Fermentative Butanol Production by Clostridia

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ABSTRACT: Butanol is an aliphatic saturated alcohol having the molecular formula of C₄H₉OH. Butanol can be used as an intermediate in chemical synthesis and as a solvent for a wide variety of chemical and textile industry applications. Moreover, butanol has been considered as a potential fuel or fuel additive. Biological production of butanol (with acetone and ethanol) was one of the largest industrial fermentation processes early in the 20th century. However, fermentative production of butanol had lost its competitiveness by 1960s due to increasing substrate costs and the advent of more efficient petrochemical processes. Recently, increasing demand for the use of renewable resources as feedstock for the production of chemicals combined with advances in biotechnology through omics, systems biology, metabolic engineering and innovative process developments is generating a renewed interest in fermentative butanol production. This article reviews biotechnological production of butanol by clostridia and some relevant fermentation and downstream processes. The strategies for strain improvement by metabolic engineering and further requirements to make fermentative butanol production a successful industrial process are also discussed.

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KEYWORDS: butanol; metabolic engineering; Clostridium; systems biotechnology

Introduction

Butanol (butyl alcohol and 1-butanol) is a four carbon primary alcohol having the molecular formula of C₄H₉OH (MW 74.12). Butanol is a colorless liquid with a distinct odor and its vapor has an irritant effect on mucous membranes and a narcotic effect in higher concentrations. Butanol is completely miscible with organic solvents and partly miscible with water. Important characteristics of butanol are summarized in Table I.

It has been estimated that 10–12 billion pounds of butanol is produced annually (Donaldson et al., 2007), which accounts for 7–8.4 billion dollar market at current price. Butanol has a projected market expansion of 3% per year (Kirschner, 2006). Half of the butanol production is used in the form of butyl acrylate and methacrylate esters used in latex surface coating, enamels and lacquers (Kirschner, 2006). Other important derivatives of butanol are butyl glycol ether, butyl acetate and plasticizers. Butanol is also an excellent diluent for brake fluid formulations and solvent for the manufacturing of antibiotics, vitamins and hormones. An important application of butanol receiving renewed
interest is its use as a direct replacement of gasoline or as a fuel additive. Butanol has sufficiently similar characteristics to gasoline to be used directly in any gasoline engine without modification and/or substitution (Table I). Butanol is superior to ethanol as a fuel additive in many regards: higher energy content, lower volatility, less hydroscopic (thus does not pick up water), and less corrosive (Dürre, 2007). Also, branched chain 4-carbon alcohols including isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol have higher octane numbers compared with \( n \)-butanol (Atsumi et al., 2008), and thus are good candidates as fuel additives.

Early industrial production of butanol was based on fermentation of the bacterium \textit{Clostridium acetobutylicum} which ferments carbohydrates and produces mainly butanol and acetone (Jones and Woods, 1986). However, increasing demand for butanol and the dramatic growth of the petrochemical industry saw biological processes replaced by more efficient chemical processes.

In recent years, high crude oil price and increasing concerns over global warming have renewed interest in biotechnological production of butanol, not only as a chemical but also as an alternative fuel. Reflecting this, a number of companies are developing bio-butanol processes. BP and DuPont, for example, recently announced a joint effort to develop a fermentative butanol process. BP has also created a subsidiary, BP Biofuels, to commercialize butanol by fermentation. Many venture companies have also been established aiming to commercialize of bio-butanol.

**Chemical Synthesis of Butanol**

Oxo synthesis, Reppe synthesis, and crotonaldehyde hydrogenation are the three most important processes for the chemical butanol industry (Fig. 1). In oxo synthesis (hydroformylation), carbon monoxide and hydrogen are added to a carbon–carbon double bond using catalysts such as Co, Rh, or Ru substituted hydrocarbonyls (Falbe, 1970) (Fig. 1a). Aldehyde mixtures are obtained in the first reaction step, which is followed by hydrogenation for the production of butanol. Depending on reaction conditions (pressure, temperature) as well as the catalyst, different isomeric ratios of butanol are obtained. In the Reppe process, propylene, carbon monoxide and water are reacted in the presence of a catalyst (Bochman et al., 1999) (Fig. 1b). The Reppe process directly produces butanol at low

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**Table I. Properties of butanol.**

<table>
<thead>
<tr>
<th>Properties</th>
<th>Butanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting point (°C)</td>
<td>−89.3</td>
</tr>
<tr>
<td>Boiling point (°C)</td>
<td>117.7</td>
</tr>
<tr>
<td>Ignition temperature (°C)</td>
<td>35</td>
</tr>
<tr>
<td>Flash point (°C)</td>
<td>365</td>
</tr>
<tr>
<td>Density at 20°C (g/mL)</td>
<td>0.8098</td>
</tr>
<tr>
<td>Critical pressure (hPa)</td>
<td>48.4</td>
</tr>
<tr>
<td>Critical temperature (°C)</td>
<td>287</td>
</tr>
</tbody>
</table>

---

**Figure 1.** Chemical synthesis of butanol. (a) Oxo synthesis, (b) Reppe process, (c) crotonaldehyde hydrogenation.
temperature and pressure. This process has not been commercially successful, however, because of costly process technology (Bochman et al., 1999). Until a few decades ago, the common route for butanol synthesis was from acetaldehyde using crotonaldehyde hydrogenation (Fig. 1c). The process consists of aldol condensation, dehydration, and hydrogenation (Bochman et al., 1999). Although rarely used today, it may again become important in the future. While other processes rely completely on petroleum, the crotonaldehyde hydrogenation process provides an alternative route from ethanol which can be produced from biomass. In this case, ethanol is dehydrogenated to form acetaldehyde from which the synthesis can proceed (Swodenk, 1983).

**General Aspects of Biological Butanol Production**

Biological production of butanol has a long history as an industrially significant fermentation process. An excellent review article by Jones and Woods (1986) on the history of acetone–butanol–ethanol (ABE) fermentation processes is available. After Pasteur discovered bacterial butanol production from his landmark anaerobic cultivation in 1861, fermentative ABE production prospered during the early 20th century and became after ethanol the second largest industrial fermentation process in the world. In 1945, two thirds of industrially used butanol was produced by fermentation in U.S. However, the ABE fermentation process had lost competitiveness by 1960s due to the increase of feedstock costs and advancement of the petrochemical industry except in Russia and in South Africa, where the substrate and labor costs were low. The ABE fermentation processes in South Africa and Russia continued to operate until the late 1980s to early 1990s (Zverlov et al., 2006). It has recently been reported that the Russian fermentation industry is concentrating on the conversion of agricultural biomass into butanol (Zverlov et al., 2006). The successful industrial-level butanol fermentation in these countries can provide guidelines to our current effort to produce butanol in large-scale.

**Microorganisms**

Butanol (and acetone, ethanol, and isopropanol) are naturally formed by a number of clostridia. In addition, clostridia can produce chiral products which are difficult to make by chemical synthesis (Rogers, 1986) and degrade a number of toxic chemicals (Francis et al., 1994; Spain, 1995). Clostridia are rod-shaped, spore-forming Gram positive bacteria and typically strict anaerobes. Solventogenic clostridia can utilize a large variety of substrates from monosaccharides including many pentoses and hexoses to polysaccharides (Jones and Woods, 1986). Complex nitrogen sources such as yeast extract are generally required for good growth and solvent production, but otherwise the nutrient requirements for the growth of clostridia are rather simple (Monot et al., 1982). As shown in Figure 2, clostridia require high redox potential to produce butanol (and ethanol) and the supply of additional reducing power results in increased butanol and ethanol formation with reduced acetone formation (Mitchell, 1998). Among many solventogenic clostridia, *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylicetonum* are primary solvent producers (Dürrre, 2005). It should be mentioned that *C. beijerinckii* NCIMB 8052, *C. saccharobutylicum*, and *C. saccharoperbutylicetonum* were originally designated as *C. acetobutylicum* (Dürrre, 2005).

