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Research review paper

Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass

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ABSTRACT

Interest in biobutanol, a sustainable vehicle fuel, is increasing due to rising oil prices and concerns of surrounding climate change and the energy crisis. However, the costs of biobutanol with conventional ABE fermentation by *Clostridium* are higher than the cost of butanol from today's petrochemical processes. Two major problems in the economic production of biobutanol are difficulty controlling the induction of a metabolic shift from acidogenesis to solventogenesis and limitations imposed by severe product inhibition. With developments in biotechnology, and the completion of genome sequencing of *Clostridium*, genetic modification is a viable method to improve the solvent yield and the butanol production ratio. The present article aims to highlight the latest research progress on overexpressing, inserting, knocking out, and knocking down genes of the key enzymes in the ABE fermentation pathway and other relative genes (such as genes coding for heat-shock proteins, operon, transcription, etc). Recombinant manipulations of these genes in *Escherichia coli* and yeast have also been reported recently, although their butanol yields are lower than in *Clostridium*. Butanol production with solventogenic clostridia from various feedstocks is also evaluated in this review.

Contents

1. Introduction

Butanol from biomass is called biobutanol. It is increasingly regarded as a source of clean energy which can be widely applied from mixing with gasoline to being used as a co-solvent in ethanol–diesel blend fuel. The ability to produce butanol from biomass sources via fermentation has existed since the early 1900s. During the two World Wars, biobutanol plants were operated in the United States, UK, China, Russia, South Africa, India, etc. But while the petroleum industry was growing, the lower cost of producing butanol from petroleum products rather than renewable feedstocks made the biobased butanol plants obsolete. Since the end of 20th Century, with

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growing concerns of global greenhouse gasses, the global energy market has been in a continual push for the development of renewable energy sources. The people's point of view on biobutanol was again changed as it is a different fuel from ethanol that can eventually replace gasoline (Durre, 2007). Compared to ethanol, butanol has a low vapor pressure, high energy density, and a gasoline-like octane rating, which allows it to be blended into existing gasoline at much higher proportions than ethanol with less compromise in the performance, mileage and organic pollution standards. In June 2006, DuPont and BP announced a joint venture to deliver advanced biofuels, initially targeting biobutanol and aiming to deliver a biobutanol production process with economics equal to ethanol production by 2010 (Ni and Sun, 2009).

For the production of biobutanol, the acetone–butanol–ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fermentations (Lee et al., 2008). However, butanol toxicity to the fermenting microorganisms limits its concentration in the fermentation broth, resulting in low butanol yields and a high cost for butanol recovery from the dilute solutions. Fortunately with the progress on modern molecular biology, more and more genes involved in the biosynthesis of organic acids and solvents were cloned and characterized to increase knowledge of fermentation metabolic process. Scientists are increasing solventogenic clostridia's tolerance to solvents such as butanol. Furthermore, some other researchers intend to genetically engineer for the ability to restrict continued sporulation. Meanwhile, there is continuing interest in a more suitable industrial organism for butanol production derived from the common view point that Clostridia are not as genetically tractable, well-characterized, and as fast in growth as *E. coli*. Also as the current industrial alcohol producer strain, *Saccharomyces cerevisiae*, is expected to be able to tolerate high concentrations of butanol by the same mechanisms by which it tolerates ethanol. Thus, genetic tools are being used to manipulate their metabolism by introducing the genes responsible for butanol production from *Clostridium acetobutylicum* into *E. coli* (Atsumi et al., 2008) or Yeast (Steen et al., 2008). Any achievement on the genetic engineering of producing strains, reducing butanol toxicity, improving yield, and simplifying downstream processing can contribute toward introducing biobutanol fermentation into industry (Edward, 2008).

2. Butanol biosynthesis pathway

2.1. Extracellular breakdown of polymeric carbohydrates from biomass sources

A major advantage of using a biomass source for fuel production is its renewability. As the fourth largest source of energy in the world after coal, petroleum and natural gas, biomass is also a carbon neutral resource over its life cycle. Biomass is a complex mixture of organic materials, but the main components of plant biomass are polymeric carbohydrates (approximately 75%, dry weight) including celluloses and hemicelluloses with lignins which function in imparting strength to the plant structure and holding the fibers together (Saxena et al., 2009). Of course, some plants store starch in seeds and roots such as corn, soybeans, and potatoes which could be used to make liquid biofuels, but that has been criticized for diverting food away from the human food chain, leading to food shortages and price rises.

