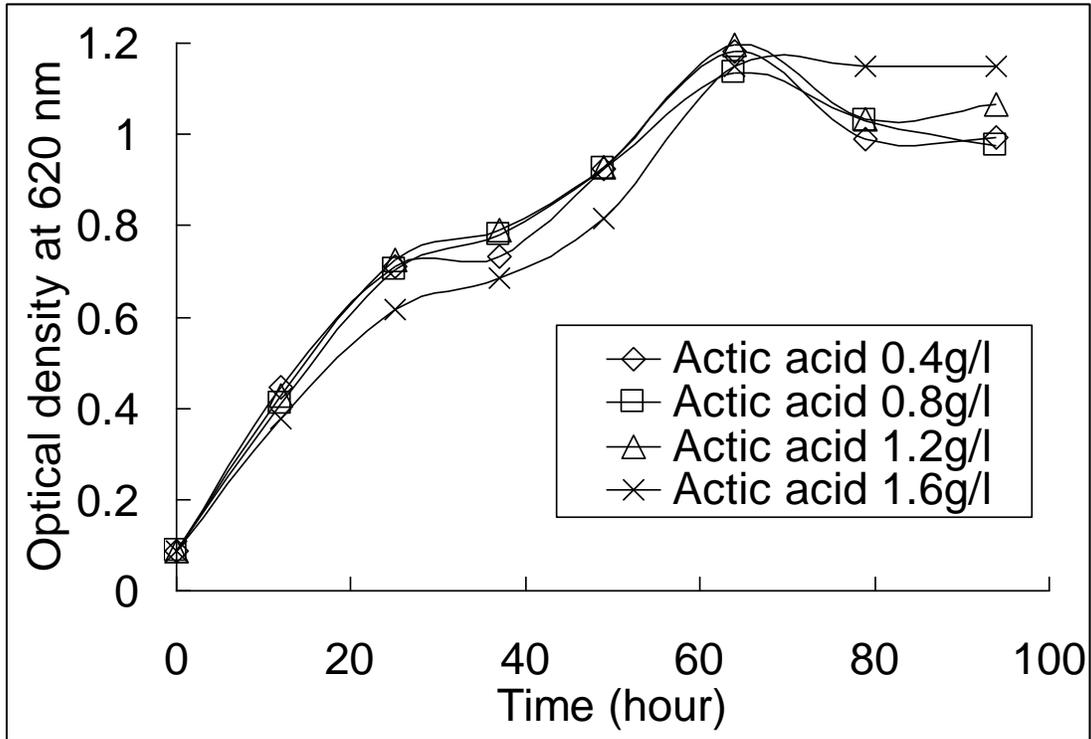


Figure 5.3 Optical density of *C. cohnii* by acetic acid

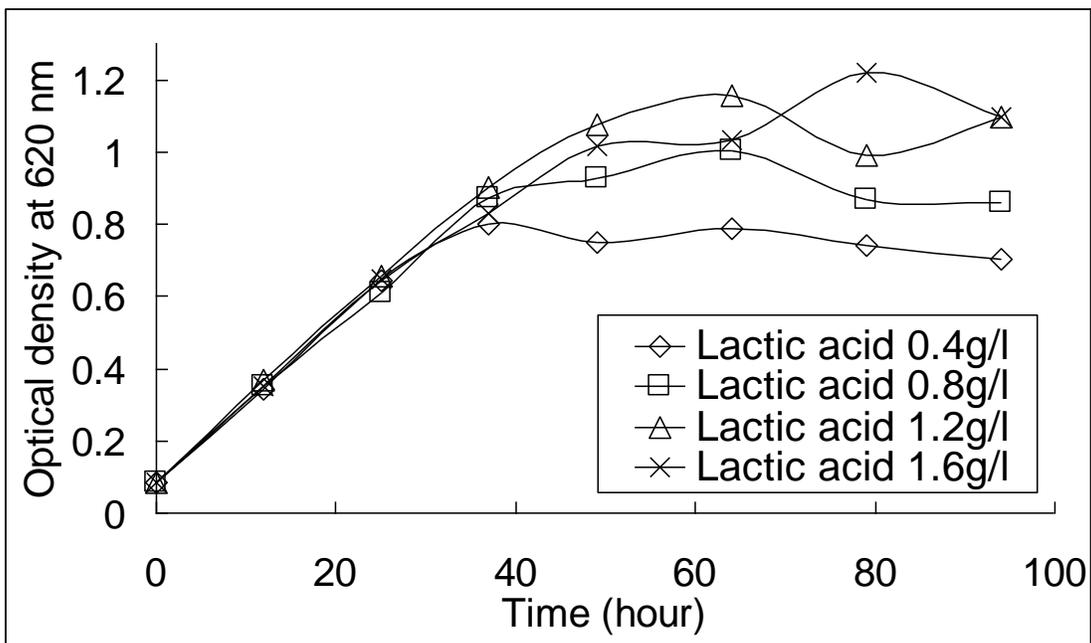


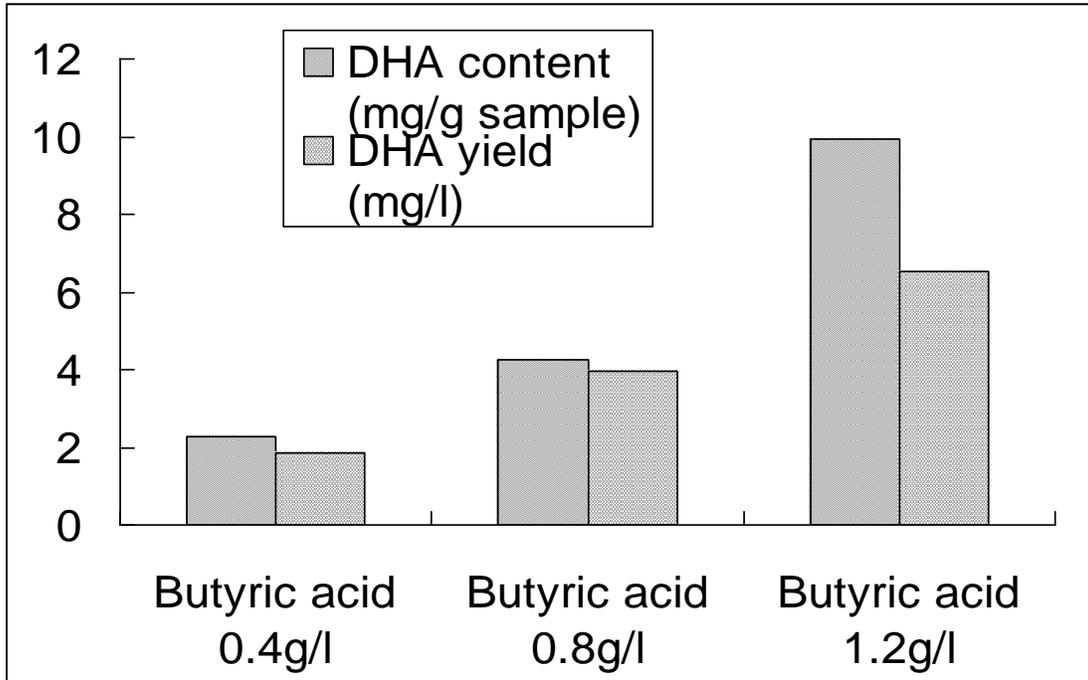
Figure 5.4 Optical density of *C. cohnii* by lactic acid

#### 5.4.1.2 DHA production

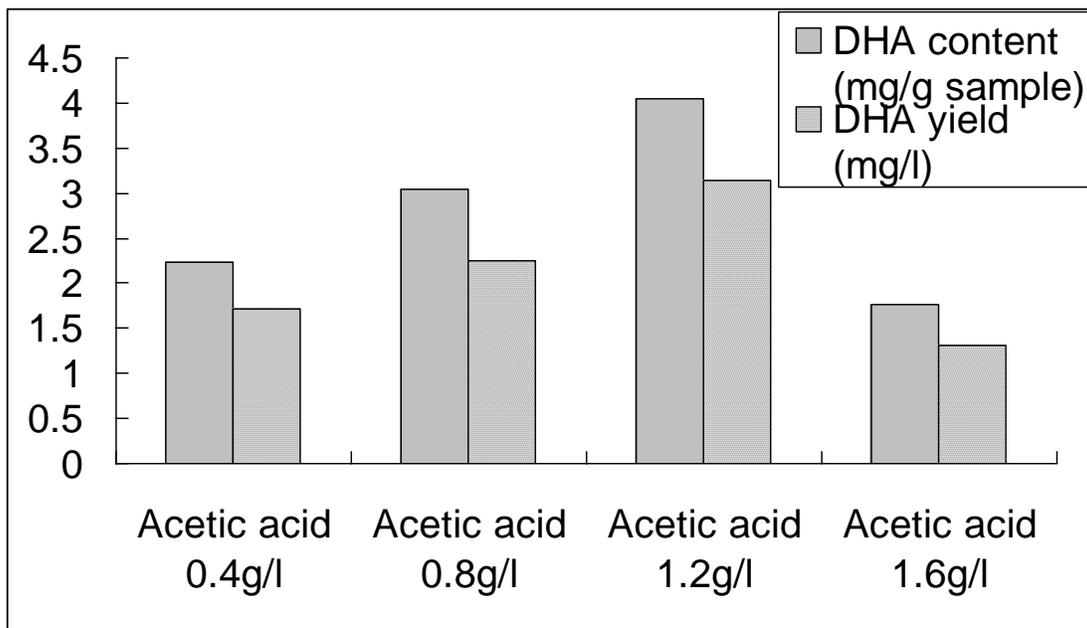
The dinoflagellate *C. cohnii* has been identified as a good producer of DHA. *C. cohnii*

can accumulate lipid with a high content of DHA (from 20% to 30% of the total lipid), which was the same as our result. The DHA yield of microalgae is related to both biomass and DHA content in the cell, which are highly related to the growth situation. In general, the growth and metabolism of microorganism are inhibited under conditional stress (carbon limitation or nitrogen limitation) and some changes in lipid composition occur to enable microalgae to survive poor environments (Yumoto, 2004). Previously reported strategies for adaptive response of microbes in terms of fatty acid changes include: (1) increasing the degree of fatty acid unsaturation, (2) shortening fatty acid chain length, and (3) increasing the proportion of branched fatty acids (Gounot and Russell, 1999). These changes in lipid composition are associated with maintaining the membrane fluidity since unsaturated, shortened and branched fatty acids have lower melting points than saturated straight long-chain fatty acids (Suutari, 1994). In *C. cohnii*, DHA is a typical unsaturated fatty acid. That is to say, if the microalgae grow relatively slowly, its DHA content will relatively high.

