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Chapter 6

The Promising Fuel-Biobutanol

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Additional information is available at the end of the chapter

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1. Introduction

In recent years, two problems roused peoples' concern. One is energy crisis caused by the depleting of petroleum fuel. The other is environmental issues such as greenhouse effect, global warming, etc. Therefore, renewable sources utilization technology and bioenergy production technology developed fast for solving such two problems. Bioethanol as one of the biofuel has been applied in automobiles with gasoline in different blending proportions (Zhou and Thomson, 2009; Yan and Lin, 2009). Biobutanol is one of the new types of biofuel. It continuously attracted the attention of researchers and industrialists because of its several distinct advantages.

1.1. Property of butanol

Butanol is a four carbon straight chained alcohol, colorless and flammable. Butanol can be mixed with ethanol, ether and other organic solvent. Butanol can be used as a solvent, in cosmetics, hydraulic fluids, detergent formulations, drugs, antibiotics, hormones and vitamins, as a chemical intermediate in the production of butyl acrylate and methacrylate, and additionally as an extract agent in the manufacture of pharmaceuticals. Butanol has a 4-carbon structure and the carbon atoms can form either a straight-chain or a branched structure, resulting in different properties. There exist different isomers, based on the location of the–OH and carbon chain structure. The different structures, properties and main applications are shown as Table 1.

Although the properties of butanol isomers are different in octane number, boiling point, viscosity, etc., the main applications are similar in some aspects, such as being used as solvents, industrial cleaners, or gasoline additives. All these butanol isomers can be produced from fossil fuels by different methods, only n-butanol, a straight-chain molecule structure can be produced from biomass.

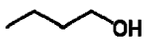
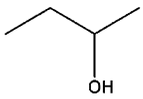
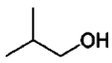
	n-Butanol	2-Butanol	iso-Butanol	tert-Butanol
Molecular structure				
Density (g/cm3)	0. 81	0. 806	0. 802	0. 789
Boiling point(°C)	118	99. 5	108	82. 4
Melting point(°C)	-90	-115	-108	25-26
Refractive index(n20D)	1. 399	1. 3978	1. 3959	1. 3878
Flash point(°C)	35	22-27	28	11
Motor octane number	78	32	94	89
Main applications	Solvents-for paints, resins, dyes, etc. Plasticizers- improve a plastic material processes Chemical intermediate -for butyl esters or butyl ethers, etc. Cosmetics- including eye makeup, lipsticks, etc. Gasoline additive	Solvent Chemical intermediate-for butanone, etc. Industrial cleaners -paint removers Perfumes or in artificial flavors	Solvent and additive for paint Gasoline additive Industrial cleaners -paint removers Ink ingredient	Solvent Denaturant for ethanol Industrial cleaners-paint removers Gasoline additive for octane booster and oxygenate Intermediate for MTBE, ETBE, TBHP, etc.

Table 1. Structures, properties and main applications of n-butanol, 2-Butanol, iso-Butanol and tert-Butanol

1.2. Advantages of butanol as fuel

Except the use of solvent, chemical intermediate and extract agent, butanol also can be used as fuel, which attracted people's attention in recent years. Because of the good properties of high heat value, high viscosity, low volatility, high hydrophobicity, less corrosive, butanol has the potential to be a good fuel in the future. The properities of butanol and other fuels or homologues are compared as Table 2. (Freeman et al., 1988; Dean, 1992)

Fuel	Octane number	Cetane number	Evaporation heat (MJ/kg)	Combustion energy(MJ/dm³)	Flammability limits (%vol)	Saturation pressure (kPa) at 38°C
Gasoline	80-99	0-10	0. 36	32	0. 6-0. 8	31. 01
Methanol	111	3	1. 2	16	6-36. 5	31. 69
Ethanol	108	8	0. 92	19. 6	4. 3-19	13. 8
Butanol	96	25	0. 43	29. 2	1. 4-11. 2	2. 27

Table 2. Properities of butanol and other fuels

Butanol appeared the good properties compared with it's homologues such as 2-butanol, iso-butanol and tert-butanol and other fuels such as Gasoline and ethanol. Actually, when ethanol is mixed with gasoline (less than 10%), there exists some disadvantages. Firstly, the heating value of ethanol is one sixth of gasoline. The fuel consumption will increase 5% if the engine is not retrofitted. Secondly, acetic acid will be produced during the burning process of ethanol, which is corrosive to the materials of vehicle. The preservative must be added when the ethanol proportion upper than 15%. Thirdly, ethanol is hydroscopic and the liquid phase separation may be occurring with high water proportion. Furthermore, ethanol as fuel cannot be preserved easily and it is more difficult in the process of allocation, storage, transition than that of gasoline.

Compared with ethanol, butanol overcomes above disadvantages and it shows potential advantages. For example, Butanol has higher energy content and higher burning efficiency, which can be used for longer distance. The air to fuel ratio and the energy content of butanol are closer to gasoline. So, butanol can be easily mixed with gasoline in any proportion. Butanol is less volatile and explosive, has higher flash point, and lower vapor pressure, which makes it safer to handle and can be shipped through existing fuel pipelines. In addition, Butanol can be used directly or blended with gasoline or diesel without any vehicle retrofit (Durre, 2007; Pfromm et al., 2010).

Actually, the first-time synthesis of biobutanol at laboratory level was reported by Pasteur in 1861 (Durre, 1998) and the industrial synthesis of biobutanol was started during 1912–1914 by fermentation (Jones and Woods, 1986). However, before 2005, butanol was mainly used as solvent and precursor of other chemicals due to the product inhibition and low butanol productivity. To bring awareness to butanol's potential as a renewable fuel, David Ramey drove his family car from Ohio to California on 100% butanol (http://www.consumerenergyreport.com /2011/02/09/reintroducing-butanol/). And then, two giant companies DuPont and BP have declared to finance development of a modernize production plant supported by research and development. (http://biomassmagazine.com/articles/2994 /eu-approves-bp-dupont-biobutanol-venture) The economy of biobutanol production also was revaluated. The research of a continuous fermentation pilot plant operating in Austria in the 1990s introduced new technologies and proved economic feasibility with agricultural waste potatoes. (Nimcevic and Gapes, 2000).