The fermentation substrate is an important factor influencing the cost of biobutanol production. Clostridia can secrete numerous enzymes that facilitate the breakdown of polymeric carbohydrates into monomers as substrates for biobutanol production (Ezeji et al., 2007b). Starch hydrolysis involves α-amylase, β-amylase, pullulanase, glucoamylase and α-glucosidase, while cellulose hydrolysis needs cellulases and β-glucosidase. Glucose is recovered from starch and cellulose. Xylose and arabinose converted from hemicellulose are subsequently broken down via the transketolase–transaldolase sequence producing fructose 6-phosphate and glyceraldehyde 3-phosphate with subsequent metabolism by the Embden–Meyerhof–Parnas (EMP) pathway (Ounine et al., 1983). Finally glucose passes through the cell membrane with the aid of glucose phosphotransferase system (PTS) and then executes ABE fermentation.

2.2. Intracellular two-stage ABE fermentation with C. acetobutylicum

The various monosaccharides produced above are transported into the cell by specific membrane-bound transport systems, and the carbohydrates are subsequently metabolized via glycolysis or the pentose phosphate pathway. *C. acetobutylicum* was firstly isolated in 1916 by Chaim Weizmann to discover how it produced acetone, butanol, and ethanol from starch using the so-called ABE process. In a typical ABE fermentation, it can be characterized by two distinct phases (Fig. 1), which are termed acidogenesis and solventogenesis, respectively (Ennis et al., 1986). Bacteria in the first acidogenesis are experiencing an exponential growth phase with active hydrogen evolution and acetic and butyric acid production. The medium pH decreases to as low as around 4.5 because of acid formation. As the accumulation of organic acids increases, the fermentation switches to solventogenesis accompanied by steady cell growth and decreased hydrogen evolution. Acetone, butanol and ethanol are produced with partial uptake of the acids formed during the first growth phase. Then along with *C. acetobutylicum*'s aging, it gradually loses its original bioactivity, and enters the cell autolysis phase forming spores until an end point is reached.

3. Enzymes involved in biobutanol synthesis of *Clostridium*

In a natural state, bacterial butanol production is carried out exclusively by members of the genus Clostridia including *C. acetobutylicum, Clostridium beijerinckii, Clostridium saccharoperbutylacetonicum, Clostridium saccharoacetobutylicum, Clostridium aurantibutyricum, Clostridium pasteurianum, Clostridium sporogenes, Clostridium. cadaveris, Clostridium tetanomorphum* and so on (Keis et al., 1995). Along with the deep research of biobutanol synthesis, acetone–butanol–ethanol fermentation has been widely studied and microorganisms capable of ABE fermentation can be viewed as a model system for understanding the regulation and genetics of complex primary metabolism. Although a number of different clostridia are continually being isolated and their abilities in butanol production are verified, *C. acetobutylicum* is still the species which is most often used for industrial production of butanol. In the following text, *C. acetobutylicum* will be taken as an example to introduce the key enzymes in the ABE fermentation system.

As seen in Table 1, there are two enzymes responsible for conversion of acetyl-CoA during the synthesis of lactate, phosphotransacetylase (PTA) and acetate kinase (AK). The Acetyl-CoA (A-CoA) node which serves as a connecting point at which several metabolic pathways intersect is also the switch point in the acid- and solvent-producing pathways. During acidogenesis, with the effect of increasing AK and PTA, ATP is produced as an important energy source for cell growth. During solventogenesis, the activity levels of AK and PTA decrease, which have unclear roles in this stage (Hartmanis et al., 1986). The activities of PTA and AK in batch fermentation of *C. acetobutylicum* were observed maximal at the early stage of the exponential phase and dropped significantly with the increase of acetate concentration in the medium (Ballongue et al., 1986). It was shown that AK was synthesized inside of this organism until acetate had accumulated to 3 g/L in the fermentation broth. Then the higher concentration of acetate caused a rapid drop in acetate kinase activity.