A typical feature of the clostridial solvent production is biphasic fermentation, as shown in Figure 2. The first phase is the acidogenic phase, during which the acids forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products. This acidogenic phase usually occurs during the exponential growth phase (Andersch et al., 1983; Hartmanis and Gatenbeck, 1984). The second phase is the solventogenic phase during which acids are reassimilated and used in the production of acetone, butanol and ethanol (or isopropanol instead of acetone in some *C. beijerinckii* strains). The transition from acidogenic to solventogenic phase is the result of a dramatic change in gene expression pattern (Dürrre et al., 1987).

Solventogenesis is closely coupled to sporulation (see Paredes et al., 2005 for a detailed review on clostridial sporulation). The transcription factor responsible for initiation of sporulation (Spo0A) also initiates solvent production in *C. acetobutylicum* (Bahl et al., 1995) by activating transcription of acetooacetate decarboxylase (*adc*), alcohol dehydrogenase (*adhE*), and CoA transferase (*ctfAB*) genes (Sullivan and Bennett 2006). Spo0A deletion mutants are severely deficient in solvent production and fails to septate, while strains with amplified Spo0A overexpress solventogenic genes but fail to produce more solvent due to an accelerated sporulation process (Harris et al., 2002).

Spo0A is a multifunctional regulator which is also involved in solvent formation and sporulation in *C. beijerinckii*, though control of solvent formation involves different mechanisms than in *C. acetobutylicum* (Harris et al., 2002; Thormann et al., 2002). Spo0A regulates the sporulation by binding the genes involved in the sporulation process (Harris et al., 2002). It is activated when it is phosphorylated by the phosphorelay two-component signal transduction system (Scotcher et al., 2005). The sigma factors affecting the transcription of the sporulation-related genes are encoded by the *spoIIA*, *sigE*, and *sigG* genes in *C. acetobutylicum* (Harris et al., 2002). The transcription factors encoded by *sinR* and *abrB* are also regulating the sporulation initiation (Scotcher et al., 2005). Scotcher et al. (2005) proposed that *abrB* might act as a regulator at the
transition between the acidogenic and solventogenic phases. The effect of Spo0A on solvent formation is a balancing act in regulating sporulation versus solvent gene expression (Harris et al., 2002). Thus selective utilization of positive effect of Spo0A on solvent formation is required for enhanced solvent production. For this purpose, it is recommended that Spo0A should be evolved to a solventogenic gene-activating regulator without the sporulation activating function.

Fermentation

Recent advances in butanol fermentation including upstream and downstream processing were evaluated by Qureshi and Blaschek (2001b). The use of excess carbon under nitrogen limitation is required to achieve high levels of solvent production (Madihah et al., 2001). Iron is an important mineral supplement since the conversion of pyruvate to acetyl-CoA involves a ferredoxin oxidoreductase iron-sulfur protein (Kim et al., 1988).

Clostridia can lose the ability to produce solvents during the cultivation. This degeneration phenomenon was found to be due to the loss of the megaplasmid pSOL1 in *C. acetobutylicum*, which could be prevented under phosphate-limited condition (Ezeji et al., 2005a). *C. beijerinckii* NCIMB 8052 does not have indigenous plasmids but degeneration still occurs (Kashket and Cao, 1993) and can be prevented by the addition of sodium acetate (Chen and Blaschek, 1999).

The pH of the medium is very important to the biphasic acetone–butanol fermentation. In acidogenesis, rapid formation of acetic and butyric acids causes a decrease in pH. Solventogenesis starts when pH reaches a critical point, beyond which acids are reassimilated and butanol and acetone are produced. Therefore, low pH is a prerequisite for
solvent production (Kim et al., 1984). However, if the pH decreases below 4.5 before enough acids are formed, solventogenesis will be brief and unproductive. Increasing the buffering capacity of the medium is a simple way to increase growth and carbohydrate utilization as well as the buffering capacity of the medium is a simple way to increase growth and carbohydrate utilization as well as butanol production (Bryant and Blaschek, 1988).

It takes 2–6 days to complete a batch fermentation depending on the condition and the type of substrate employed. The final total concentration of solvents produced ranges from 12 to 20 g L⁻¹ in batch fermentation, which can be separated from the fermentation broth by distillation. Classical fed-batch and continuous cultivation do not seem to be economically feasible, because of solvent toxicity and the biphasic nature of acetone–butanol fermentation, respectively. To overcome this problem, fed-batch culture has been coupled with an in situ recovery process (Ezeji et al., 2004a,b), and multistage continuous fermentation has been conducted (Godin and Engasser, 1990).

Fermentation Substrates

Butanol has been produced from various raw materials including molasses, whey permeate, and corn (Ezeji and Blaschek, 2007; Ezeji et al., 2007c; Jones and Woods, 1986; Qureshi et al., 2008). Substrate cost is a major factor impacting economics of butanol production. Early studies on the various alternative fermentation substrates for the ABE fermentation have been reviewed (Jones and Woods, 1986). Here we focus on recent reports on the utilization of various substrates in solvent production.

An economic assessment of butanol production from corn using the hyper-butanol-producing strain of C. beijerinckii BA101 has been conducted (Qureshi and Blaschek, 2001a). It showed that an improvement of butanol yield by 19.0% (0.42–0.50 g butanol per g glucose) would reduce the butanol price by 14.7% (US$ 0.34/kg to 0.29/kg). Spray-dried soy molasses (SDSM) was used to produce ABE by C. beijerinckii BA101 in batch cultures (Qureshi et al., 2001). Higher concentrations of SDSM resulted in poor solvent production due to the presence of excessive salt and inhibitory components. However, when supplemented with 25.3 g/L glucose, a 113% (10.7–22.8 g/L) increase in ABE production was achieved with 80 g/L SDSM. These studies suggest that SDSM can be a useful substrate for producing solvents if it is used with additional sugar, such as glucose or sucrose. Butanol has also been produced from low cost waste products such as starch-based waste packing peanuts and agricultural waste, highlighting the versatility of C. beijerinckii BA101 (Jesse et al., 2002). Low-grade glycerol has also been used to produce butanol in a chemostat culture of C. acetobutylicum, resulting in the high yield and productivity of 0.34 mol/mol and 0.42 g/L/h, respectively (Andrade and Vasconcelos, 2003). Another low cost substrate explored is corn fiber xylan (Qureshi et al., 2006). Hydrolysis of corn fiber xylan, fermentation, and recovery of ABE were performed in a single, integrated system with a high yield of 0.44 g ABE per g substrate and a high productivity of 0.47 g/L/h. Recently, a 214% increase in butanol productivity was achieved in batch culture of C. beijerinckii P260 using wheat straw hydrolysate (WSH) as a carbon source (Qureshi et al., 2007). A recent report describes the impact of the components of agricultural hydrolysates on butanol production; furfural and hydroxymethyl furfural were found to have stimulatory effect on the growth of C. beijerinckii BA101 and butanol production (Ezeji et al., 2007b).

Mono- and di-saccharide uptake occurs mainly by the phosphotransferase (PTS) system (Table II) with galactose being the only sugar reported to be transported by a non-PTS mechanism (Mitchell, 1996). Also, solvent-producing clostridia are capable of efficiently utilizing pentose sugars including xylose and arabinose (Andrade and Vasconcelos, 2003).