2. Production methods of butanol

Butanol can be obtained using chemical technologies, such as Oxo-synthesis and aldol condensation. It is also possible to produce butanol in the process of fermentation by bacteria and butanol as one of the products called biobutanol. The most popular bacteria species used for fermentation is *Clostridium acetobutylicum*. Because the main products of this process containing acetone, butanol and ethanol, the fermentation is called ABE fermentation (Qureshi and Maddox, 1995).

2.1. Chemical process

Butanol can be produced by chemical synthesis. One process is Oxo-synthesis, which involves the reaction of propylene with carbon monoxide and hydrogen in the presence of cobalt or rhodium as the catalyst. The mixture of n-butyraldehyde and isobutyraldehyde are obtained and then the mixture can be hydrogenated to the corresponding n-butanol and isobutyl alcohols (Park, 1996).The reactions are as following:

$$CH_3CH{-}CH_2{+}CO + H_2 \rightarrow CH_3CH_2CH_2CHO{+}\left(CH_3\right)_2CHCHO \quad (1)$$

$$\begin{aligned} &CH_3CH_2CH_2CHO + H_2 \rightarrow CH_3CH_2CH_2CH_2OH \ (a) \\ &\left(CH_3\right)_2CHCHO{+}H_2 \rightarrow \left(CH_3\right)_2CHCH_2OH \quad (b) \end{aligned} \quad (2)$$

When using cobalt as the catalyst, the reaction processes at 10~20MPa and 130~160°°C, the products ratio of n-butyraldehyde and isobutyraldehyde is 3. Rhodium as the catalyst used in industry from 1976 and the reaction processes at 0.7-3MPa and 80-120°°C.The products ratio of n-butyraldehyde and isobutyraldehyde can reach 8-16. Hydrogenaration processes by using the catalyst of nickel or copper in gaseous phase or nickel in liquid phase. Some by-products can be transferred into butanol at high temperature and high pressure that will enhance the product purity.

Another route is aldol condensation, which involves the reaction of condensation and dehydration from two molecules of acetic aldehyde. And then, the product crotonaldehyde was transformed into n-butanol by hydrogenation at 180°°C and 0.2MPa. The reaction is as following: $CH_3CH{=}CHCHO{+}2H_2 \text{—} \rightarrow CH_3CH_2CH_2CH_2OH$

Comparing the two processes, Oxo-synthesis route has the advantages of materials easily obtained, comparable moderate reaction conditions, enhanced ratio of n-butanol to isobutyl alcohol. So, Oxo-synthesis process is the main industrial route for n-butanol production. There are also some other fossil oil derived raw materials such as ethylene, propylene and triethylaluminium or carbon monoxide and hydrogen are used in butanol production (Zverlov, et al., 2006).

2.2. Biological process

Except the chemical ways, butanol can also be obtained from biological ways with the renewable resources by the microorganism through fermentation. The *Clostridia* genus is very common for butanol synthesis under anaerobic conditions, and the fermentation products are often the mixture of butanol, acetone and ethanol. A few kinds of *Clostridium* can utilize cellulose and hemicellulose with the ability of cellulolytic activities (Mitchell et al., 1997; Berezina et al. 2009).

Compared with the chemical ways for butanol production, biological ways has the distinct advantages. For example, it can utilize the renewable resources such as wheat

straw, corn core, switch grass, etc. Furthermore, biological process has high product selectivity, high security, less by-products. Furthermore, the fermentation condition of butanol production is milder than that of chemical ways and the products are easier to separate. The process of biobutanol production with Lignocellulosic feedstocks is as following (Fig. 1):