For butyrate formation, phosphate butyryltransferase (PTB) and butyrate kinase (BK) are essential. PTB catalyzes the interconversion of butyryl-CoA and butyryl-P and enables the conversion of butyryl-CoA to butyrate together with butyrate kinase. The enzyme has eight subunits of equal molecular weight (Wiesenborn et al., 1989b).

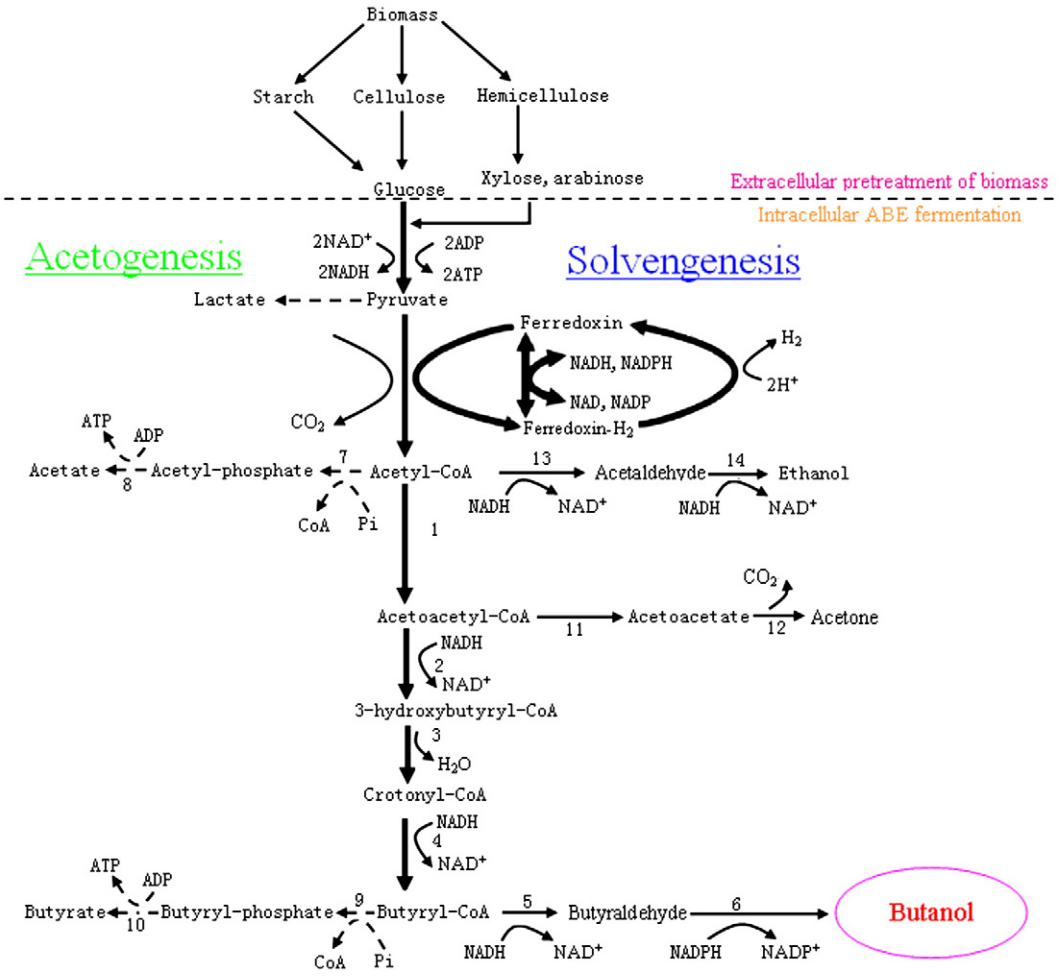

Fig. 1. Butanol biosynthesis pathway in *C. acetobutylicum*. Circled numbers refer to the enzymes employed: 1. acetyl-CoA acetyltransferase (thiolase); 2. β-hydroxybutyryl-CoA dehydrogenase; 3. 3-hydroxybutyryl-CoA dehydratase (crotonase); 4. butyryl-CoA dehydrogenase; 5. butyraldehyde dehydrogenase; 6. butanol dehydrogenase; 7. phosphate acetyltransferase; 8. acetate kinase; 9. phosphate butyryltransferase; 10. butyrate kinase; 11. acetoacetate decarboxylase; 12. CoA-transferase; 13. coenzyme A (CoA)-acylating; and 14. NAD(P)H alcohol dehydrogenase.