Solvent Toxicity

One of the most critical problems in ABE fermentation is solvent toxicity. Clostridial cellular metabolism ceases in the presence of 20 g/L or more solvents (Woods, 1995). This limits the concentration of carbon substrate that can be used for fermentation resulting in low final solvent concentration and productivity. The lipophilic solvent butanol is more toxic than others as it disrupts the phospholipid components of the cell membrane causing an increase in membrane fluidity (Bowles and Ellefson, 1985). Moreira et al. (1981) had attempted to elucidate the mechanism of butanol toxicity in C. acetobutylicum. They found that 0.1–0.15 M butanol caused 50% inhibition of both cell growth and sugar uptake rate by negatively affecting the ATPase activity. Increased membrane fluidity causes destabilization of the membrane and disruption of membrane-associated functions such as various transport processes, glucose uptake,

| Table II. The PTS systems reported in solventogenic clostridia. |
|---------------|--------------|---------------|--------------|
|               | C. beijerinckii (NCIMB 8052) | C. acetobutylicum (ATCC 824) |
| Fructose      | Mitchell (1996)               | —*                        |
| Maltose       | —                           | —*                        |
| Lactose       | —                           | Yu et al. (2007)           |
| Glucitol      | Mitchell (1996), Tangney et al. (1998a) | —*                        |
| Mannitol      | Mitchell (1996)               | —                          |

*Not reported.
and membrane-bound ATPase activity (Bowles and Ellefson, 1985). Also, butanol is the only solvent produced to the level that becomes toxic to the cells during the fermentation of clostridia (Jones and Woods, 1986). It has been known that the addition of 7–13 g/L of butanol to culture medium results in a 50% inhibition of growth, while the addition of acetone and ethanol up to 40 g/L reduced growth by 50% (Jones and Woods, 1986).

Biotechnological production problems such as solvent toxicity have traditionally been addressed by random mutagenesis and process optimization. Development of C. beijerinckii BA101 strain, which is a more solvent tolerant strain derived from C. beijerinckii NCIMB 8052 by random mutagenesis, is one such example (Qureshi and Blaschek, 2001c). 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). Also, integration of an in situ solvent recovery process with fermentation has been used as a means to overcome the solvent toxicity problem. It is expected that a more solvent tolerant strain of clostridia will be developed by metabolic and cellular engineering using the systems approach (Lee et al., 2005). So far, metabolic engineering approaches have been explored more extensively for C. acetobutylicum ATCC 824, which are reviewed below.

Metabolic Engineering of C. acetobutylicum ATCC 824

The objectives of metabolic engineering for butanol producing clostridia include enhanced butanol production with respect to the final concentration and productivity, increased butanol (solvent) tolerance, extended substrate utilization range, engineering the metabolic network for better medium design, increased butanol yield on carbon source, and selective production of butanol instead of mixed acids/solvents production.

Genetic Engineering Tools: Shuttle Vectors, Transformation Techniques, and Gene Knock-Out Systems

For successful metabolic engineering of C. acetobutylicum, it is necessary to have efficient genetic engineering tools for metabolic pathway manipulation. Several B. subtilis–C. acetobutylicum and E. coli–C. acetobutylicum shuttle vectors were developed in the early 1990s (Lee et al., 1992; Minton et al., 1993). Merzmelstein et al. (1992) made a breakthrough in metabolic engineering of C. acetobutylicum ATCC 824. Since C. acetobutylicum ATCC 824 possesses a strong restriction system encoded by Cac824I (recognizing 5’-GCNGC-3’), which prevents efficient transformation of recombinant plasmid prepared in E. coli. Thus, they developed a B. subtilis–C. acetobutylicum shuttle vector pFNK1, which allowed higher transformation efficiency. Using this shuttle vector, the acetocetate decarboxylase (adc), and the phosphotransbutyrylase (ptb) genes were successfully expressed at elevated levels in strain ATCC 824. The development of an in vivo methylation system was an important step (Mermelstein and Papoutsakis, 1993). Methylation of the shuttle vectors with φ3T methyltransferase (encoded by B. subtilis phage φ3T) prior to transformation greatly reduces or prevents the degradation of the transforming plasmid DNA by the attack of a strong restriction system (Cac824I) present in C. acetobutylicum (Mermelstein and Papoutsakis, 1993). The copy number of commonly used plasmids in C. acetobutylicum is around 7–20 copies per cell, which seem to be suitable for metabolic engineering purposes (Lee et al., 1993).

The characteristics of many clostridial promoters were identified using reporter systems based on lacZ gene from Thermoaerobacterium thermosulfurigenes (encoding β-galactosidase) (Tummal et al., 1999) and the lucB gene from Photinus pyralis (encoding luciferase) (Feustel et al., 2004). A promoter probe vector for C. perfringens was constructed by using the promoterless chloramphenicol acetyltransferase gene (catP) as a reporter, and used to select a promoter of the phospholipase C gene (Matsushita et al., 1994). Another promoter probe vector for C. perfringens, which has also been constructed by using catP, was successfully validated by inserting the promoter region of the alpha-toxin gene into the upstream of the catP (Bullifent et al., 1995). An alternative gene expression reporter system based on the E. coli gusA gene (encoding β-glucuronidase) have also been developed (Laurence et al., 2004).

Metabolic engineering of clostridia has been hampered by the lack of an efficient gene knock-out system; only five genes (buk, pta, adhE, solR, spo0A) have been knocked-out in C. acetobutylicum (Green and Bennett, 1996; Green et al., 1996; Harris et al., 2002; Nair et al., 1999) with low reproducibility. Recently, an efficient gene knock-out system was developed for clostridia (Heap et al., 2007). The new system is a clostridial version of group II introns gene targeting system. Mobile group II introns of Lactococcus lactis, Ll. LtrB, show a retrohoming mechanism in both Gram-positive and Gram-negative bacteria (Cousineau et al., 1998). Further analyses of Ll. LtrB have revealed that DNA target sites are recognized by base-pairing between intron RNA in the RNP complex (intron-encoded protein and excised intron RNA complex) and DNA target sites, which results in reverse splicing (Mohr et al., 2000). In addition to this, target-site recognition rules were also reported based on comprehensive experimental results; key nucleotides are directly recognized by the RNP complex and strict spacing is required among the target site elements (Mohr et al., 2000). As a consequence, gene targeting is controllable (Karberg et al., 2001) and it is possible to predict the target sites and design primers for intron
targeting (Perutka et al., 2004). A commercial knock-out system, Targetron (Sigma–Aldrich, St. Louis, MO), has been developed based on these rules. Heap et al. (2007) adapted Ll. LtrB to design the clostron system and made several mutant strains of clostridia in less than 2 weeks. This clostron system is the most efficient gene knock-out system for clostridia at least for now.

Metabolic Engineering of C. acetobutylicum

Several examples of metabolic engineering of C. acetobutylicum have been reported over the years (Table III), mainly by the research groups of E. Terry Papoutsakis and George Bennett. The first successful metabolic engineering example was the amplification of the acetone formation pathway in C. acetobutylicum. As shown in Figure 2, the acetone producing pathway is coupled with that leading to the formation of the precursor of butanol, butyryl-CoA, from butyrate. In the fermentation of recombinant C. acetobutylicum with amplified adc (encoding acetoacetate decarboxylase) and ctfAB (encoding CoA transferase) genes, the acetone-forming enzymes became active earlier, which led to earlier induction of acetone formation. This resulted in increased final concentrations of acetone, butanol, and ethanol by 95%, 37%, and 90%, respectively, compared to its parental strain (Mermelstein et al., 1993).

In another study, Nair et al. (1994) amplified the adhE gene (encoding aldehyde dehydrogenase) without success in increasing solvent production. The strain lacking pSOL1 but expressing the adhE gene was found to produce butanol and ethanol (Nair and Papoutsakis, 1994). As shown in Figure 2, AdhE is responsible for the formation of both acetdehyde and butyraldehyde. However, the effect of its overexpression on solvent formation appears to depend on the physiological state of the cell (Table III). When the control strain produces less than 10 g/L of butanol, the adhE gene overexpression results in increased butanol production (Nair and Papoutsakis, 1994; Nair et al., 1994). On the other hand, when the control strain produces more than 10 g/L of butanol, overexpression of the adhE gene leads to production of more ethanol than butanol.