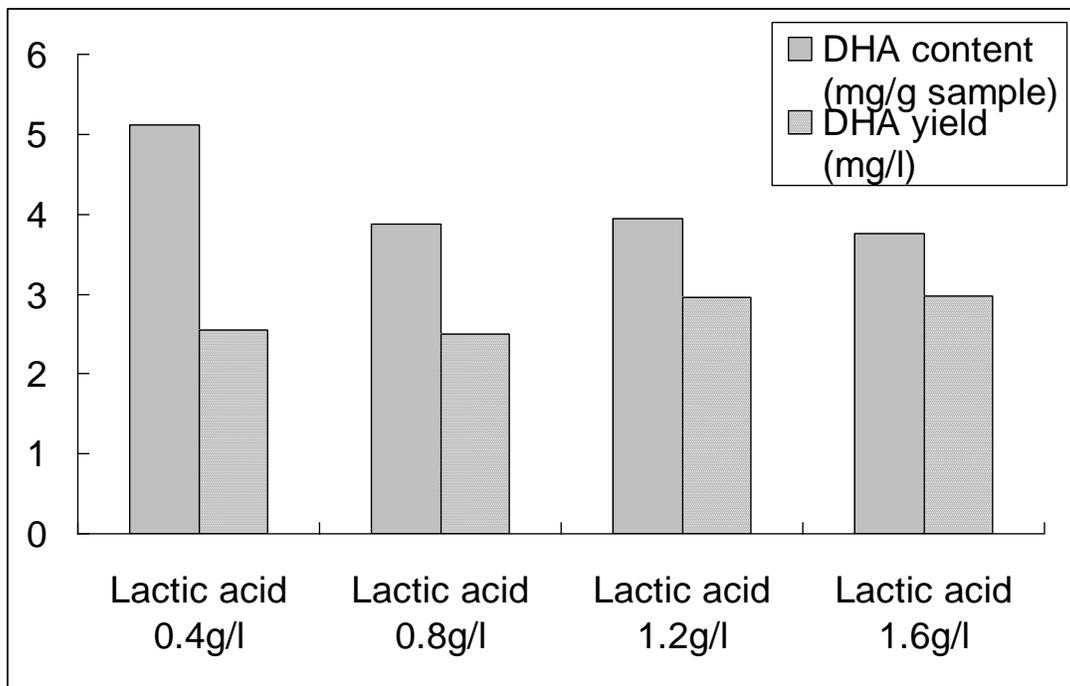
Both DHA content and DHA yield increased as butyric acid concentration increased from 0.4g/l to 1.2g/l. The best concentration for DHA yield was 1.2g/l butyric acid. Although microalgae in 0.8g/l butyric acid had a higher biomass, but the DHA content in 1.2g/l was much higher than that in 0.8g/l, due to low growth rate caused by substrate inhibition (Figure 5.5). As for the acetic acid, both DHA content and DHA yield increased as acetic acid increased from 0.4g/l to 1.2g/l and then decreased as the acetic acid concentration increased to 1.6g/l (Figure 5.6). The best concentration for DHA yield was 1.2g/l, because at end of the cultivation, compared with 1.2g/l, microalgae in 1.6g/l acetic acid had a higher growth rate yet lower DHA content. The DHA content decreased as the lactic acid concentration increased from 0.4g/l to 1.6g/l (Figure 5.7). *C. cohnii* in 0.4g/l lactic acid had a slow growth rate due to substrate inhibition and high DHA content. The DHA yield increased as the lactic acid increased from 0.4g/l to 1.2g/l. DHA yield of 1.2 g/l lactic acid was just a slightly higher than that of 1.6g/l lactic acid. *C. cohnii* in 1.2g/l had a much higher biomass than that of other concentration, so the best concentration was still also 1.2g/l. Compared with three organic acids, the butyric acid was the best for DHA yield, for it could reach 6.53mg/l in 1.2g/l butyric acid, which was much higher than that from acetic acid and lactic acid.



**Figure 5.5 DHA production from *C. cohnii* by butyric acid**



**Figure 5.6 DHA production from *C. cohnii* by acetic acid**



**Figure 5.7 DHA production from *C. cohnii* by lactic acid**

### ***5.4.2 Fermentation of two organic acids combination***

#### ***5.4.2.1 Cell growth in acid mixtures***

The result showed that acetic acid tended to be beneficial for biomass accumulation. When either butyric acid or lactic acid was mixed with acetic acid, the OD could reach 1.3 or above (Figure 5.8 & Figure 5.9). When acetic acid was mixed with butyric acid, no difference in biomass was seen, regardless of the butyric acid concentration. Except when the acetic acid: lactic acid ratio was 1:4, the biomass growth was almost the same. However, when butyric acid and lactic acid were mixed, the growth rate decreased as the butyric acid concentration increased. When Lactic acid is 0.96g/l and butyric acid 0.24 g/l, the biomass reached the highest point. This indicates that when lactic acid was mixed with butyric acid, higher lactic acid is better for biomass growth (Figure 5.10).