Butyryl-P is formed with an optimized pH value of 8.1 which is also optimal for PTB in the physiological direction, while in the reverse direction (butyryl-CoA forming), the enzyme is less sensitive and shows a broad pH optimum ranging from 7.5 to 8.7. BK catalyzes the reaction by which butyryl-P is converted to butyrate with concomitant ADP phosphorylation and the reverse reaction by which butyrate is converted to butyryl-P uses ATP as the phosphate donor (Sullivan et al., 2010).

In the butanol synthetic pathway, six enzymes are needed to complete the conversion of acetyl-CoA to butanol including thiolase, also called acetyl-CoA acetyltransferase (THL), β-hydroxybutyryl-CoA dehydrogenase (BHBD), 3-hydroxybutyryl-CoA dehydratase (CRT, also termed crotonase), butyryl-CoA dehydrogenase (BCD), Butyraldehyde dehydrogenase (BAD) and Butanol dehydrogenase (BDH). Thiolase plays a key role in the production of both acids and solvents, as it effectively controls the flow of carbon into these pathways by catalyzing the condensation of two molecules of acetyl coenzyme A into one acetoacetyl-CoA, which will later on be converted to acetone, buayrate or butanol. Regulation of thiolase activity is important for maintaining the C2:C4 acid ratio, which has a profound effect on the energy management of the cell. Meanwhile, THL may play an indirect role in acid uptake (Papoutsakis and Bennett, 1993). Butyraldehyde dehydrogenase, which is NADH-dependent, catalyzes the conversion of butyryl-CoA to butyraldehyde, a key intermediate in the formation of butanol, accompanied by the oxidation of NAD(P)H and the release of CoA. Butanol dehydrogenase catalyzes the final step in butanol synthesis with the reduction of butyraldehyde to butanol at the expense of a reduced NAD(P). The assay performed with butanol dehydrogenase from *C. acetobutylicum* ATCC 824 (Welch et al., 1989) clearly revealed that the enzyme has two different coenzyme requirements: the NADH-dependent butanol dehydrogenase (NADH–BDH) with higher activity at lower pHs and the NADPH-dependence with higher activity at basic pHs. BCD catalyzes the α, β desaturation of acyl-CoA substrates.

CoA-transferase and acetoacetate decarboxylase are two important enzymes involved in the acetone synthetic pathway. The former enzyme seems to be a heterotetramer with two α- and two β-subunits, coding for the genes of *ctfA* and *ctfB* respectively (Wiesenborn et al., 1989a). The formation of acetoacetate is catalyzed by a CoA-transferase which transfers CoA from aceacetyl-coA to either acetate or butyrate. Acetate and butyrate harvested during the growth phase reduce the pH value of the cell culture and the acidic condition at the stationary phase is widely regarded as a trigger of a shift in metabolism which consequently results in solvent production (Jones and Woods, 1986). The uptake of acetate and butyrate by CoA-transferase from *C. acetobutylicum* serves is a detoxification mechanism by reducing the inhibitory effects of these acids on cell growth. Acetoacetate decarboxylase (AADC) is another critical enzyme in this pathway which catalyzes the conversion from acetone to acetoacetyl-CoA. The acetoacetate formed via CoA-transferase is subsequently decarboxylated by AADC to yield acetone and carbon dioxide. Thus the formation of acetone was coupled with the uptake of acetate and

Table 1
The list of key enzymes of the butanol synthetic pathway in *C. acetobutylicum*.