Further studies were carried out to understand the regulation involved in solvent formation by characterizing the fermentation performance of the solR (encoding the regulator of solvent production genes) mutant strain (Harris et al., 2001). SolR was suggested to be a putative DNA-

### Table III. Fermentation performance of metabolically engineered C. acetobutylicum strains.*

<table>
<thead>
<tr>
<th>Up-regulated</th>
<th>Down-regulated</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Acetone</th>
<th>Butanol</th>
<th>Ethanol</th>
<th>Total solvent</th>
<th>BE/A*</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>adc, ctfAB</td>
<td>4.2</td>
<td>6.7</td>
<td>4.5</td>
<td>9.5</td>
<td>0.74</td>
<td>14.7</td>
<td>2.28</td>
<td>50% higher yield (g/L) of solvents on glucose</td>
<td>Mermelstein et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>adhE</td>
<td>3.2</td>
<td>3.2</td>
<td>3.8</td>
<td>5.5</td>
<td>0.7</td>
<td>16.4</td>
<td>1.63</td>
<td>BE/A increased</td>
<td>Nair et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>pSOL1 adcE</td>
<td>6.4</td>
<td>14.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.6</td>
<td>N/A</td>
<td>Acetone was not produced/adhE is responsible for ABE production</td>
<td>Nair and Papoutsakis (1994)</td>
</tr>
<tr>
<td></td>
<td>pSOL1 adhE</td>
<td>6.1</td>
<td>8.7</td>
<td>0</td>
<td>6.2</td>
<td>0.4</td>
<td>6.6</td>
<td>N/A</td>
<td>Butyryl-P level is critical for butanol production</td>
<td>Green et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>pSOL1 solR</td>
<td>6.7</td>
<td>9.5</td>
<td>4.6</td>
<td>9.7</td>
<td>0.5</td>
<td>14.8</td>
<td>2.2</td>
<td>Improved yields of solvent on glucose</td>
<td>Nair et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>pSOL1 solR</td>
<td>6.8</td>
<td>8.7</td>
<td>3.8</td>
<td>6.3</td>
<td>4.5</td>
<td>26.9</td>
<td>5.8</td>
<td>High butanol concentration achieved</td>
<td>Harris et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>pSOL1 solR</td>
<td>3.6</td>
<td>3.6</td>
<td>5</td>
<td>11.7</td>
<td>0.73</td>
<td>17.4</td>
<td>2.5</td>
<td>Controlling regulator of solvent producing genes. High butanol concentration achieved</td>
<td>Harris et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>adhE solR</td>
<td>4.1</td>
<td>5.6</td>
<td>14.6</td>
<td>4.4</td>
<td>24.6</td>
<td>3.4</td>
<td>2.4</td>
<td>Butyrate asRNA regulation</td>
<td>Tummala et al. (2003a)</td>
</tr>
<tr>
<td></td>
<td>adhE solR</td>
<td>5.1</td>
<td>8.2</td>
<td>17.6</td>
<td>2.1</td>
<td>27.9</td>
<td>2.4</td>
<td>2.4</td>
<td>Highest ethanol production</td>
<td>Tomas et al. (2003b)</td>
</tr>
<tr>
<td>Wild type</td>
<td>cfB</td>
<td>6.8</td>
<td>10</td>
<td>0.3</td>
<td>1.2</td>
<td>0.2</td>
<td>1.7</td>
<td>4.7</td>
<td>Solvent tolerant strain</td>
<td>Tomas et al. (2003b)</td>
</tr>
<tr>
<td>Control</td>
<td>adhE</td>
<td>4</td>
<td>4.4</td>
<td>4.4</td>
<td>10.4</td>
<td>0.2</td>
<td>15</td>
<td>2.4</td>
<td>Butyrate asRNA regulation</td>
<td>Tummala et al. (2003a)</td>
</tr>
<tr>
<td></td>
<td>pSOL1 adhE</td>
<td>7</td>
<td>0</td>
<td>1.4</td>
<td>10</td>
<td>8.8</td>
<td>20.2</td>
<td>13.4</td>
<td>Highest ethanol production</td>
<td>Tomas et al. (2003b)</td>
</tr>
<tr>
<td></td>
<td>pSOL1 groESL</td>
<td>4.5</td>
<td>3.5</td>
<td>6</td>
<td>13</td>
<td>1.1</td>
<td>20.1</td>
<td>2.4</td>
<td>Butyrate asRNA regulation</td>
<td>Tomas et al. (2003b)</td>
</tr>
</tbody>
</table>

*Up-regulated and down-regulated mean amplification and removal of the corresponding gene/plasmid, respectively. Control means the strain harboring the vector only. The concentrations of fermentation products are given in g/L.

a.(Butanol + ethanol)/acetone (g/g).
binding transcriptional repressor that negatively regulates solventogenic metabolism (Nair et al., 1999). Thus, SolR is induced in acidogenesis and suppressed in solventogenesis. In the solR inactivated strain, the expression of the solventogenic genes are induced earlier and maintained throughout the solventogenic phase. However, Thomann et al. (2002) reported that the aforementioned result was incorrect because the DNA clone used to complement the phenotype of the solR knock-out also contained the binding site of a regulator, possibly Spo0A or another regulator, which affected the sol operon expression by a regulator titration mechanism (Scotcher and Bennett, 2005). They suggested that Spo0A, rather than SolR, is a putative regulator of the sol operon. Further studies are needed to clarify the detailed control mechanism of solventogenesis. Nonetheless, removing the solR gene region (which contains the putative Spo0A binding motif) allowed enhanced butanol production (Table III).

There have been studies examining the effects of knocking out the butyrate forming enzymes on metabolic characteristics. The buk and pta genes, which encode butyrate kinase and phosphotransacetylase, respectively, were inactivated to redirect carbon flow from acid formation to solvent formation. The PJC4BK strain, which is a buk inactivated strain, produced 10% more butanol than the parental strain (Green et al., 1996). Under more optimized condition, the PJC4BK strain was able to produce higher concentrations of solvents: 16.7 g/L butanol, 4.4 g/L acetone, and 2.6 g/L ethanol (Harris et al., 2000).

Desai and Papoutsakis (1999) examined the effectiveness of anti-sense RNA (asRNA) for reducing the activities of butyrate forming enzymes in C. acetobutylicum. They designed two different asRNAs, one for buk and the other for ptb, and successfully reduced the butyrate kinase and phosphotransbutyrylase activities by 85% and 70%, respectively. This result shows the potential of asRNA for the strain improvement through the fine-tuning of gene expression. While the strain transformed with the buk-asRNA produced 50% and 35% higher final concentrations of acetone and butanol, respectively, the strain transformed with the ptb-asRNA exhibited 96% and 75% lower final acetone and butanol concentrations, respectively. The results obtained with the strain transformed with the buk-asRNA were similar to those obtained with the buk-inactivated strain (PJC4BK), which showed 65–75% lower butyrate-formation flux yet produces more solvents than the wild-type strain, as mentioned above. Continued work with the asRNA approach revealed that CoA transferase, rather than acetoacetate decarboxylase, is the rate-limiting enzyme in the acetone formation pathway (Tummala et al., 2003a).