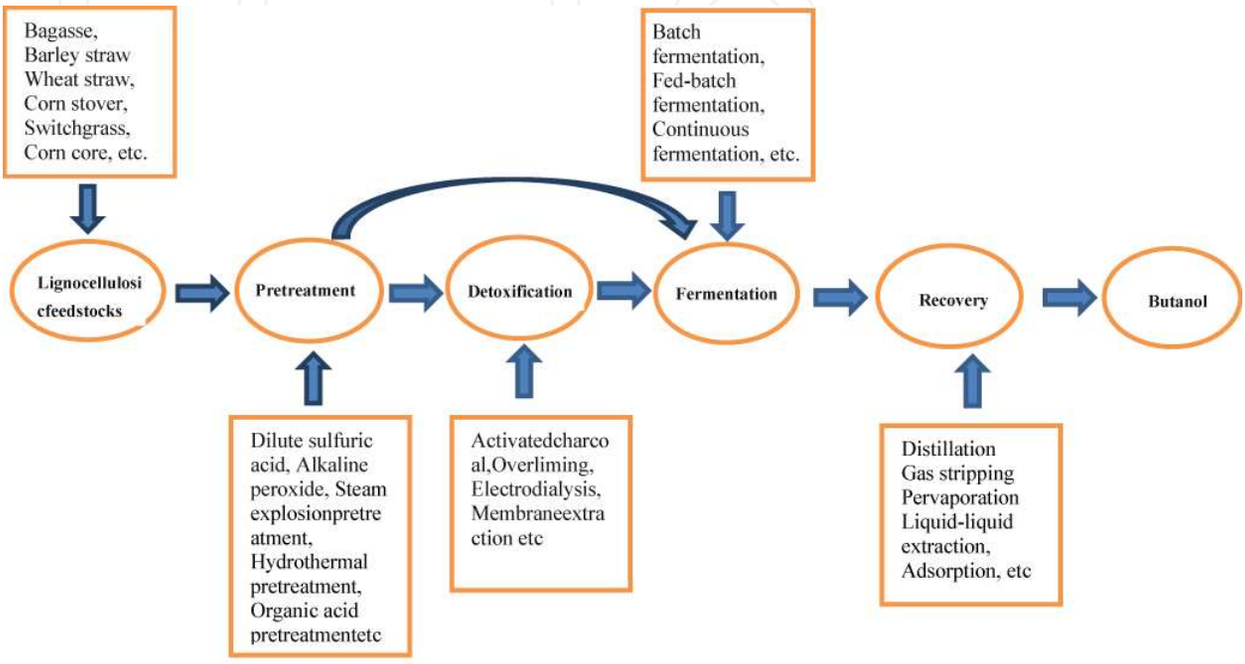


Figure 1. Butanol production process from lignocellulosic feedstocks

For the first step, biomass containing lignocellulosics should be pretreated before they were used as the substrate for the fermentation, except for a few high cellulase activity strains (Ezeji and Blaschek, 2008). The pretreatment methods are different according to the different types of biomass used. There often use dilute sulfuric acid pretreatment, alkaline peroxide pretreatment, steam explosion pretreatment, hydrothermal pretreatment, organic acid pretreatment etc. Some inhibitors such as acetic acid, furfural, 5- hydroxymethyl furfural, phenols etc. that need to be further detoxified. The ordinary detoxification methods are using activated charcoal (Wang et al., 2011), overliming (Sun and Liu, 2012; Park et al., 2010), electrodialysis (Qureshi et al., 2008c), membrane extraction (Grzenia et al., 2012) to remove the inhibitors. This step is determined by different feed stock and different pretreatment methods. After the fermentation, the desired product is recovered and purified in the downstream process. Biological ways has been set up for many years while it was inhibited for industrial application for economic reasons. So, as an alternative fuel, biomass feedstock for biobutanol production must be widely available at low cost (Kent, 2009). Therefore, by using agricultural wastes for butanol production such as straw, leaves, grass, spoiled grain and fruits etc are much more profitable from an economic point of view. Recently, other sources

such as algae culture (Potts et al., 2012; Ellis et al., 2012) also is studied as one substrate for butanol production.

3. Biobutanol production by fermentation

3.1. Microbes

Clostridium is a group of obligate, Gram positive, endospore-forming anaerobes. There are lots of strains used for ABE fermentation in different culture collections, such as ATCC (American Type Culture Collection), DSM (German Collection of Microorganisms, or Deutsche Sammlung Von Mikroorganismen), NCIMB (National Collections of Industrial & Marine Bactria Ltd), and NRRL (Midwest Area National Center for Agriculture Utilization Research, US Department of Agriculture). The different strains share similar phonotype such as main metabolic pathway and end products. Molecular biology technology offers efficient method for classification. The butanol-producing clostridium can be assigned to four groups according to their genetic background, named *C. acetobutylicum, C. beijerinckii, C. saccharoperbutyl acetonicum*, and *C. saccharobutylicum*, respectively. *C. acetobutylicum* is phylogenetically distinct from the other three groups.

The common substrate for the solvent production by these strains is soluble starch. The original starch-fermenting strains belong to *C. acetobutylicum*. A recently isolated butanol-producing strain *C. saccharobutylicum* showed high hemicellulotic activity (Berezina et al., 2009). All of the four group strains can ferment glucose-containing medium to produce solvent. In 4% glucose TYA medium, *C. beijerinckii* gave the lowest solvent yield (28%), while the solvent yield was upper than 30% compared to the other three groups (Shaheen et al., 2000). In standard supplement maize medium (SMM), *C. acetobutylicum* is the best strain for maize fermentation, and the total solvent concentration can reach 19g/L. The solvent yield was 16, 14, and 11 for that of *C. beijerinckii, C. saccharoperbutyl acetonicum*, and *C. saccharobutylicum* respectively. However, *C. acetobutylicum* can't ferment molasses well and it produces bright yellow riboflavin in milk, which is different from other groups and easy identified. The best molasses-fermenting strains belong to *C. saccharobutylicum* and *C. beijerinckii* (Shaheen et al., 2000). *C. saccharoperbutyl acetonicum* can utilize sugar, molasses and maize. Comparing to *C. acetobutylicum*, *C. beijerinckii* was more tolerant to acetic acid and formic acid (Cho et al., 2012), which suggests the advantage when using lignocellulosic hydrolysate treated with acetic and formic acid as substrate.

There are also some *C. beijerinckii* strains produce isopropanol instead of acetone (George et al., 1983). Some microorganisms can produce biobutanol from carbon monoxide (CO) and molecular hydrogen (H_2), including acetogens, *Butyribacterium methylotrophicum*, *C. autoethanogenum*, *C. ljungdahlii* and *C. carboxidiworans*. The *C. carboxidivorans strain P7(T)* genome possessed a complete Wood-Ljungdahl pathway gene cluster which is responsible for CO, hydrogen fixation and conversion to acetyl-CoA(Fig.2) (Bruant et al., 2010).

