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## **Biobutanol: An outlook of an academist and industrialist**

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## **Abstract**

The gradual shift of transportation fuels from oil based fuels to the alternative fuel resources and worldwide demand for energy has been the impetus for research to produce alcohol biofuels from renewable resources. Current bioethanol and biodiesel can, however, not cover an increasing demand for biofuels. Hence, there is an extensive need for advanced biofuels with superior fuel properties. The present review is focused on the developments of biobutanol, which is regarded to be superior to bioethanol in terms of energy density and hygroscopicity. Although acetone-butanol-ethanol (ABE) fermentation is one of the oldest large-scale fermentation processes, butanol yield by anaerobic fermentation remains sub-optimal. For sustainable industrial scale butanol production, a number of obstacles need to be addressed including choice of feedstock, low product yield, product toxicity to production strain, multiple end-products and downstream processing of alcohol mixtures. Metabolic engineering provides a means for fermentation improvements. Different strategies are employed in the metabolic engineering of *Clostridia* that aim to enhance the solvent production, improve selectivity for butanol production, and increase the tolerance of *Clostridia* to solvents. The introduction and expression of a non-clostridial butanol producing pathway in *E. coli* is most promising strategy for butanol biosynthesis. Several rigorous kinetic and physiological models for fermentation have been formulated, which form useful tool for optimization of the process. Due to the lower butanol titers in the fermentation broth, simultaneous fermentation and product removal techniques have been developed to improve production economics. With the use of new strains, inexpensive substrates, and superior reactor designs, economic ABE fermentation may further attract an attention of researchers all over the world. The present review is attempting to provide an overall outlook on discoveries and strategies that are being developed for commercial *n*-butanol production.

**Keywords:** ABE, Advanced fuel, Biobutanol, *Clostridium*, Metabolic pathway, Economics

## 1. Introduction

The rapid depletion and environmental aspects of fossil fuels and highly fluctuating market prices has made the quest for alternative fuels with a high priority. Over the past more than two decades, several alternative liquid fuels have been investigated, which can either completely replace the petroleum derived fuels (gasoline and diesel) or be blended with petroleum fuels at some proportions, without modifying the vehicle engines.<sup>1-3</sup> First generation biodiesel although a popular biofuel, fails to replace petro-diesel completely, unless the significant changes in the engine are configured.<sup>4,5</sup> Low energy content (or heat of combustion) of ethanol influences the economy of the blended fuel. Moreover, given its solubility characteristics, ethanol is likely to separate from gasoline in the presence of water, which introduces some operational problems. Butanol isomers (*n*- or iso-butanol), overcome the most of the limitations which are mentioned here. Besides, to be a potential biofuel, butanol is also a valuable C4 feedstock for chemical synthesis (esters, ethers, acetates, and plasticizers), as well as a solvent. The current international price of bulk grade butanol is approximately \$4 per gallon (liquid fuels) with a worldwide market of 350 million gallons per year.<sup>6</sup> The conventional chemical processes for butanol synthesis includes the oxo process, wherein synthesis gas is reacted with propylene and hydrogenated subsequently to produce butanol. Table 1 lists and compares some common properties of various alternative liquid fuels. It can be seen that the properties of butanol match more closely with gasoline than for the other listed fuels.

Apart from the merits enlisted in the table 1, butanol also offers some advantages over ethanol and methanol i.e. (a) It can be blended to varied ratio with gasoline as well as diesel directly in the refinery without any additional infrastructure. (b) Easy transportation and less corrosion through pipelines because of low vapor pressure. (c) The air: fuel ratio for butanol is close to that of gasoline which is permissible in existing vehicle engines. The complete replacement of gasoline by butanol would require an enhancement of the air: fuel ratio, but blends up to 20% of butanol can be directly used in engines without any modifications.<sup>7</sup> (d) The heat of vaporization of butanol is slightly higher than that of gasoline. Hence, the butanol-blended engine does not observe the cold start problem as seen in methanol or ethanol blended gasolines.<sup>7</sup> (e) Low solubility of butanol in water reduces the potential for groundwater contamination.