The solvent tolerance and stress response of C. acetobutylicum involves the overexpression of many genes including molecular pumps, chaperones (e.g., groES, dnaKJ, hsp18, and hsp90), and genes involved in sporulation, fatty acid synthesis and transcriptional regulators (Tomas et al., 2004). Overexpression of the heat shock proteins GroES and GroEL allowed an increase of the final solvent concentration, presumably by stabilizing solventogenic enzymes (Tomas et al., 2003b). Genomic library screening has been used to identify individual genes capable of improving solvent tolerance (Borden and Papoutsakis, 2007). The candidate list of 16 included 4 transcriptional regulators of which one was able to increase solvent tolerance by 81%. In general, targeting transcriptional regulators may be the most efficient strategy to realize complex phenotypes that requires intensive reprogramming of the cellular metabolism. As illustrated by Alper et al. (2006) for ethanol tolerance in yeast, this may be realized by altering global transcription regulators.

**Systems-Level Analysis of C. acetobutylicum**

In 2001, the complete genome sequence of C. acetobutylicum was published (Nölling et al., 2001). The C. acetobutylicum ATCC 824 genome consists of a 3,940,880 bp chromosome and a 192,000 bp megaplasmid pSOL1. A total of 3,740 and 178 ORFs were identified on the chromosome and megaplasmid, respectively. C. acetobutylicum has distinctive families of proteins involved in sporulation, anaerobic energy conversion, and carbohydrate degradation, which are well matched to the physiological characteristics of C. acetobutylicum. For butanol formation, two mechanisms have been identified in this strain; one is related to solventogenesis (ABE forming process) and the other is alcohologenesis (butanol and ethanol forming process). The genes involved in alcohologenesis remain unidentified. It is currently believed that the enzymes encoded by the adhE (aldehyde/alcohol dehydrogenase; CAP0035), pdc (pyruvate decarboxylase; CAP0025), and edh (ethanol dehydrogenase; CAP0059) genes are associated with this metabolism.

Having the complete genome sequence, genome-wide transcriptome studies on C. acetobutylicum have already been performed (Alsaker et al., 2004; Paredes et al., 2004; Tomas et al., 2003a, 2004; Tummala et al., 2003b), and proteome studies are underway (Schaffer et al., 2005; Sullivan and Bennett, 2006). Alsaker et al. (2004) examined gene expression profiles during the cultivation and confirmed many of the previously reported transcriptional mechanisms and controls. Some of the key results are shown in Figure 3. Interestingly, the strain lacking the megaplasmid pSOL1 showed increased expression of the genes involved in glycolysis, and butyryl-CoA and butyrate formation during solventogenesis indicating that loss of pSOL1 leads to the change in primary metabolism. Sullivan and Bennett (2006) recently conducted proteome profiling of C. acetobutylicum. Among 2,081 protein spots, 23 proteins were analyzed in detail. As expected, the levels of stress proteins such as GroES, GroEL, and Hsp18 and Adc were up-regulated during solventogenesis. On the other hand, the levels of butyryl-CoA dehydrogenase, some proteins in glycolysis, and AtpD (ATP synthase subunit δ) were down-regulated.
Figure 3 illustrates combined metabolic flux and transcriptome analyses during acidogenic and solventogenic phases. Desai et al. (1999) constructed a stoichiometric model for the flux analysis of acid and solvent formation pathways. As expected, higher acetate and butyrate forming fluxes were observed in acidogenesis compared with those in solventogenesis. The direction of the butyrate forming pathway was reversed in solventogenesis. During solventogenesis, the fluxes toward acetone, butanol and ethanol formation were greatly elevated. In addition, fluxes linked to the acetone forming pathway (e.g., uptake fluxes of acetate to acetyl-CoA and butyrate to butyryl-CoA) increased in solventogenesis. Transcriptome profiling strongly supported that this flux pattern was driven by phase-dependent gene expression (Alsaker et al., 2004). In acidogenesis, the expression of the *buk* and *ptb* genes was up-regulated. The expression of the *ak* and *pta* genes was induced during the transition from acidogenesis to solventogenesis. The expression of the genes related to solvent formation, such as the *adc*, *ctfAB*, *adhE*, and *bdhAB* genes, was significantly up-regulated in solventogenesis.

The level of butyryl-P appears to be important for butanol production. When the *buk* gene was inactivated, more butanol was produced (Desai and Papoutsakis, 1999; Harris et al., 2000). However, the *ptb*-inactivated strain exhibited low acetone and butanol production (Desai and Papoutsakis, 1999). It was reported that the initiation of butanol formation corresponded to the time when butyryl-P concentration reached its peak. It was suggested that the concentration of butyryl-P should be higher than 60–70 pmol g DCW$^{-1}$ for butanol production (Zhao et al., 2005). The higher butyryl-P peak concentration corresponds to higher butanol formation fluxes. Supporting this finding, the butyryl-P peak concentration never exceeded 50 pmol g DCW$^{-1}$ in the non-butanol producing strain lacking the megaplasmid pSOL1 (Zhao et al., 2005).

As is the case with other organisms sequenced, much experimental verification is required for complete understanding of the genes, pathways, and metabolic and regulatory characteristics. Nonetheless, having a complete genome sequence in our hands, we can use new genome-wide information to develop novel strategies for strain optimization.
Many solventogenic clostridia transport carbon sources into the initial levels of acids produced by exponential phase (Formanek et al., 1997). Although switching mechanism and produces butanol during the fermentation characteristics. Butanol and acetone than its parent strain and has distinct very efficient into solvents (Formanek et al., 1997). While the strain hydrolyzes fiber-rich agricultural residues rather inefficiently (Ezeji et al., 2007a).

Recently, the complete genome sequence of C. beijerinckii NCIMB 8052 was reported and the final NCBI review was completed in June 2007 (Ezeji et al., 2007a). The strain contains a single 6,000,632 bp circular chromosome. Based on the genome sequence of C. beijerinckii NCIMB 8052, it should be possible to conduct systems biotechnological studies, which will bring about a great improvement in the performance of C. beijerinckii.

In silico genome-scale metabolic networks constructed based on the complete genome sequence can be used for virtual experiments under various genotypic and environmental conditions. The results can provide strategies for metabolic engineering, including genes to knock-out and amplify, to achieve desired metabolic performance. Considering the greater difficulty of engineering clostridia compared with E. coli, in silico simulation before actual metabolic engineering experiments will be beneficial. This approach was demonstrated successfully for Mannheimia succiniciproducens, a succinic acid producing bacterium recently sequenced, by using the results of the genome-scale metabolic flux analysis to understand underlying physiological characteristics appropriate for succinic acid over-production (Hong et al., 2004). Thus, a similar approach can be taken to enhance butanol production with reduced by-products formation by clostridia.

C. beijerinckii BA101 and Process Development

Random mutagenesis is a classical yet powerful way to enhance strain performance. Many industrial strains have been generated by random mutation and subsequent selection. The C. beijerinckii BA101 strain was generated by treating C. beijerinckii NCIMB 8052 with a mutagen N-methyl-N9-nitro-N-nitrosoguanidine and selectively enriching the candidate mutants on the nonmetabolizable glucose analog 2-deoxyglucose (Formanek et al., 1997). C. beijerinckii BA101 is able to produce twofold more butanol and acetone than its parent strain and has distinct fermentation characteristics.

C. beijerinckii BA101 has an altered acid-to-solvent switching mechanism and produces butanol during the exponential phase (Formanek et al., 1997). Although the initial levels of acids produced by C. beijerinckii BA101 are similar to its parental strain, the levels decrease dramatically during solventogenesis. Thus, the C. beijerinckii BA101 seems to be able to convert butyric and acetic acids very efficiently into solvents (Formanek et al., 1997). While many solventogenic clostridia transport carbon sources into the cell using the phosphoenolpyruvate-dependent PTS, C. beijerinckii BA101 and its parental strain use both PTS and non-PTS transport systems simultaneously; non-PTS systems are predominant during solventogenesis where the PTS is repressed (Bahl et al., 1982). Using maltodextrins, soy molasses, agricultural waste, and packaging peanuts as carbon sources, C. beijerinckii BA101 was able to produce 18.6 g/L (26.1), 18.3 g/L (22.8), 9.8 g/L (14.8), and 15.7 g/L (21.7) of butanol (total solvent), respectively (Ezeji et al., 2004a). However, the strain hydrolyzes fiber-rich agricultural residues rather inefficiently (Ezeji et al., 2007a).