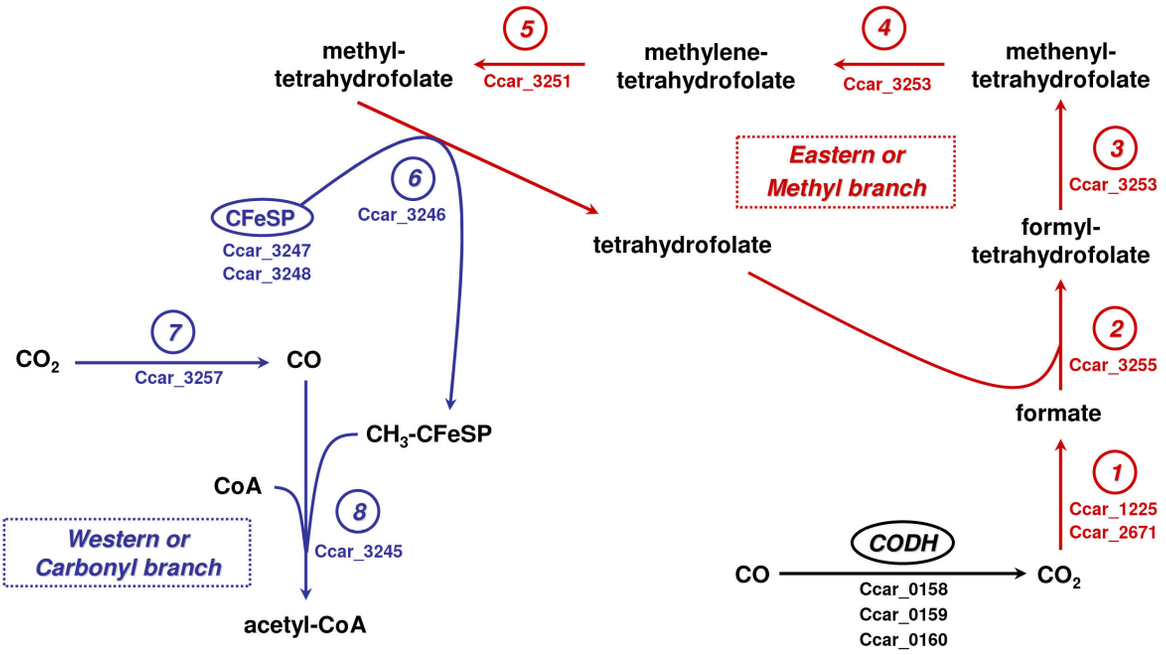


Figure 2. Wood-Ljungdahl pathway in Carboxdivorans Strain P7T. (Bruant et al. 2010, http://creativecommons. org/licenses/by/3. 0/)Wood-Ljungdahl pathway key enzymes and protein identified in C. carboxidivorans strain P7T. 1, formate dehydrogenase; 2, formate-tetrahydrofolate ligase; 3 and 4, bifunctionalmethenyl-tetrahydrofolatecyclohydrolase/methylene-tetrahydrofolate dehydrogenase (NADP+); 5, 5, 10-methylene-tetrahydrofolate reductase; 6, 5-methyl-tetrahydrofolate:- corrinoid iron-sulfur protein methyltransferase; 7, carbon monoxide dehydrogenase; 8, acetyl-CoA synthase; CFeSP, corrinoid iron-sulfur protein; CODH, additional carbon monoxide dehydrogenase complex. Reactions from the western branch are indicated in blue, those from the eastern branch are indicated in red. The corresponding genes in strain P7T genome are indicated below the enzyme.

3.2. Metabolic pathway

The ABE producing strains can hydrolyze starch to glucose or other hexose by amylases. Glucose was firstly converted to pyruvate through the Embden-Meyerhoff pathway (EMP, or glycolysis). Pyruvate was then cleaved to acetyl-CoA by pyruvate ferredoxin oxidoreductase. Acetyl-CoA is the common precursor of all the fermentation intermediate and end products. The enzyme activity and the coding genes have been widely assayed and described in butanol-producing strains (Dürre et al., 1995; Gheshlaghi et al., 2009).

The ABE fermentation process can be divided into two successive and distinct phase as acidogenesis phase and solvetogenesis phase. The acidogenesis phase is accompanied with cell exponential growth and pH drop, accumulation of acetate and butyrate. Solventogenesis phase begins with endospore forming and the cells entering stationary state. The products of acidogenesis phase include acetate and butyrate. Acetate forms from Acetyl-CoA, which is catalyzed by two enzymes, phosphotransacetylase (PTA, or phosphate acetyltransferase, endoced by *pta* gene) and acetate kinase (AK, encoded by *ak* gene). The butyrate synthesis is a little complicated with more steps. At first, two molecular of acetyl-CoA is catalyzed by thiolase (thl, or acetyl-CoA acetyltransferase, encoded by *thl* gene) and transforms into one molecular C4 unit acetoacetyl-CoA, which is another important node and precursor of buty-

rate, acetone, and butanol synthesis. The acetoacetyl-CoA is subjected to three enzymes in turn and another C4 unit butyryl-CoA is the intermediate product. The three enzymes are hydroxybutyryl-CoA dehydrogenase (encoded by *hbd* gene) (Youngleson et al., 1995), crotonase (CRT, or hydroxybutyryl-CoA dehydrolase, encoded by *crt* gene), and butyryl-CoA dehydrogenase (BCD, encoded by *bcd* gene). Accordingly, three encoded genes coexist in the BCS operon with additional two genes coding for the α and β subunit of electron transfer protein (Bennett and Rudolph, 1995). Butyryl-CoA was then catalyzed by phosphotransbutylase (PTB, or phosphate butyltransferase, encoded by *ptb* gene) and butyrate kinase (BK, encoded by *bk* gene) to form butyrate during acidogenesis phase.

As the organic acid accumulation, pH drop to the lowest point during the fermentation. This leads to the switch of acidogenesis phase to solventogenesis phase. Acetate and butyrate are reassimilated and participate in the solvent formation. Under the catalyzing of CoA transferase (CoAT, two unit encoded by *ctfα* and *ctfβ*), acetate and butyrate was transformed into acetyl-CoA and butyryl-CoA respectively again. The alcohols formation share the same key enzymes, NAD(P)H dependent aldehyde/alcohol dehydrogenases (encoded by *adh*1 and *adh*2 gene) (Chen, 1995). In addition, Butanol owns its unique butanol dehydrogenase (encoded by *bdh* gene) (Welch et al., 1989). The formation of acetone from acetoacetyl-CoA is a two-step reaction. Acetoacetyl-CoA is catalyzed to acetoacetate by CoA transferase. Acetone is produced after a molecular CO_2 released from acetoacetate by decarboxylase (AADC, encoded by *aadc* gene) (Janati-Idrissi et al., 1988; Cary et al., 1993). Both acid reassimilation and acetone formation utilize CoA transferase, however, the butyrate uptake was not concomitant with the production of acetone (Desai et al., 1999). The metabolic pathway accompanied by electron transfer and reduction force forming. The main ABE fermentation pathway was illustrated in Fig.3.