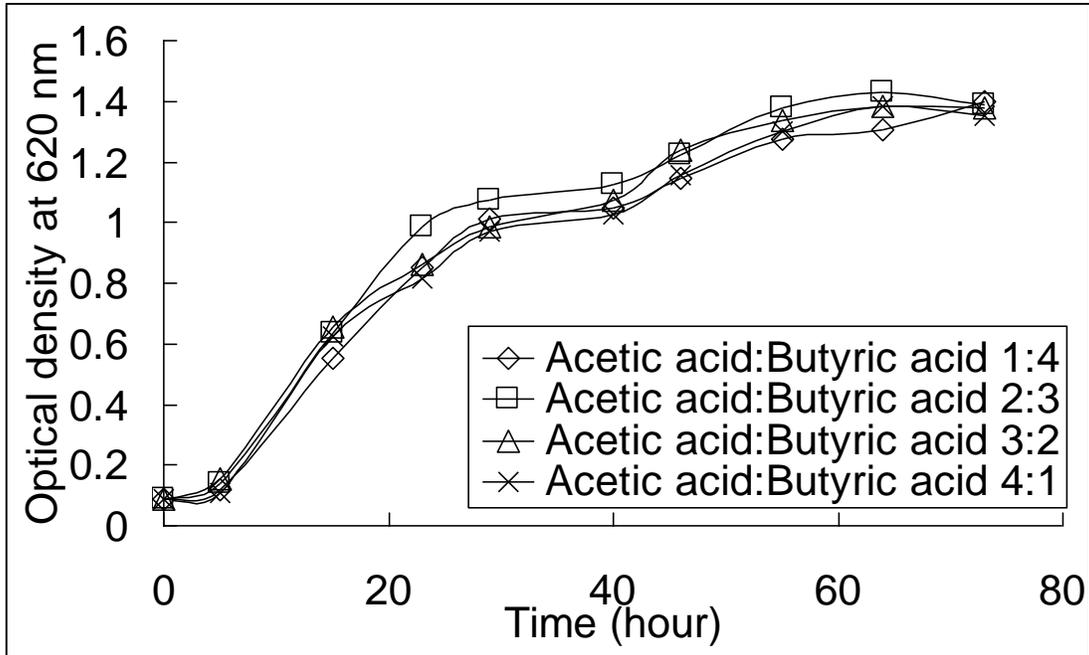


Figure 5.8 Optical density of *C. cohnii* by butyric acid/acetic acid mixture

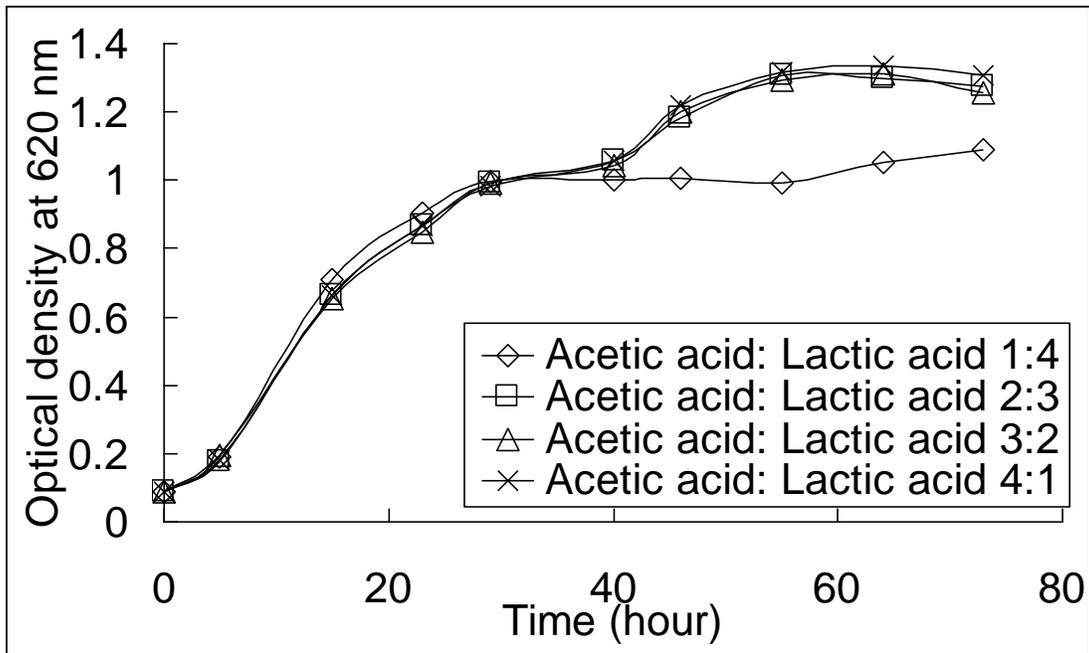
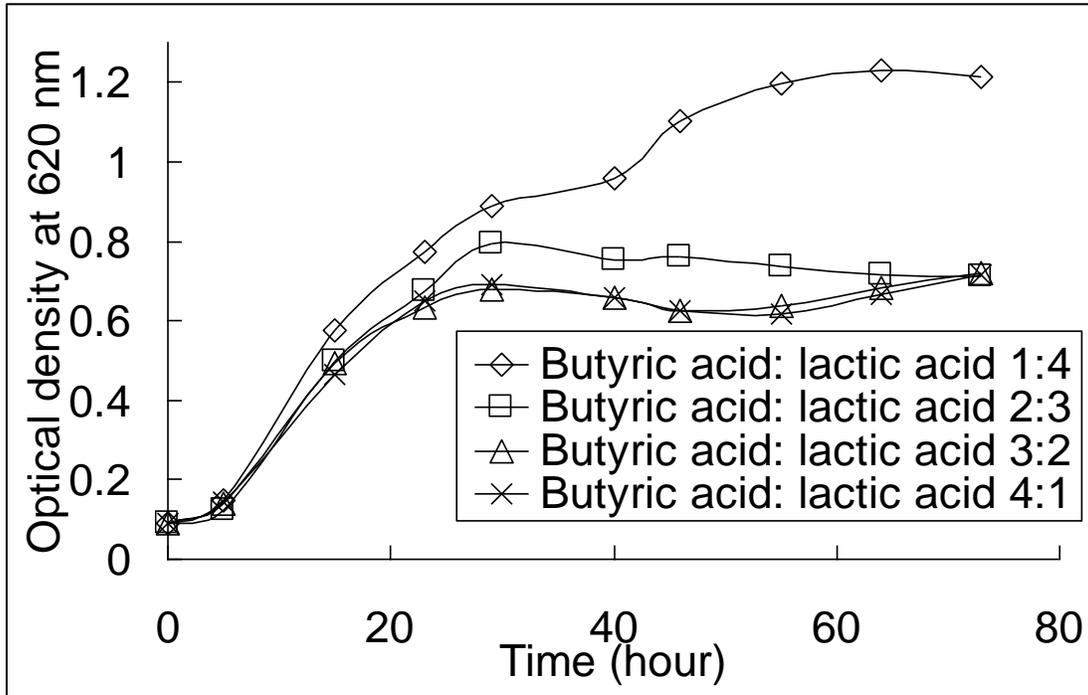