Pathway	Key enzyme	ab.	Gene	EC no.	Mr (kDa)	Reference
Lactate synthetic pathway	Phosphate acetyltransferase	PTA	*pta*	2.3.1.8	36.2	Boynton et al. (1996b)
	Acetate kinase	AK	*ack*	2.7.2.1	44.3	Boynton et al. (1996b)
Butyrate synthetic pathway	Phosphate butyryltransferase	PTB	*ptb*	2.3.1.19	264	Hartmanis (1987)
	Butyrate kinase	BK	*buk*	2.7.2.7	85	Hartmanis (1987)
Butanol synthetic pathway	Acetyl-CoA acetyltransferase	THL	*thiL*	2.3.1.9	41	Stim-Herndon et al. (1995)
	β-hydroxybutyryl-CoA dehydrogenase	BHBD	*hbd*	1.1.1.35 (157)	30.5	Boynton et al. (1996a)
	Enoyl-CoA hydratase (crotonase)	CRT	*crt*	4.2.1.17	158	Waterson et al. (1972)
	Butyryl-CoA dehydrogenase	BCD	*bcd-etfB-etfA*	1.3.99.2	33	Boynton et al. (1996a)
	Butyraldehyde dehydrogenase	BAD	*aad*	1.2.1.57	56	Palosaari and Rogers (1988)
	Butanol dehydrogenase	BDH	*bdhAB*	1.1.1	42	Petersen et al. (1991)
Acetone synthetic pathway	Acetoacetate decarboxylase	AADC	*adc*	4.1.1.4	28	Peterden and Bennett (1990)
	CoA-transferase	CoAT	*CtfA–B*	2.8.3.9	93	Palosaari and Rogers (1988)
Ethanol synthetic pathway	Acetaldehyde dehydrogenase	ALDH	*aad*	1.2.1.10	96	Palosaari and Rogers (1988)
	NAD(P)H alcohol dehydrogenase	ADH	*adh*	1.1.1.2	44	Youngleson et al. (1988)

butyrate. The activity of this decarboxylase was almost 40-fold higher in solvent-producing cells (4.93 U/mg) as compared to acid-producing cells (0.13 U/mg) (Andersch et al., 1983).

There are two enzymes in the ethanol synthetic pathway called coenzyme A-acylating aldehyde dehydrogenase and NAD(P)H alcohol dehydrogenase. The former enzyme is also known as an important enzyme at the junction in the pathway leading to the production of either acids (acetate or butyrate) or solvents (acetone, butanol or ethanol) during the growth of *C. acetobutylicum* ATCC 824. The latter enzyme catalyzes the reduction of acetaldehyde to ethanol. The EDH activities are 5 to 8 fold higher in the solvent-producing phase than the ones in the acid-producing phase (Vasconcelos et al., 1994).

4. Construction of *Clostridium* strains by genetic engineering

The completion of the genome sequences of Clostridia allows us to have a substantial understanding of cellular regulation and genetics (Nolling et al., 2001). We could now get vast amounts of available information and physiological characteristics which can contribute to potential genetic tools. Genetic modification is a viable method to improve solvent production, especially the butanol production ratio. Because of the key problems in *clostridium* fermentation, genetic modification of *Clostridium* is widely used by inserting some heterogenetic genes or overexpressing or knocking out/down some relative endogenous genes to improve butanol production.

4.1. Genetic modification of crucial enzymes involved in biobutanol synthesis

A genome-scale metabolic network consists of 422 intracellular metabolites involved in 552 reactions and includes 80 membrane transport reactions (Senger and Papoutsakis, 2008). Most of the metabolic reactions are catalyzed by enzymes. As we know, the enzymes (such as PTB, BK, PTA, AK, etc.) are more active in acidogenesis and only acids (butyric and acetic) are produced at that period. With the accumulation of acids, the activities of these enzymes decrease. Meanwhile, the enzymes (such as CoA, THL, BDH, etc.) in the solvent synthetic pathway begin to be active and the pH value stabilizes or increases slightly because most acids are taken up and converted to solvents (butanol, acetone and ethanol).

Because of the essential functions those enzymes have, over the past decades many scientists have made many efforts to improve the biobutanol production by inserting, knocking out or eliminating the key genes coding for the corresponding enzymes. Mermelstein et al. (1993) constructed a recombinant *C. acetobutylicum* strain with a pFNK6 plasmid, in which it contains an ace operon including *adc* and *ctfA/B* genes. Compared to its control strain, the final concentrations of acetone, butanol and ethanol were increased by 95%, 37% and 90%, respectively (Mermelstein et al., 1993). Harris et al. (2000) constructed a stain, PJC4BK, in which *buk* was inactivated. When the pH was greater than or equal to 5, the stain appeared to be a super solvent producer yielding 16.7 g/L of butanol, 4.4 g/L of acetone and 2.6 g/L of ethanol. Butyrate levels were low throughout all fermentations, never exceeding 20 mM (Harris et al., 2000).