Solventogenic genes *aad*, *ctfA*, *ctfB* and *adc* constitute the *sol* operon (Durre et al., 1995). In some conditions, butanol producing strains lose the ability to produce solvents after repeated subculturing, called as degenerated (DGN) strain. In *C. acetobutylicum* ATCC 824, the plasmid pSOL1 carrying the *sol* operon was found missing during degenerating process (Cornillot et al., 1997). For *C. saccharoperbutyl acetonicum* strain N1-4, the *sol* genes maintained in degenerated DGN3-4 strain, while the *sol* operon was hardly induced during solventogenesis. Extract from the culture supernatants of wild-type N1-4 is enough to induce the transcription of the *sol* operon in DGN3-4 (Kosaka et al., 2007). It suggested that the degeneration maybe caused by the incompetence of the induction mechanism of the *sol* operon. The transcription of *sol* operon may be under the control of the quorum-sensing mechanism in *C. saccharoperbutyl acetonicum*.

Though the metabolic pathway is clear, the underlying regulation mechanism is poorly understood, such as the phase switch of fermentation, the relationship between solventogenesis and sporulation. Answering these questions is critical to improve the efficiency of butanol producing fundamentally. Proteomics and transcriptomics can provide more unknown details, which will be helpful for solving these problems (Sivagnanam et al., 2011; Sivagnanam et al., 2012).

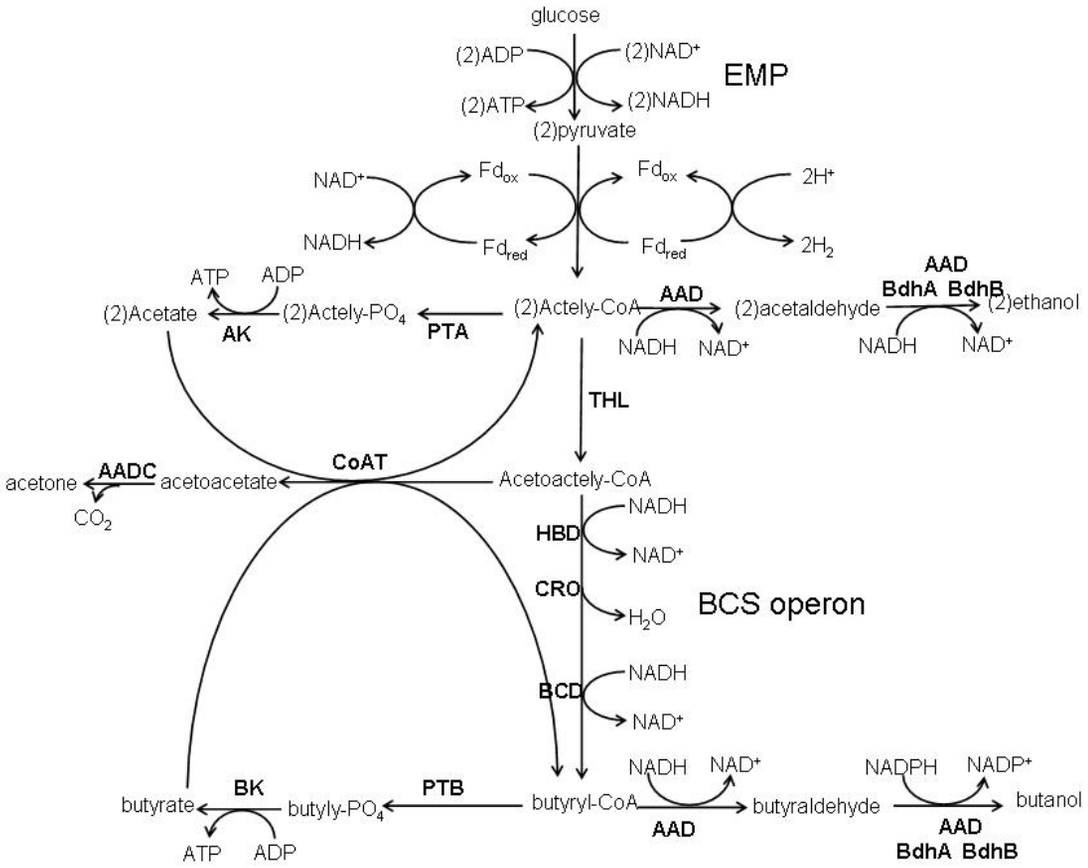


Figure 3. Metabolic pathway of Acetone-butanol-ethanol fermentation. EMP: Embden-Meyerhoff pathway (glycolysis); AK, acetate kinase; PTA, phosphotransacetylase; CoAT, CoA transferase; AADC, acetoacetate decarboxylase; THL, thiolase; BK, butyrate kinase; PTB, phosphotransbutylase; HBD, hydroxybutyryl-CoA dehydrogenase; CRO, crotonase; BCD, butyry-CoA dehydrogenase; AAD, aldylde/ alcohol dehydrogenase; BdhA, butyryl-CoA dehydrogenase A; BdhB, butyryl-CoA dehydrogenase B.

3.3. Metabolic engineering

The increasing genetic knowledge provides feasible technique for the strain modification. Many efforts have been made to construct the strain with high butanol tolerance, superior butanol yield, productivity and less byproduct. The process can be classified into pathway-based construction and regulation-based construction.

Except butanol, acetone and ethanol are main products in ABE fermentation. The byproduct, especially acetone is low valuable and undesirable. Blocking the expression key enzyme gene for acetone is thought perfect to decrease the split flux and enhance butanol yield. However, the results were not ideal as expected. Knocking out the C. acetobutylicum EA 2018 *adc* gene, the acetone is still produced in low level (Jiang et al., 2009). In *C. beijerinckii* 8052, the strain with *adc* gene disruption produced similar acetone with the original wild type strain (Han et al., 2011). To block acetate and acetone pathway by knocking out gene

adc and *ctfA* reduced solvent production (Lehmann et al., 2012). These results demonstrated that the butanol metabolic mechanism is more complicated than expected.

Acetate and butyrate are produced during acidogenesis, and then they are transformed into acetyl-CoA and butyl-CoA to participate the solvent formation during solventogenesis phase. It seems an ineffective loop. In fact, the "inefficiency" loop is necessary for acid accumulation and switching to solventogenesis, at the same time, energy and reduction force were reserved. Disruption of acetate and butyrate pathway didn't enhance butanol production. Knocking out acetate biosynthetic pathway gene by Clos Tron had no significant influence on the metabolite distribution (Lehmann et al., 2012). Disruption of *ptb* gene blocked the butyrate synthesis and led to acetate and lactate accumulation. Some mutant strain without *bk* gene even can't survival (Sillers et al., 2008). It indicated that the pathways seeming useless were necessary for butanol synthesis. What's more, it is not possible to improve performance by decrease acid formation.