Different routes of biosynthesis of butanol from various biological substrates such as sugars, starch, and biomass are available.<sup>6</sup> Biologically produced *n*-butanol is popularly known as 'biobutanol'. The most prevalent and historical route to biobutanol production is by acetone–butanol–ethanol (ABE) fermentation with the solvent-producing strains of *Clostridium* sp.<sup>8,9</sup> The

conventional substrates for the ABE fermentation have been corn starch or molasses; however, as reviewed subsequently in this paper, several alternative substrates have also been considered.

### ***1.1 Chemical Synthesis of Butanol***

Oxo synthesis, reppe synthesis, and crotonaldehyde hydrogenation are considered as the three most important processes for the chemical butanol industry.<sup>8,9</sup> Oxo synthesis (hydroformylation), involves the addition of carbon monoxide and hydrogen to a carbon–carbon double bond using catalysts such as Co, Rh, or Ru substituted hydrocarbonyls.<sup>10</sup> In the Reppe process, propylene, carbon monoxide and water are reacted in the presence of a catalyst to produce butanol at low temperature and pressure.<sup>11</sup> Crotonaldehyde hydrogenation consists of aldol condensation, dehydration, and hydrogenation.<sup>11</sup>

## **2. Biobutanol**

Fermentative ABE production was prospered during the early 20<sup>th</sup> century after Pasteur discovered it from anaerobic cultivation in 1861. In 1945, two third of industrially used butanol was produced by fermentation in U.S.A and the ABE production evolved as the second largest industrial fermentation process in the world after the ethanol. However, due to progression of the petrochemical industry, low solvent yields and the increase in the feedstock cost, the ABE fermentation process had lost its competitiveness by 1960s.<sup>12</sup> Research and development of the ABE process continued and during the 1980s and 1990s the studies at pilot plants were carried out in several countries. Few countries such as China, South Africa and Russia developed its ABE process industry with *Clostridium* strains from starch based feedstocks.<sup>13</sup> In the past years China has restarted the ABE production including at least 11 production plants which are in the operation and others are under construction or at the start up stage.<sup>14,15</sup>

### ***2.1 Applications of Butanol***

An estimation of 10–12 billion pounds of butanol is produced annually, which accounts for 7–8 billion \$ market at current price.<sup>16</sup> Butanol has a projected market growth of 3% per year.<sup>17</sup> The most important application of butanol receiving renewed attention is its use as a fuel additive or as a direct replacement of gasoline. In an automotive fuel market, it offers substantial advantages over petroleum gasoline. Butanol has been shown to be compatible with OEM (original equipment manufacturer) gasoline engines without modification and exhibits comparable performance.<sup>18</sup> It is also compatible with the existing fuel pipeline infrastructure. Under current US Environmental

Protection Agency (EPA) regulations, butanol can be added as an oxygenate to gasoline in concentrations up to 11.5% (v/v). Butanol is safer to handle than gasoline, less flammable (lower vapor pressure and higher flash point) and is miscible with gasoline in any ratio. It is non-hygroscopic, non-corrosive, and proves an overall low order of toxicity.<sup>19</sup> Butanol has a volumetric energy density of 29.2 MJ.l<sup>-1</sup>, which is comparable to that of gasoline (32.5 MJ.l<sup>-1</sup>), and much higher than that of ethanol (19.2 MJ.l<sup>-1</sup>) (Table 1). Motor gasoline consumption in the USA is about 370 million gallons per day representing a huge fuel market.<sup>20</sup> The Energy Independence and Security Act of 2007 mandates that the volume of renewable fuels sold in the USA must be 30 billion gallons by 2020, of which 10.5 billion gallons must be of lignocellulosic origin.<sup>21</sup>

The other direct application of butanol is as an industrial solvent or co-solvent, mainly for surface coatings (resins, lacquers, varnishes, waxes). About 50% of butanol production is used in the form of butyl acrylate and methacrylate esters used in latex surface coating, enamels and lacquers.<sup>17</sup> 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.