Acetone, ethanol, and butanol are the dominant products of the ABE process. The ratio of butanol, acetone and ethanol is approximately 3:1:6. As a by-product, acetone accounted for about 30%. Improving the ratio of butanol and the ratio of substrate utilization were studied. Tummala et al. (2003) downregulated genes coding acetoacetate decarboxylase and coenzyme A transferase by antisense RNA. Despite effective downregulation, there was no redirection of the carbon flux towards butanol synthesis. On the contrary, the butanol level was drastically decreased. Another attempt was made to increase the butanol/acetone ratio through overexpression of the alcohol–aldehyde dehydrogenase gene (*aad*) and downregulation of CoA by antisense RNA against *ctfB* (the second CoA gene on the polycistronic *aad-ctfA-ctfB* message). Again, the butanol level was drastically reduced in the resultant mutant, while the ethanol concentration was 23-fold higher than the control, which demonstrated that asRNA against *ctfB* degraded the entire sol operon (*aad–ctfA–ctfB*) transcript. However, the butanol/acetone ratio of this engineered strain (4.89) was more than two fold greater than that of the control strain (1.83) (Tummala et al., 2003). Jiang et al. (2009). disrupted the acetoacetate decarboxylase gene (*adc*) in *C. acetobutylicum* EA 2018 to increase the butanol ratio by eliminating the production of other by-products, such as acetone. The butanol ratio increased from 70% to 80.05%, while acetone production reduced to approximately 0.21 g/L in the adc-disrupted mutant (Jiang et al., 2009).

4.2. Genetic modification to improve the stain's solvent tolerance

One of the most critical problems in ABE fermentation is solvent toxicity. Clostridial cellular metabolism will cease after the solvent concentration reaches 20 g/L. The production of butanol also restrained the microorganism growth. It has been known that a 50% inhibition of growth could happen when the concentration of butanol is as high as 7–13 g/L in the culture medium (Moreira et al., 1981). This indicates that a concentrated product cannot be obtained with the wild strain. Therefore, how to conquer this problem is one of existing areas of study. Although during the last few years, the final total butanol production has been greatly increased by physical methods such as separating the noxious solvent from the fermentation broth and biological methods may provide more opportunity for production improvement.

Previously, scientists screened out stains with high tolerance to the solvent by random mutagenesis such as *C. beijerinckii* BA101 derived from *C. beijerinckii* NCIMB 8052 (Lyanage et al., 2000). Gaining a tolerant strain is more feasible by genetic modification. Spo0A is a

multifunctional regulator. Alsaker et al., (2004) revealed that overexpression of *spo0A* by DNA microarray technology increased the tolerance and prolonged metabolism in response to butanol stress. Tomas et al. (2004) also constructed an engineered stain with overexpressed *spo0A*. The results show that the butanol production is higher than the *spo0A* inactivated strain (Tomas et al., 2004). But strains with *spo0A* overexpression failed to produce more solvent due to an accelerated sporulation process (Harris et al., 2002). Nowadays, more and more genes correlating to solvent tolerance are found, although the action mechanism is still not clear. Borden and Papoutsakis (2007) inserted the CAC0003 and CAC1869 genes to build strain 824(pCAC0003) and strain 824(pCAC1869), respectively. Comparing with the plasmid control strain, 824(pCAC0003) and 824 (pCAC1869) exhibited 13% and 81% increases in butanol tolerance (Borden and Papoutsakis, 2007). Meanwhile, antisense RNA has also been used to improve butanol tolerance. A butanol-tolerant mutant strain of *C. beijerinckii* NCIMB 8052 was constructed by using antisense RNA targeting the *gldA* gene which encodes glycerol dehydrogenase (Lyanage et al., 2000).

The overexpression of some non-enzyme proteins such as GroEL, a class I heat-shock protein, also could increase the solvent tolerance (Tomas et al., 2003). Narberhaus et al. (1992) identified a cluster of heat-shock genes in the *dnaK* gene region of *C. acetobutylicum*, including *grpE, dnaK, dnaJ* and a new heat-shock gene coding an unknown heat-shock protein. DnaK, the protein product of the *dnaK* gene, may aid the tolerance of *C. acetobutylicum* to solvent, given that it is induced during the onset of solventogenesis (Narberhaus et al., 1992). The improvement of solvent tolerance allowed for an increase of the final solvent concentration, presumably by stabilizing solventogenic enzymes.