The genes participate in butanol synthesis including of *thl*, BCS operon, and *add*, *bdh*. Overexpression these genes are thought useful to increase the butanol yield. Overexpression of *aad* gene alone could enhance butanol production (Nair and Papoutsakis, 1994; Tummala et al., 2003). Transformed strain M5 (*sol* operon deficient because of lose of plasmid pSOL) with a plasmid carrying *aad* gene restored butanol-producing capability (Nair and Papoutsakis, 1994). Overexpression of *aad* gene and down-regulated *ctf* gene increased the butanol and ethanol production. To boost the butyryl-CoA pool, the strain with both *thl* and *aad* overexpression was constructed. However, butyrate and acetone concentration were increased, not butanol. The *thl* overexpression with *ctf* knock down didn't change the product significantly (Sillers et al., 2009). So, the metabolic is more complicated than it seems. Theoretical analyses also suggested alteration single solvent-associated gene is not sufficient to increase butanol yield (Haus et al., 2011).

Low butanol tolerance of the strains is another problem of butanol production. Although butanol synthesis is spontaneous in clostridium, the wild type strains can't endure high butanol concentration upper than 2%. Butanol stress influence gene expression of amino acid, nucleotide, glycerolipid biosynthesis and the cytoplasmic membrane composition (Janssen et al., 2012). Cells have heat shock response system will protect it from heat or other stress (Bahl, Müller et al. 1995). Overexpression of grosESL improved the strain tolerance and butanol titer (Tomas et al., 2003).

The utilization of xylose and other carbon sources was inhibited by glucose is a phenomenon called as Carbon catabolite repression (CCR). CCR limited the efficiency of butanol fermentation with lignocellulosic material as substrate. The utilization rate of pentose was improved efficiently by knocking out pleiotropic regulator gene *ccpA*, *glcG* (responsibility for phosphoenoopyruvate-dependent phophotransferase system, PTS) and overexpressing the genes of xylose utilization (Ren et al., 2010; Xiao et al., 2012). By heterogonous expression transaldolase gene talA in ATCC 824, the xylose utilization was improved significantly (Gu et al., 2009). Knocking out xylose repressor gene *XylR* also increased the fermentation efficiency (Xiao et al., 2012).

There also some strategies aim at the upstream regulation. Global transcription machinery engineering (gTME) is thought to be a promising method to improve the butanol-producing performance (Alper et al., 2006; Papoutsakis, 2008). By regulating the transcription factor, the gTME strategy is thought to be able to change the metabolic strength and direction. gTME has been shown an efficient solution to improve substrate utilization, product tolerance, and production in yeast (Alper et al. 2006) and *E. coli* (Chen et al., 2011). In butanol-producing *Clostridium*, the metabolic pathway have been described clearly, however, the mechanism of metabolism regulation is still not fully understood. This situation keeps the gTME strategy away from butanol-producing strains. Much effort should be devoted on the proteomics and transcriptomics etc. that will increase more details behind the appearance of ABE fermentation. A true gTME strategy will bring fresh and effective innovation to the butanol fermentation.

The concept of metabolic engineering is to develop strains as "cell factory" which is efficient for desired products production from renewable sources (Na et al., 2010). Some microbes attracted interests because they are more tolerant to butanol than *Clostridium*, although these bacteria haven't natural solvent-producing ability. Some kinds of Lactic acid bacteria can grow in 3-4% butanol (Liu et al., 2012) after long term adaption, that makes them promising host for butanol producing. The synthetic biology strategy has been implemented by constructing the whole butanol-producing pathway in *Escherichia coli*, *Bacillus subitilis*, *Saccharomyces cerevisiae* and *Pseudomonas putida* (Shen and Liao, 2008; Nielsen et al., 2009). This strategy deserves further attempts in spite of the poor final butanol concentration.

3.4. Fermentation application

ABE fermentation can be conducted as batch, fed-batch, and continuous under anaerobic conditions. Batch fermentation is the simplest mode. The substrate is typical 40-80g/L and the efficiency decreased as substrate concentration upper than 80g/L (Shaheen et al, 2000). With optimized physiological and nutritional parameters, 20g/L n-butanol was obtained by *C. beijerinckii* ATCC 10132 in 72h (Isar and Rangaswamy, 2012). Fed-batch fermentation was adopted to avoid substrate inhibition. However, because of product inhibition, the substrate feeding seems ineffective. The solvent must be removed from the broth to decrease the product toxicity. The solvent can be removed by several ways such as liquid-liquid extraction, perstraction, gas-stripping, and pervaporation etc. (Qureshi and Maddox, 1995; Qureshi and Blaschek, 2001b). The whole systemic technique of high productivity was constructed by continuous feeding combined with product removal (Qureshi et al., 1992), such as using membrane reactor (Qureshi et al., 1999a). With these techniques, the fermentation can be continuing for a long time and resulting in higher productivity. To improve the utilization efficiency of cells, the immobilization system is used (Huang et al., 2004; Qureshi et al., 2000; Lienhardt et al., 2002). Comparing with the free cell system, the immobilization system is easier to separate cells from product, can reach high cell concentration and productivity, and can decrease nutrient depletion and product inhibition.

Co-culture is another important way for butanol fermentation (Abd-Alla and El-Enany, 2012). *C. beijerinckii* NCIMB 8052 was entangled with ATCC 824 and thought as *C. acetobuty-*

licum before the 16S rDNA based method was exploited (Johnson and Chen, 1995). These data implied that they could be cocultured before isolation. A microflora of four strain isolated from hydrogen-forming sludge of sewage performed a little high solvent yield (Cheng et al., 2012). Different strains possess various advantages, either with larger carbon substrate, higher butanol yield, or with high substrate and product tolerance. The co-culture should possess potential benefits and be harnessed fully after all the details are disclosed for each individual strain.