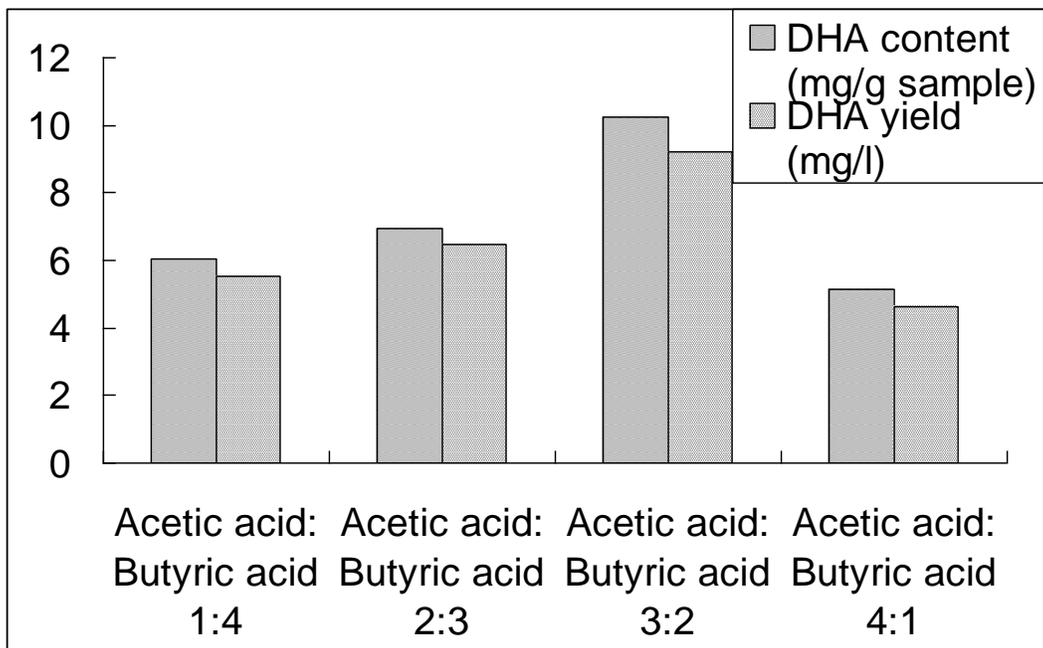
Figure 5.9 Optical density of *C. cohnii* by acetic acid/lactic acid mixture