Advanced Fermentation Techniques

Batch reactors are often preferred in the bioindustry due to simple operation and reduced risk of contamination. However, the productivity achievable in a batch reactor is low due to the lag phase, product inhibition as well as down time for cleaning, sterilizing, and filling. The preparation time and lag phase can be eliminated using continuous culture and the problem of product inhibition can be solved using an in situ product removal system. It should be noted that single stage continuous fermentation is not feasible due to the complexity of butanol production in clostridia. To avoid substrate inhibition and to increase cell mass, fed-batch fermentation has been applied to the butanol production. Furthermore, immobilized cell reactors and cell recycle reactors have also been applied to butanol production, in order to increase productivity.

C. beijerinckii BA101 was immobilized onto clay brick particles and the fermentation was performed for ABE
production (Qureshi et al., 2000). At a dilution rate of 2 h⁻¹, a solvent productivity of 15.8 g/L/h was achieved with a yield of 0.38 g/g and concentration of 7.9 g/L (Table IV). As expected, the yield and concentration increased with lower dilution rate. The process suffered from the fact that only a fraction of the biomass was in the solventogenic state and a significant amount of biomass was present as inactive biomass, spores (Qureshi et al., 2004). Therefore, it is suggested that sporulation should be blocked to achieve higher productivity. Nevertheless, immobilized cell continuous reactors is a strong candidate for an industrial fermentation process.

Membrane cell recycle reactors are another option to improve productivity. A hollow-fiber ultrafiltration was applied to separate and recycle cells in a continuous fermentation (Pierrot et al., 1986). At a dilution rate of 0.5 h⁻¹, cell mass, solvent concentration, and solvent productivity of 20 g/L, 13 g/L, and 6.5 g/L/h, respectively, were achieved (Table IV). However, fouling of the membrane with the fermentation broth was a major obstacle of this system. Lipnizki et al. (2000) suggested a way to overcome this problem by allowing only the fermentation broth to undergo filtration by using the immobilized cell system.

Recovery and In Situ Separation

High product recovery cost is another problem in biological butanol production. Besides the traditional distillation process, several other processes including pervaporation, adsorption, liquid–liquid extraction, gas stripping, and reverse osmosis have been developed to improve recovery performance and reduce costs (Dürre, 1998). The traditional recovery process employing distillation suffers from a high operation cost due to the low concentration of butanol in the fermentation broth. To solve this problem and the solvent toxicity problem at the same time, in situ recovery systems have been employed. From an economic point of view, reverse osmosis is most preferable. However, it has disadvantages of membrane clogging or fouling. In contrast, liquid–liquid extraction has high capacity and selectivity, although it can be expensive to perform (Dürre, 1998). Thus, there are advantages and disadvantages of using each recovery system, which need to be thoroughly examined.

Gas stripping is a simple but efficient way to recover butanol from the fermentation broth (Fig. 4a). The fermentation gas is bubbled through the fermentation broth then passed through a condenser for solvent recovery. The stripped gas is then recycled back to the fermentor and the process continues until all the sugar in the fermentor is utilized. Gas stripping enables the use of a concentrated sugar solution in the fermentor (Qureshi and Blaschek, 2001d) and a reduction in butanol inhibition and high sugar utilization (Maddox et al., 1995). Gas stripping was applied to a batch reactor to recover solvents from the fermentation broth of C. beijerinckii BA101 (Ezeji et al., 2003). A 161.7 g/L sugar solution was successfully fermented and 75.9 g/L total solvent produced in the integrated process. Fed-batch fermentation was also integrated with gas stripping to reduce substrate inhibition and increase cell mass (Ezeji et al., 2004a). In this system, 500 g glucose was consumed and 233 g solvent was produced with the productivity of 1.16 g/L/h and the yield of 0.47 g/g (Table IV). In the case of continuous fermentation integrated with gas stripping, 460 g of total solvent was produced from 1,163 g glucose with the productivity of 0.91 g/L/h.

Liquid–liquid extraction is another efficient technique to remove solvents from the fermentation broth. This approach takes advantage of the differences in the distribution coefficients of the chemicals. Because butanol is more soluble in the extractant (organic phase) than in the

### Table IV. Performance of integrated fermentation and recovery processes.

<table>
<thead>
<tr>
<th>Process</th>
<th>Total solvent productivity (g/L/h)</th>
<th>Yield (g/g)</th>
<th>Solvent concentration (g/L)</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous immobilized reactor</td>
<td>15.8</td>
<td>0.38</td>
<td>7.9</td>
<td>Highest productivity</td>
<td>Qureshi et al. (2000)</td>
</tr>
<tr>
<td>Continuous membrane cell recycle reactor</td>
<td>6.5</td>
<td>0.35</td>
<td>13</td>
<td>20 g/L of cell mass</td>
<td>Pierrot et al. (1986)*</td>
</tr>
<tr>
<td>Batch</td>
<td>0.34</td>
<td>0.42</td>
<td>24.2</td>
<td>59.8 g/L of glucose</td>
<td>Evans and Wang (1988)*</td>
</tr>
<tr>
<td>Batch with pervaporation</td>
<td>0.5</td>
<td>0.42</td>
<td>32.8</td>
<td>78.2 g/L of glucose</td>
<td>Evans and Wang (1988)*</td>
</tr>
<tr>
<td>Fed-batch with pervaporation</td>
<td>0.98</td>
<td>0.43</td>
<td>165.1</td>
<td>384 g of glucose</td>
<td>Groot et al. (1984)**</td>
</tr>
<tr>
<td>Batch with gas stripping</td>
<td>0.61</td>
<td>0.47</td>
<td>75.9</td>
<td>161.7/L of glucose</td>
<td>Ezeji et al. (2003)</td>
</tr>
<tr>
<td>Fed-batch with gas stripping</td>
<td>1.16</td>
<td>0.47</td>
<td>233</td>
<td>500 of glucose</td>
<td>Ezeji et al. (2004a)</td>
</tr>
<tr>
<td>Continuous with gas stripping</td>
<td>0.91</td>
<td>0.4</td>
<td>460</td>
<td>1,163 of glucose</td>
<td>Ezeji et al. (2004b)</td>
</tr>
<tr>
<td>Continuous membrane bioreactor with liquid–liquid extraction</td>
<td>3.08</td>
<td>0.3</td>
<td>14.5</td>
<td>7 g/L of cell mass</td>
<td>Eckert and Schügerl (1987)**</td>
</tr>
<tr>
<td>Batch</td>
<td>0.34</td>
<td>0.42</td>
<td>24.2</td>
<td>59.8 g/L of glucose</td>
<td>Qureshi and Blaschek (1999)</td>
</tr>
<tr>
<td>Batch with pervaporation</td>
<td>0.69</td>
<td>0.42</td>
<td>51.5</td>
<td>121.2 g/L of glucose</td>
<td></td>
</tr>
</tbody>
</table>

*The strains employed are: 1, C. acetobutylicum; 2, C. beijerinckii LMD; others, C. beijerinckii BA101.
fermentation broth (aqueous phase), it is selectively concentrated in the extractant. Common extractants employed include decanol and oleyl alcohol (Evans and Wang, 1988). Liquid–liquid extraction has critical problems, however, such as the toxicity of the extractant to the cell and emulsion formation. These problems can be overcome if the fermentation broth and the extractant are separated by a membrane that provides surface area for butanol exchange between the two immiscible phases; this is termed “Perstraction” (Fig. 4b) (Ezeji et al., 2007a). C. acetobutylicum has been cultivated in a continuously operated membrane bioreactor connected to a four-stage mixer-settler cascade (Eckert and Schügerl, 1987). In this system, butanol was extracted with n-decanol (extractant) from the cell-free fermentation broth, which was re-fed into the fermentor. This system enabled production of solvents with a high productivity of 3.08 g/L/h. Among the several extractants reported, oleyl alcohol, being a good extractant, is the most promising for C. beijerinckii BA 101 because it is relatively non-toxic (Ezeji et al., 2007a).