4. Separation of butanol product

Because butanol has a higher boiling point than water, therefore, distillation is not suitable for butanol recovery. Other processes such as adsorption, pervaporation, membrane pertraction, reverse osmosis and gas stripping have been developed to improve recovery performance and reduce costs (Oudshoorn et al., 2009; Ezeji et al., 2004b).

4.1. Adsorption process

Adsorption is the technology operating easily for the butanol separation. Butanol can be adsorpted by the adsorbents in the fermenter and then the butanol was obtained by desorption. A variety of materials can be used as adsorbents for butanol recovery and silicalite is the common one used (Qureshi et al., 2005b; Ezeji et al., 2007). Silicalite is a form of silica with a zeolite-like structure and hydrophobic properties, it can selectively adsorb small organic molecule like C1–C5 alcohols from dilute aqueous solutions (Zheng et al., 2009). However, adsorption separation process is not suitable on an industrial or semi-technical scale because the capacity of adsorbent is very low.

4.2. Butanol recovery by membrane reactor

Immobilization of microorganisms in the membrane or using membrane reactors is another option of butanol removal. The productivity can be enhanced obviously by this way. Huang et al. reported the continuous ABE fermentation by immobilized *C. acetobutylicum* cells with the fibrous as carrier and a productivity of 4.6 g/L/h was obtained (Huang et al., 2004). Qureshi et al. studied the butanol fermentation by immobilized *C. beijerinckii* cells with different carriers such as clay brick, the reactor productivity was enhanced to 15.8 g/(lh) (Qureshi and Blaschek, 2005a). Although the butanol productivity increased by using immobilized cell fermentation, leakage of cells from the matrices is a frequent problem for the industrial application. There still some other problems such as poor mechanical strength and increase mass transfer resistance etc.

4.3. Butanol recovery by gas stripping

Gas stripping seems to be a promising technique that can be applied to butanol recovery combined with ABE fermentation. When the gas (ordinary N_2 or CO_2) are bubbled through the fer-

mentation broth, it captures the solvents. The solvents then condensed in the condenser and are collected in a receiver. Ezeji applied gas stripping on the fed-batch fermentation, 500 g glucose was consumed and 233 g/l solvent was produced with the productivity of 1.16 g/(Lh) and the yield of 0.47 g/g.When combined with continuous fermentation with gas stripping, 460g/l solvent was obtained with 1163g glucose consuming (Ezeji et al., 2004a; Ezeji et al., 2004b).

4.4. Butanol recovery by pervaporation

Pervaporation is a membrane-based process that allows selective removal of volatile compounds from fermentation broth. The membrane is placed in contact with the fermentation broth and the volatile liquids or solvents diffuse through the membrane as a vapor which is recovered by condensation. A vacuum applied to the side of permeate. Polydimethylsiloxane membranes and silicon rubber sheets are generally used for the pervaporation process. Selection of a suitable polymer forming the active part of the membrane is a key factor in this case. In the batch fermentation, Evans and Wang increased the solvent concentration and productivity from 24.2g/l and 0.34g/(lh) to 32.8g/l and 0.5g/(lh) with pervaporation (Evans and Wang, 1988). Groot et al. applied pervaporation on the fed-batch fermentation and the solvent productivity and concentration reached 0.98g/lh and 165.1g/l (Groot et al., 1984). The Reverse osmosis is another recovery technique that based on membranes. Before the reverse osmosis is carried out, the suspended vegetative organisms must be removed using the hollow-fiber ultra-filter. After the pretreatment, reverse osmosis starts to dewater the fermentation liquor by rejecting solvents but allowing water to pass through the membrane. And then, the products are concentrated (Zheng et al., 2009).

4.5. Liquid–liquid extraction

Liquid–liquid extraction can be used to remove solvents from the fermentation broth. In this process, the water-insoluble organic extractant is mixed with the fermentation broth. Butanol is more soluble in the organic (extractant) phase than in the aqueous (fermentation broth) phase. So, butanol can be selectively concentrated in the organic phase. As the extractant and fermentation broth are immiscible, the extractant can easily be separated from the fermentation broth after butanol extraction. (Qureshi and Blaschek, 1999a). However, there still some problems with liquid–liquid extraction such as toxicity of extractant, extraction solvent losing, the formation of an emulsion, etc. Oleyl alcohol as a good extractant with relatively low-toxic has been used widely by the researchers (Karcher et al., 2005; Ezeji, 2006).

4.6. Application of ionic liquids

The butanol extraction process using conventional solvents may be useful, but the solvents used are often volatile, toxic and dangerous. In recent years, a growing interest in ionic liquids(IL) which also can be used in butanol recovery. Ionic liquids are organic salts present in the liquid state at room conditions, have very low vapor pressure and low solubility in water. Hence, Ionic liquids is valuable solvent in the extraction process from aqueous solutions (Fadeev and Meagher, 2001; Garcia-Chavez et al., 2012). Ionic liquids as the non-volatile, environment friendly solvents have been used in various chemical processes. With the development of the technology, ionic liquids extraction would be more promising for butanol recovery.

5. Biobutanol production from renewable resources

Biobutanol is no doubt a superior candidate renewable energy facing the exhausted fossil-energy. The clostridium can incorporate simple and complex soluble sugar, such as corn, molasses, cassava, and sugar beet. The ABE fermentation is also a solution to deal with agriculture residue, spoilage material, and domestic organic waste (Table 3). Additionally, using renewable resources is also ideal for environment problem solving.