4.3. Genetic modification to restrict the sporulation

The accumulation of solvent, which will create adverse circumstances for cell growth, may stimulate the bacteria to form spores. Sporulation makes the active cells enter a dormant state where they lose the ability to produce solvents. So, additional effort on non-sporulation or the delay of the sporulation is carried out by many researchers. *spo0A* also has been shown to affect the expression of genes at the transition from the acidogenic phase to the solventogenic phase in solventogenic Clostridia (Harris et al., 2002). It controls the initiation of sporulation, the development of competence for DNA uptake and many other stationary phase-associated processes (Harris et al., 2002). *abrB*, which was described as an inhibitor of sporulation that acts in opposition to *spo0A*, regulates catabolite repression in carbon-limited media and acts as both a positive and negative regulator of cell competence at various stages in the life cycle. Scotcher et al. (2005) constructed 824 (pMSpo) to overexpress *spoIIE* and 824(pASspo) to decrease *spoIIE* expression via antisense RNA targeted against *spoIIE* respectively. Strain 824 (pASspo) prolonged solventogenesis characterized by increased production of ethanol (225%), acetone (43%) and butanol (110%) (Scotcher et al., 2005).

5. Attempts on making use of non-food biomass to improve the butanol production

In the past few years, several carbon sources, such as glucose and different kinds of starches including corn, sago, wheat, tapioca and potato starch, have been utilized for laboratory biobutanol production, which make biobutanol's price prohibitive and also competes for food uses. Compared to the chemical synthesis method, biobutanol production must be executed on the premise of cheaper and renewable substrates. Among the total polysaccharides, researchers focus on celluloses and hemicelluloses which are the most abundantly renewable and available resources on the planet (Koukiekolo et al., 2005).

Raw material can only be used by the microorganism for ABE fermentation after being degraded to monosaccharides or other small molecules such as glucose, xylose, arabinose, galactose, glucuronic acid, acetic acid, ferulic acid, and so on (Koukiekolo et al., 2005; Ezeji et al., 2007b). Though *C. acetobutylicum* contains an apparently complete cellulosome, it is still not able to uptake cellulose and hemicelluloses effectively (Desvaux et al., 2005). One possible reason is that the genes encoding related enzymes or function-limiting proteins are silenced or have an essential mutation. Another possible reason is that there may not be a way to transport the enzymes and sugar. Because the whole process is not yet clear, it is more interesting to search for a genetic mechanism for making use of non-food biomass to improve the butanol production.

Sabathe and Soucaille made the A mini-CipA (the *cipA* gene encodes the *C. acetobutylicum* scaffolding protein CipA) polypeptide consisting of a family 3a-cellulose-binding domain and two cohesin domains overexpressed in *C. acetobutylicum*, yielding the in vivo formation of a minicellulosome (Sabathe and Soucaille, 2003). This effort suggests that the *Clostridium* can acquire a complete, functional, and efficient cellulosome that can be in principle associated with cellulose degradation. Though in fact no evidence shows that cellulose could be degraded, this study demonstrated the feasibility. Another effort sought to insert the heterologous genes coding mini-cellulosomes from *Clostridium cellulolyticum* and *Clostridium thermocellum* in *C. acetobutylicum* (Perret et al., 2004; Mingardon et al., 2005). First, they respectively produced two functional scaffoldins (miniCipC1, a truncated form of CipC from *C. cellulolyticum* and the hybrid scaffoldin Scaf 3 from *C. thermocellum*). And then it was made sure that the functional protein correctly express, fold and bind to the scaffold. Both proteins were correctly matured and secreted in the medium, and their various domains were found to be functional. These studies suggest that *C. acetobutylicum* is a suitable platform host for the expression, assembly and secretion of heterologous (mini) cellulosomes.