**Figure 5.10 Optical density of *C. cohnii* by butyric acid/lactic acid mixture**

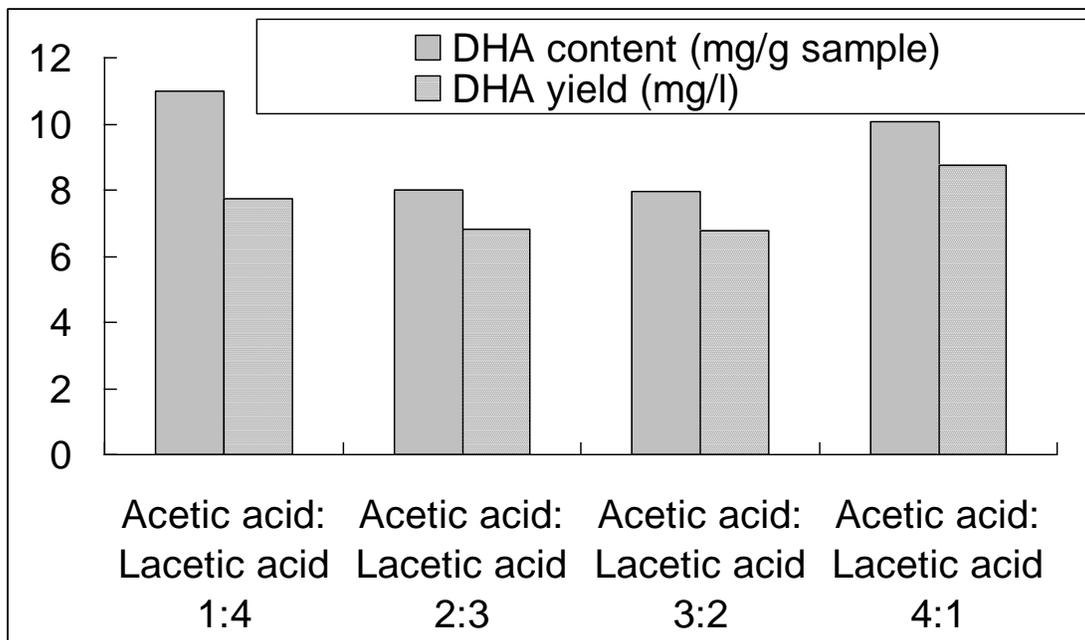
#### 5.4.2.2 DHA production

The principle of DHA yield in mixture organic acid cultivation is the same with cultivation in single organic acid. The DHA yield of microalgae is related to both biomass and DHA content in the cell and the DHA content has an opposite trend to the growth rate. When concentrations of acetic acid was 0.72g/l and butyric acid 0.48g/l, the mixture was best for DHA accumulation (Figure 5.11). Therefore, acetic acid and butyric acid would influence each other in both biomass growth and DHA accumulation.



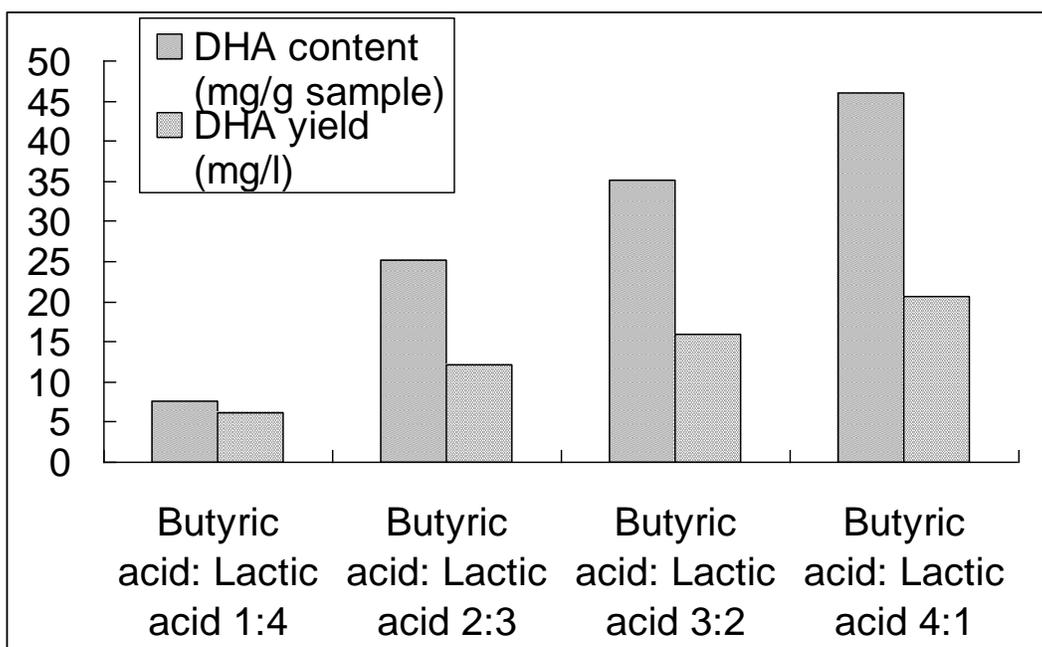
**Figure 5.11 DHA production from *C. cohnii* by acetic acid/butyric acid mixture**

In addition, when lactic acid was 0.96g/l and acetic acid 0.24 g/l, the DHA content was best (Figure 5.12). That is, the lactic acid was better for DHA accumulation. But the DHA content trend was a little different from the trend of butyric acid-acetic acid mixture. From this trend we can see, when acetic acid or lactic acid concentration was higher, the DHA content was higher, which might be because when acetic acid was mixed with lactic acid, both organic acids exerted little influence on each other utilization. Acetic acid and lactic acid did not intensely influenced by each other and their function was more likely to be additive effect.