Pervaporation is a membrane-based process that allows selective removal of volatile compounds from fermentation broth (Fig. 4c). 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. Both liquid and solid pervaporation membranes have been used. A liquid membrane containing oleyl alcohol was used in pervaporation of dilute aqueous butanol solutions (Matsumura et al., 1988). The selectivity of this liquid membrane was better than that of a silicon rubber membrane. When pervaporation using an oleyl alcohol liquid membrane was employed for the pretreatment of butanol purification, the energy requirement was just one tenth of that of conventional distillation. To develop a stable membrane having a high degree of selectivity, Qureshi et al. (1999) synthesized a silicon-silicalite-1 composite membrane which showed a 2.2-fold improvement in selectivity. Using this membrane in an integrated batch-pervaporation process with C. beijerinckii BA101, a twofold increase in the total solvent concentration (from 24.2 g/L in control batch to 51.5 g/L in batch-pervaporation) was achieved (Qureshi and Blaschek, 1999). The pervaporation condition did not affect the growth of C. beijerinckii BA101. Since the membrane permeate contains acetone, butanol, and ethanol, distillation is still required for further purification. Pervaporation was also applied to a fed-batch reactor resulting in increased solvent productivity from 0.35 g/L/h (batch reactor) to 0.98 g/L/h (fed-batch reactor) due to the reduction in product inhibition (Qureshi and Blaschek, 2000). Recently, the overall solvent productivity in continuous fermentation of C. acetobutylicum was increased up to 2.34 g/L/h by integrating with a pervaporation system using an ionic liquid polydimethylsiloxane ultrafiltration membrane (Izák et al., 2008).

It should be emphasized that the recovery and purification processes are directly affected by the performance of fermentation, which in turn is affected by the strain characteristics. For example, when a strain is metabolically engineered to produce butanol without or much less acetone and ethanol, the purification process will be considerably simplified. When the butanol tolerance of a strain is increased by metabolic engineering, this will also facilitate the recovery process as higher butanol concentration can be
achieved during the fermentation. Thus, the overall process needs to be optimized from strain development to fermentation to downstream processes. This will lead to the reduction in overall production costs. For example, an engineered strain capable of producing butanol to a high concentration with high productivity will result in significant reduction in the direct fixed capital costs (e.g., costs of fermentor and recovery units) and associated depreciation costs because smaller fermentor can be used to produce a desired amount of butanol.

### Systems Metabolic Engineering

Several engineered strains of *C. acetobutylicum* have been developed before the complete genome sequence was available. Thus, the engineering targets were limited to those genes that were directly related to acid or solvent formation or those of stress proteins. In the case of the *C. beijerinckii* BA101 mutant strain, process development rather than further strain engineering has been the primary direction of study. Now, based on the complete genome sequence, it is possible to carry out a more systematic strain improvement process. As mentioned earlier, genome-wide transcriptome and proteome studies on *C. acetobutylicum* have already been performed (Alsaker et al., 2004; Paredes et al., 2004; Schaffer et al., 2005; Sullivan and Bennett, 2006; Tomas et al., 2003a, 2004; Tummala et al., 2003b). It is expected that those genome-wide studies will also be conducted for *C. beijerinckii* BA101, the genome sequence of which is now available. Although the transcriptome and proteome studies on *C. acetobutylicum* have so far been mainly focused on understanding the complex cellular physiology and metabolism, they will be extended for the applications in strain improvement as demonstrated for other microorganisms (Lee et al., 2005). In this way, new targets of regulatory as well as metabolic pathways can be identified for metabolic engineering of clostridia to achieve desired goals.

As a part of systems biotechnology, in silico modeling and simulation can also be used as an efficient tool for metabolic pathway engineering of clostridia. In fact, the first such stoichiometric models for quantitative analysis of metabolic fluxes were reported for *C. acetobutylicum* in the pioneering work by Papoutsakis (1984). As the complete genome sequence is now available, a genome-scale metabolic model can be constructed and used for more accurate simulation of metabolism. The effects of genetic and/or environmental perturbations on cellular metabolism can be predicted by in silico simulation and the results can be used to design strategies for strain improvement. As recently demonstrated for the development of an engineered *E. coli* strain capable of efficiently producing L-valine and L-threonine (Lee et al., 2007; Park et al., 2007), systems metabolic engineering will allow the development of optimal butanol producing strains in the near future by combining the results of omics and computational studies.

### Patent Review

Since the 1910s, numerous patents on butanol production have been reported. Here, we briefly review the key patents on the use of clostridia for butanol production which have been reported since mid 1980s (Table V). A continuous process for the production of butanol employing *C. acetobutylicum* was invented by Levy (1984). An improved continuous fermentation process employing *C. acetobutylicum* was reported by Heady and Frankiewicz (1985). In this process, vigorous fermentation could be performed for extended period of time by using butanol-absorbing material and cycling the fermentation broth. A novel asporogenic strain of *C. acetobutylicum*, which is a better solvent producer, was obtained by growing a spore-forming strain in a continuous culturing reactor (Lemme and Frankiewicz, 1985). The yield of butanol could be increased by more than 20% by co-culturing *C. pasteurianum* and *C. butylicum*, which favor production of butyric acid and butanol, respectively (Bergstrom and Foutch, 1985). Butyric acid produced by *C. pasteurianum* in the first stage is converted to butanol by *C. butylicum* in the second stage. The yield of butanol could be increased by more than 20% by co-culturing at least two different species including *C. pasteurianum* and *C. butylicum* (Bergstrom and Foutch, 1985). The yield of butanol could be increased by providing sufficient carbon monoxide dissolved in aqueous medium (Datta and Zeikus, 1985). It has been shown that butanol can be produced by direct fermentation of a low cost carbon source, such as corn fiber and xylan by the asporogenic strain of *C. acetobutylicum* ATCC 39236 (Datta and Lemmel, 1987). *C. acetobutylicum* mutant strain IFP904 showing increased resistance to butanol was isolated by treating cells with a mutagen nitrosoguanidine. The resulting strain was used to produce butanol to a higher concentration (Hermann et al., 1988). The production rate of butanol and the final concentration of butanol could be increased by 20% and 12%, respectively, in *C. acetobutylicum* by the addition of a suitable fluorocarbon to the culture medium (Husted et al., 1988). It has been found that butanol and butoxyacetaldehyde can be separated from each other by distillation under a pressure of not more than 0.66 bar. Butoxyacetaldehyde is a valuable starting material for the preparation of dyes, plastics, fragrance materials, resins and elastomers (Driscoll et al., 1991). It is produced by dehydrogenation or oxydehydrogenation of ethylene glycol monoalkyl ethers (alkyl glycols). A high concentration of butanol (20.3 g/L) could be obtained by employing asporogenic strains of *C. acetobutylicum* in an improved fermentation process which combines continuous and batch fermentation processes (Glassner et al., 1991). In another invention, a high concentration of butanol (20.2 g/L) could be achieved by employing the asporogenic *C. acetobutylicum* ATCC 55025 strain, which was developed by treating the parent strain *C. acetobutylicum* ATCC 4259 with ethyl methane sulfonate (Jain et al., 1993). It has been reported that butanol can be produced by anaerobically fermenting...
carbohydrates with multiple strains of microorganisms including *C. acetobutylicum* ATCC 824, *C. thermobutyricum* ATCC 49875 and *C. tyrobutyricum* ATCC 25755 (Ramey, 1998). An efficient method of recovering butanol from fermentation medium using silicalite pervaporation membrane was reported (Meagher et al., 1998). The method of producing high levels of butanol using a fermentation process employing a mutant strain of *C. beijerinckii* has been reported (Blaschek et al., 2002). In this patent, batch fermentation of the mutant strain *C. beijerinckii* BA101 enabled production of 20.6 g/L of butanol while the parent strain *C. beijerinckii* NCIMB 8052 produced 11.7 g/L of butanol. A continuous fermentation process combined with gas stripping using the *C. beijerinckii* BA101 strain has been reported (Ezeji et al., 2005b). The concentrations of cells and carbohydrates were controlled while solvents were removed by gas stripping, resulting in more complete glucose utilization (from 45.5 to 1163.4 g) and more butanol production (from 11.8 to 251.3 g/L). Recently, a new *Clostridium* strain (*C. carboxidivorans* ATCC BAA-624) producing butanol was reported. This strain is capable of producing butanol by directly fermenting CO with a yield of 0.03 mol per mol carbon content (Lewis et al., 2007). It has been reported that *C. acetobutylicum* ATCC 824 strain, transformed with an antisense RNA vector targeted for *spoIIE* gene, was able to produce 153 mM butanol, which is a 132% increase compared to the control strain. This result suggests that inhibition of sporulation enables continued solventogenesis, thus improving solvent production (Bennett and Scotcher, 2007). The invention providing an efficient procedure for the replacement of a target DNA sequence by homologous recombination in clostridia has recently been published (Soucaille et al., 2008). This invention should be useful for the metabolic engineering of clostridia as it allows efficient gene deletion and replacement on the chromosome.