Recently, researchers have focused on the use of agricultural residues (such as corn stover, corn fiber and fiber-rich distillers dried grains and solubles) and other low cost biomass for biobutanol production with *C. beijerinckii* BA101. Up to now, the Clostridia could utilize glycerol and a number of other hexoses, pentoses and oligosaccharides like cellobiose, lactose, raffinose, mannose, xylose and arabinose (Keis et al., 2001; Andrade and Vasconcelos, 2003). Though sometimes glucose should be added, raw materials could be used in the industrial production such as excess sludge (Kobayashi et al., 2005), agricultural residues (Ezeji et al., 2007a), wheat straw hydrolysate (Qureshi et al., 2007; Qureshi et al., 2008), hydrolyzed agricultural waste, corn fiber (Qureshi et al., 2006), domestic organic waste and so on. In the past, the ABE plants in Russia were the only industrial plants which used hydrolyzates of lignocellosic waste for butanol fermentation (Zverlov et al., 2006).

6. Efforts on *E. coli* and *S. cerevisiae* as alternative hosts for biobutanol synthesis

Though the biobutanol production by clostridia fermentation is up to 20.9 g/l in batch fermentation (Chen and Blaschek, 1999), clostridia are not ideal because of the relative lack of genetic tools to manipulate their metabolism, their slow growth, their intolerance to butanol above 1–2% and oxygen, and their production of butyrate, acetone and ethanol as by-products. Considering the rapidly expanding genomic information, molecular biology techniques, and high-throughput tools, metabolic engineers have made significant progress in constructing non-native organisms for the production of fuel-grade compounds beyond the scope of what native organisms can produce. A number of different organisms such as *S. cerevisiae*, *E. coli* and *Ralstonia eutropha* are being studied for biobutanol production.

Among these organisms, *E. coli* is now the most mature genetic tool as it grows quickly and genetic tools for its modification are well developed (Atsumi et al., 2008; Zheng et al., 2009). Over the last few years, many scientists have gotten into this work. Atsumi and Liao inserted the genes (*thl, hbd, crt, bcd, etf*AB and *adh*E2) coding the six enzymes of the butanol synthetic pathway of clostridia into *E. coli*. Under anaerobic conditions, 139 mg/L of butanol was finally produced (Atsumi and Liao, 2008). In the same way, Inui et al. inserted different combinations of the genes (*thiL, hbd, crt, bcd–etfB–etfA*, and *adhel* or *adhe*) into *E. coli*. Under anaerobic condition, The *E. coli* stains BUT1 with adhe1 and BUT2 with adhe were shown to produce 296 mg/L and 1184 mg/L butanol, respectively (Inui et al., 2008). Though the titer is lower than clostridia, it still showed the feasibility to get a high butanol production using *E. coli* as a host.

As another non-native butanol producer, *S. cerevisiae* has inherent tolerance to solvents with widespread use for industrial production of ethanol, and the ability to withstand oxygen, unlike clostridia, and might become an ideal host for industrial butanol production. In Steen's research group at the University of California, Berkeley, after related enzymes were genetic modified, the best combination achieved was a butanol titer is around 2.5 mg/L (Steen et al., 2008).

7. Conclusions

Butanol from biomass is a better renewable fuel for replacing gasoline than ethanol. Clostridia can secrete numerous enzymes that facilitate the breakdown of polymeric carbohydrates into monomers for biobutanol production. In a typical ABE fermentation with *C. acetobutylicum*, it can be characterized by two distinct phases: acidogenesis and solventogenesis, (Fig. 1). Table 1 lists the key enzymes and their genes acting on the butanol synthetic pathway in *C. acetobutylicum*. Genetic modification is a viable method to improve the solvent production and butanol production ratio. In order to construct an engineered bacteria with a high butanol production, we may choose the following 4 methods: inserting or knocking out/down the genes coding critical enzymes, regulating some genes to enhance the strain's solvent tolerance, inserting some enzyme genes from other bacteria to improve the ability to break down cheaper substrate and performing genetic modification to restrict or delay the sporulation. What should be noted is that *C. acetobutylicum* cannot uptake non-food biomass like cellulose and hemicelluloses effectively, although it contains an apparently complete cellulosome. Scientists' efforts are searching for the genetic mechanism involved in controlling related enzymes in charge of degrading polysaccharides. Some researchers instead of studying *C. acetobutylicum*, are working with *S. cerevisiae* and *E. coli* to make an effective biobutanol production system. A new biobutanol industry has begun to emerge with all above research progress.

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