**Figure 5.12 DHA production from *C. cohnii* by acetic acid/lactic acid mixture**

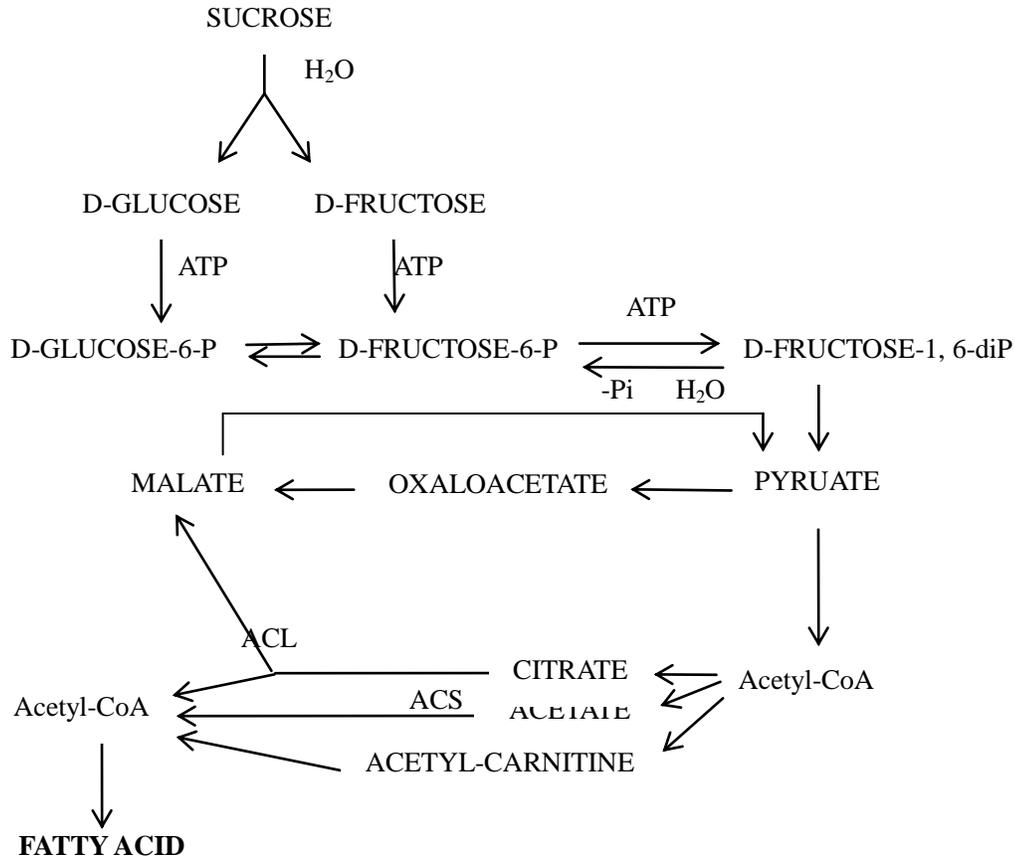
On the other hand, when lactic acid was mixed with butyric acid, due to low growth rate, the DHA content of this kind of mixture was relatively higher than other mixtures, which led to higher DHA yield (Figure 5.13). As a result, we presumed that lactic acid and butyric acid could influence each other in metabolic pathway greatly.



**Figure 5.13 DHA production from *C. cohnii* by butyric acid/lactic acid mixture**

Although there have been no previous reports on microalgae utilizing organic acids as an

energy source, the metabolic pathway of microalgae using glucose is very clear. The utilization of glucose process in microalgae could be explained by acetyl-CoA metabolism. In brief, the main flux of carbon from glucose to cytosolic acetyl-CoA involves glycolysis to produce pyruvate, transport of pyruvate into the mitochondrion, conversion of pyruvate into citrate, transport of citrate into the cytosol and cleavage of citrate by ATP: citrate lyase to yield acetyl-CoA (Sijtsma, 2004) as described in Figure 5.14. In addition, it has been previously found that acetyl-CoA may be supplied in the cytosol in a more direct way by cultivation of the organism on C<sub>2</sub> compounds, like acetic acid and ethanol. The conversion of acetic acid into acetyl-CoA involves a one-step enzymatic reaction catalyzed by the enzyme acetyl-CoA synthetase. The utilization of ethanol by *C. cohnii* could suggest the presence of an alcohol dehydrogenase, which converts ethanol to acetaldehyde, and an acetaldehyde dehydrogenase, which converts acetaldehyde to acetate (Sijtsma, 2004). Referring to biochemistry and subcellular location of acetyl-CoA metabolism in yeasts (Pronk & PRONK, 1996), this difference may be that during growth on glucose, export of acetyl-CoA from the mitochondrial matrix to the cytosol is required to make it available for lipid synthesis, while acetic acid can be directly activated to acetyl-CoA by the action of acetyl-Coenzyme A synthetase, and then this would curtail the need for translocation of acetyl-CoA from the mitochondrial matrix. In our experiments, the acetic acid serves as a C<sub>2</sub> compound, lactic acid a C<sub>3</sub> compound and butyric acid as a C<sub>4</sub> compound. Therefore, acetic acid can be used more efficiently with a higher biomass, and while culturing with a mixture of lactic acid and butyric acid, the two organic acids may compete for the same enzyme for degradation, so this kind of mixture has a low efficiency with a low biomass production. Further studies on the molecular mechanism and metabolic pathways of organic acid mixtures are needed.