### Butanol Production by Other Microorganisms

The lack of efficient genetic engineering tools including a gene knock-out system for *C. acetobutylicum* has hampered further strain improvement. As described earlier, much effort is exerted to develop genetic engineering tools for clostridia. In the mean time, Liao and collaborators recently
reported metabolic engineering of *E. coli* for butanol production. The mutant *E. coli* BW25113 (ΔadhE ΔldhA ΔfdrBC Δfrr Δpta) strain overexpressing the *crt*, *bcd*, *etfAB*, *hbd* and *adhE2* genes of *C. acetobutylicum*, and *atoB* gene of *E. coli* was able to produce 552 mg/L butanol using 2% (w/v) glycerol as a carbon source (Atsumi et al., 2007). More recently, *E. coli* JM109 strain overexpressing the *crt*, *bcd*, *etfAB*, *hbd*, *adhE* and *thiL* genes of *C. acetobutylicum* was developed. This engineered *E. coli* strain was able to produce 16 mM butanol using 4% (w/v) glucose as a carbon source (Inui et al., 2008).

In another recent study, branched-chain alcohols, which have higher octane numbers compared with their straight-chain counterparts, have been produced by metabolic engineering of *E. coli* (Atsumi et al., 2008). 2-keto acids, the intermediates of highly active amino acid biosynthetic pathway in *E. coli*, were converted to various higher alcohols including isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol by overexpressing 2-keto acid decarboxylase (KDC) from *L. lactis* and alcohol dehydrogenase 2 (Adh2) from *Saccharomyces cerevisiae*. To improve the production of the desired alcohol, the pool of specific 2-keto acid was increased by genetic modification. As a result, 86% of the theoretical maximum yield per glucose was achieved in the case of isobutanol. A recently published international patent by DuPont describes the production of butanol in *E. coli*, *Bacillus subtilis* and *S. cerevisiae*. By overexpressing the *thl*, *hbd*, *crt*, *bcd*, *ald* and *bdhAB* genes of *C. acetobutylicum* involved in butanol biosynthesis, 0.8 mM, 0.19 mM and 0.01 mM butanol could be produced by engineered *E. coli*, *B. subtilis* and *S. cerevisiae*, respectively (Donaldson et al., 2007).

**Conclusions and Future Prospects**

As the overall competitiveness of the bioprocess highly depends on the strain performance, successful metabolic
engineering is crucial to enhance butanol production. Solvent toxicity problems can be reduced by in situ recovery systems, but these systems can add substantial capital and operating costs. The cost of in situ recovery decreases with higher operating concentration of solvent, and thus solvent tolerant strains would greatly improve process economics.

The specific and volumetric butanol productivities and the butanol yield on carbon source are linked to each other, and can be increased by optimizing metabolic fluxes. The target genes to be engineered to achieve these goals can be identified by genome-scale metabolic flux analysis and omics studies (Lee et al., 2005). For this purpose, genome-scale metabolic network of clostridia needs to be constructed. As the complete genome sequences for both Clostridium acetobutylicum and C. beijerinckii are available, it is just a matter of time and interest. Genome breeding can also be a good tool for metabolic engineering (Ohnishi et al., 2002). By genome comparison between C. beijerinckii BA101 and its parental strain C. beijerinckii NCIMB 8052, the mutated genes of C. beijerinckii BA101 involved in enhanced solvent production and different fermentation characteristics can be identified, and subsequently used for further enhancement of solvent production. In addition to engineering the metabolic genes, one can attempt to engineer global regulators, transcription factors, chaperones, and other cellular proteins that may increase butanol production and butanol tolerance. For example, Spo0A, may be a good target for metabolic engineering towards enhanced solvent production, as it is a multifunctional global regulator which activates the expression of genes involved in solvent formation and sporulation. As sporulation interrupts solvent production, an engineered Spo0A, which activates and represses genes involved in solvent production and sporulation, respectively, is needed for possible enhancement of solvent production.

Compared to strain development, the development of fermentation processes has been explored extensively, but needs further improvements. Development of new fermentation and recovery processes based on the metabolically engineered strain will be essential for a bio-based butanol process to become economically feasible.

It should be noted that one of the most critical factor not only for butanol production but also for whole bioindustry is securing low price substrates for the processes. To compete with the conventional fossil resource-based chemical industry, the biotechnology industry needs a reliable, cost-effective raw materials infrastructure. The cost-effectiveness of biomass production and the efficient storage and transport of harvested biomass resources will be critical elements of securing raw materials. Environmental impacts and sustainability are also important issues. There is a cautious prediction that agricultural crop production may not match future industrial demand. According to Paster et al. (2003), while the demand for biomass resources has increased by 2–3 folds in 10–30 years, corn productivity alone has increased by about 1.5% per year over the past decade. More importantly, these food and agricultural crops should be discouraged if we consider the regional food shortage and rapid hike in their price. As an alternative plan, many research projects have been initiated for the efficient use of lignocellulosic biomass, which should be accomplished in the future. Sucrose from sugar cane is also an excellent substrate in certain regions of the world.

Much improvement is still needed to make a biobutanol process economically competitive. However, it is likely to become a competitive process by systems-level metabolic engineering of strains based on the recent availability of complete genome sequences and new metabolic engineering tools for clostridia. An optimal bioprocess for butanol production can be developed by integrating the fermentation and downstream processes with strain development (Fig. 5). Based on the genome sequence, a genome-scale metabolic model can be constructed. This model should go through validation process by comparing the results with those obtained from actual fermentation experiments. Transcriptome and proteome profiling can be performed under various genotypic and environmental conditions for identifying target genes and pathways to be manipulated. Gene knock-out simulations can be performed with the genome-scale metabolic model to identify combinatorial target genes to be manipulated. Integration of all these results will lead to the development of a superior strain capable of producing much more butanol. Of course, one will repeat this research cycle as shown in Figure 5 until the desired level is achieved. Taken together, bio-based butanol production is likely to become an economically feasible process in the coming years.

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References


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