Raw materials	Bacterial strain	Fermentation process	ABE concentration (g/l)	ABE Yield (g/g)	ABE productivity (g/lh)	References
Barley straw	*C. beijerinckii*	Batch fermentation	26.64	0.43	0.39 g/lh	Qureshi et al., 2010a
Wheat straw	*C.beijerinckii*	simultaneous saccharification and fermentation combined with gas stripping	21.42	0.41	0.31	Qureshi et al., 2008a
Corn fiber	*C. beijerinckii*	Batch fermentation	9.3	0.39	0.10	Qureshi et al., 2008b
Corn stover	*C. beijerinckii*	Batch fermentation	26.27	0.44	0.31	Qureshi et al., 2010b
Rice straw	*C. saccharoperbutylacetonicum*	Batch fermentation	13	0.28	0.15	Soni et al.(1982)
Bagasse	*C. saccharoperbutylacetonicum*	Batch fermentation	18.1	0.33	0.3	Soni et al.(1982)
Switch grass (*Panicum virgatum*)	*C. beijerinckii*	Batch fermentation	14.61	0.39	0.17	Qureshi et al., 2010a
Domestic organic waste	*C. acetobutylocum*	Batch fermentation	9.3	0.38	0.08	Claassen et al., 2000
Sago	*C. saccharobutylicum*	Batch fermentation Continuous Fermentation ($D=0.11h^{-1}$)	16.38 7.74±0.55	0.33 0.29	0.59 0.85	Liew et al., 2005
Defibrated-sweet potato-slurry (DSPS)	*C. acetobutylocum*	Batch fermentation Continuous Fermentation, immobilized cell ($D=0.129\ h^{-1}$)	5.87 7.73	0.29 0.195	0.12 1	Badr et al., 2001
Cassava	Co-culture of *B. Subtilis* and *C. butylicum*	Batch fermentation	9.71	~0.21	0.135	Tran et al., 2010

Table 3. Butanol production with different raw materials

Food-based substrate arouses many problems. The cost of butanol from glucose was four fold higher than that from sugarcane and cellulose materials (Kumar et al., 2012). For the cellulose-based substrate, the crystal structure of cellulose is hard to use for normal ABE fermentation clostridium. The pretreatment of cellulose is costly, complex, and often leads to new environment problems. For example, using corn as substrate, the cost is 0.44-0.55 US $/kg butanol by the hyper-butanol producing strain *C. beijerinckii* BA101 (Qureshi and Blaschek, 2000) by continuous fermentation combined with butanol separation. The cost

reached 0.73-1.07 US$/kg when grass-rooted plant was used as substrate (Qureshi and Blaschek, 2001a). A promising solution is co-culture of butanol-producing and cellulolytic strains. However, many obstacles must be cleared before the system is constructed. It's difficult for different strains to play a role in turn in the substrate medium. Firstly, strain with high hydrolysis activity must be obtained. Secondly, the procedure must also be optimized.

Some strains can use CO_2, H_2, and CO as substrate (Tracy et al., 2012). The celluloses substrate can be transformed into CO ($_2$) and H_2 firstly. The simple substrates then are used by *C. carboxidivorans* to produce butanol. The more simple and feasible process is still need to be further explored for different substrates.

6. The promising application and prospect of biobutanol

Due to the excessive exploitation, the fossil fuels are facing scarce and they cannot be generated. On the other hand, most of the carbon emissions result from fossil fuel combustion. Reducing the use of fossil fuels will considerably reduce the amount of carbon dioxide and other pollutants produced. Renewable energy has the potential to provide energy services with low emissions of both air pollutants and greenhouse gases. Currently, renewable energy sources supply over 14% of the total world energy demand. Biofuels as the important renewable energy are generally considered as sustainability, reduction of greenhouse gas emissions, regional development, social structure and agriculture, and security of supply (Reijnders, 2006). Biodiesel and bioethanol are presently produced as a fuel on an industrial scale, including ETBE partially made with bioethanol, these fuels make up most of the biofuel market (Antoni et al., 2007).

Biobutanol also has a promising future for the excellent fuel properties. It has been demonstrated that n-butanol can be used either 100% in unmodified 4-cycle ignition engines or blended up with diesel to at least 30% in a diesel compression engine or blended up with kerosene to 20% in a jet turbine engine in 2006 (Schwarz et al., 2006). The production of biobutanol from lignocellulosic biomass is promising and has been paid attention by many companies. Dupont and BP announced a partnership to develop the next generation of biofuels, with biobutanol as first product (Cascone, 2007). In 2011, Cobalt Technologies Company and American Process Inc. (API) have been partnering to build an industrial-scale cellulosic biorefinery to produce biobutanol. Additionally, the companies agreed to jointly market a GreenPower+ biobutanol solution to biomass power facilities and other customers worldwide. The facility is expected to start ethanol production in early 2012 and switch to biobutanol in mid-2012. The annual production of biobutanol is estimated to 470, 000 gallons. (http://www.greencarcongress.com/2011/04/cobalt-20110419.html, http://www.renewableenergyfocususa.com/view/17558/cobalt-and-api-cooperate-on-biobutanol/) Gevo, Inc. signed a Joint Development Agreement with Beta Renewables, a joint venture between Chemtex and TPG, to develop an integrated process for the production of bio-based isobutanol from cellulosic, non-food biomass, such as switch grass, miscanthus, agriculture residues and other biomass will be readily available. (http://www.greencarcongress.com/biobutanol/). Syntec company also is currently developing catalysts to produce bio-butanol

from a range of waste biomass, including Municiple Solid Waste, agricultural and forestry wastes. (http://www.syntecbiofuel.com/butanol.php). Utilization the waste materials improve the economy of butanol production that makes biobutanol great potential to be the next new type of biofuel in spite of the existing drawbacks.

7. Conclusions

Biobutanol production has only recent years booming again after long time of silence. Quite a lot of progress has been made with the technology development of metabolic engineering in enhancing solvent production, increasing the solvent tolerance of bacteria, improving the selectivity for butanol. Fortunately, *Clostridia* have been tested being able to consume lignocellulosic biomass for ABE fermentation. The complex regulation mechanism of butanol synthesis is still need to be further study. For the strain improvement, for example, constructing better butanol tolerance strains, more suitable hosts and genetic methods are required to be set up. Furthermore, more efficient techniques for removing the inhibitors in the lignocellulosic hydrolysate need to be developed. In addition, from the economic point of view, the integrated system of hydrolysis, fermentation, and recovery process also are important to be further developed to reduce the operation cost of butanol synthesis.

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