**Figure 5.14** Metabolic pathways of *Cryptocodinium* (Sijtsma, 2004)

## ■ 5.5 Conclusions

This study demonstrated the possibility of DHA production from *C. cohnii* using three organic acids, butyric acid, acetic acid, and lactic acid. The DHA yield was highly related to both biomass yield and DHA content in the cell, whereas low growth rate always could bring high DHA content. Both DHA content and DHA yield increased as butyric acid concentration increased from 0.4g/l to 1.2g/l. As for the acetic acid, both DHA content and DHA yield increased as acetic acid increased from 0.4g/l to 1.2g/l and then decreased as the acetic acid concentration increased to 1.6g/l. The DHA content decreased as the lactic acid concentration increased from 0.4g/l to 1.6g/l. The DHA yield increased as the lactic acid increased from 0.4g/l to 1.2g/l. DHA yield of 1.2 g/l lactic acid was just a slightly higher than that of 1.6g/l lactic acid. 1.2g/l was the best concentration for DHA yield in three single organic acid media. In two organic acids mixture media, acetic acid tended to be beneficial for biomass accumulation, regardless whether butyric acid or lactic acid was mixed with acetic acid, the OD could reach 1.3

or above. When butyric acid was mixed with lactic acid, it could give out the highest DHA yield, due to mutual influence between butyric acid and lactic acid.

## • Chapter 6- Conclusions and future work

### ■ 6.1 Conclusions

A unique two-step fermentation to produce DHA was proposed. The two core parts of the fermentation are low value agricultural residues such as corncob fermentation by *C. tyrobutyricum* to produce organic acids mixture, in which butyric acid was the main product. Then organic acid mixture fermentation by *C. cohnii* produced DHA. We completed a preliminary explore on this issue.

The effect of glucose and xylose on the yield of butyric acid produced by *C. tyrobutyricum* was investigated, separately. Cell growth of *C. tyrobutyricum* increased with increasing initial glucose or xylose concentration, but was not affected significantly when the concentration was above 55g/l for glucose or 35g/l for xylose. Butyric acid yield increased from 2.628g/l to 27g/l as glucose concentration increased from 5g/l to 75g/l, whereas butyric acid yield in xylose increased from 3.24g/l to 16.29024g/l as xylose initial concentration increased from 5g/l to 75g/l, but the conversion rate from xylose or glucose to butyric acid decreased as the sugar concentration increased. The xylose to glucose ratio in their mixture did not significantly affect cell growth or butyric acid yield.

The effect of arabinose on the yield of butyric acid produced by *C. tyrobutyricum* was also studied. As for butyric acid production, compared with glucose or xylose, the arabinose was in a low efficiency, with butyric acid output of 2.25g/l in 10g/l arabinose and a long lag period of about 3-4 d. However, we found that a low concentration of arabinose can be used as a nutritional supplement to improve the fermentability of a mixture of xylose and glucose. The conversion rate of sugar to butyric acid increased as the supplement arabinose increased: 48.4% for medium without supplement, 49.375% for medium supplement with 2g/l arabinose, and 57.94% for medium with 5g/l arabinose.

The effect of the mixture of the above sugars on butyric acid production was also studied. In three-sugar mixture, we could find that 9.46g/l butyric acid was produced, which was from 14.52g/l (from 15g/l glucose and 15g/l xylose) to 8.3115g/l (from 20g/l glucose) or 9.007g/l (from 20g/l xylose). That is, arabinose can promote butyric acid productivity, but is less effective than the 30g/l glucose/xylose mixture. Also, in three-sugar mixture, it is easy to conclude that glucose was the favored substrate, followed by xylose.

In order to obtain low cost xylose, corncobs were hydrolyzed and this xylose-rich product was used to culture *C. tyrobutyricum*. The results showed that at end of the 9 d fermentation, the concentration of butyric acid from corncob hydrolysis reached 10.56 g/l, and the mimic medium reached 11.3 g/l. This suggests that corncob hydrolysate can be used as a carbon source for butyric acid production by *C. tyrobutyricum*, although some inhibitory effects were found on cell growth with corncob hydrolysate.

The effect of butyric acid, lactic acid and acetic acid on the yield of DHA produced by *C. cohnii* was also investigated, separately. The DHA yield was highly related to both biomass and DHA content in the cell, whereas low growth rate always could bring high DHA content. It was found that 1.2g/l was the best concentration for DHA yield in three single organic acid media. In two organic acids mixture media, acetic acid tended to be beneficial for biomass accumulation, regardless whether butyric acid or lactic acid was mixed with acetic acid, the OD could reach 1.3 or above. When butyric acid was mixed with lactic acid, the highest DHA yield was achieved, due to significantly increased DHA content from mutual influence between butyric acid and lactic acid.

## ■ 6.2 Future work

Based on research work completed, this project can be further developed in all following studies:

- (1) Understand the inhibitory effect of lignocellulose hydrolysates on *C. tyrobutyricum* growth and butyric acid yield.
- (2) Culture *C. cohnii* in three organic acids mixture to further study the effect of organic acid concentration and type on DHA yield.
- (3) Culture *C. cohnii* with the organic acid mixture produced by *C. tyrobutyricum* to integrate the two processes.
- (4) Develop and optimize a fed-batch culture process to increase the productivity of both microorganisms to reach the goal of higher DHA yield.

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