## 2.2 Microorganisms

Butanol (along with, acetone, ethanol, and isopropanol) are naturally formed by a number of *Clostridia*.<sup>22</sup> *Clostridia* are typically strict anaerobes and rod-shaped, spore-forming Gram positive bacteria. Solventogenic *Clostridia* can utilize a large variety of substrates from monosaccharides including many pentoses and hexoses to polysaccharides.<sup>23</sup> Among many solventogenic *Clostridia*, *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* are primary solvent producers.<sup>24</sup> *C. beijerinckii* NCIMB 8052, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* were originally designated as *C. acetobutylicum*.<sup>24</sup>

The *Clostridial* solvent production is biphasic fermentation, which is detailed in subsequent sections of this paper (Figure 1). The first phase is the acidogenic phase, during which the acids forming pathways are activated, to produce acetate, butyrate, hydrogen, and carbon dioxide as major products. The acidogenic phase usually occurs during the exponential growth phase of the *Clostridia*.<sup>25,26</sup> The latter phase is the solventogenic phase wherein acids are reassimilated and used in the production of acetone, butanol and ethanol (or isopropanol in some *C. beijerinckii* strains). The transition from acidogenic to solventogenic phase is the result of an intense change in gene expression pattern.<sup>27</sup> The biosynthetic pathways involving acidogenic and

solventogenic phase for *n*-biobutanol production are discussed scrupulously in subsequent sections of this paper.

### **2.3 Biomass for biobutanol**

Low conversion rates of conventional fermentative technologies which in turn results in the economically unviable large-scale production is the key reason for less attracted stronger market interest of biobutanol. However, a number of biotechnology ventures with proprietary approaches to microbial strain selection and engineering, feedstock handling, bioreactor management, and product separation have emerged as a new generation of butanol producers (Butamax, Gevo, Green Biologics, etc.). These are established as major forces in the renewable market. Biomass is the fourth largest source of the energy in the world after coal, petroleum and natural gas, and it is also a carbon neutral resource over its life cycle. Although, some plants are rich sources of starch such as corn, soybeans, and potatoes and could be used to make liquid biofuels, this has been criticized for diverting food away from the human food chain, leading to food versus feed/energy war. The fermentation feed substrate is also an important factor influencing the cost effectiveness of the biobutanol production.

The major advantage of using biomass substrates for fuel production is its renewability. Biomass is a complex mixture of organic materials, but the main components of plant biomass are polymeric carbohydrates (approximately 75% of dry weight) including celluloses and hemicelluloses with lignins which function in imparting strength to the plant structure and holding the composition together.<sup>28</sup> *Clostridia* can secrete numerous enzymes that facilitate the breakdown of polymeric carbohydrates into sugar monomers for biobutanol production.<sup>29</sup> However, acidic or enzymatic hydrolysis of lignocellulosic materials is essential to convert them into monosaccharides before using them as substrates in ABE fermentation. *Clostridia* are not cellulolytic bacteria and hence cannot utilize cellulose as carbon source. However, cellulosomes are multienzyme complexes having high activity against crystalline cellulose are being analyzed in *Clostridia*.<sup>30</sup> The lignocellulosic biomass is the most abundant renewable resource on the earth for biofuel production.<sup>31,32</sup> Countries like India alone generates over 370 million tonnes of biomass every year directly from plants, rice husk from rice mills, saw dust from saw mills, bagasse from sugar mills etc.<sup>33</sup> Experimentally, the potential of numerous lignocellulosic feedstocks like wood forestry residues, corn stover, wheat straw, corn fibers, barley straw, and switchgrass has been tested for ABE fermentation.<sup>34-42</sup>

## **2.4 Biochemistry of the ABE fermentation**

In a natural state, bacterial butanol production is carried out exclusively by the members of the genus *Clostridia* including *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, *C. saccharoacetobutylicum*, *C. aurantibutyricum*, *C. pasteurianum*, *C. sporogenes*, *C. cadaveris*, *C. tetanomorphum*.<sup>43</sup> The most commonly considered substrates for the *Clostridial* cultures include fibrous biomass containing hemicellulose and cellulose (e.g., wheat straw, rice straw); starchy biomass (such as ground corn and whey permeate); and fruits and vegetables containing fructose, glucose and xylose as the basic components. Laboratory-based media for the culture growth have normally been semi-defined and defined wherein the main carbohydrate source is supported by various vitamins and minerals depending on the microbial cultures. The optimum temperature for the ABE fermentation is between 30 to 40 °C, while the initial pH of the fermentation broth, is 6.8–7, and it drops down to 4.5–5 during acidogenic phase.

## **3. Fermentative production of biobutanol**

A typical ABE fermentation using *C. acetobutylicum* yields acetone, butanol and ethanol in the ratio of 3:6:1. A high concentration of acetyl-CoA is needed to increase the overall solvent production and hence the quantity of acetyl-CoA plays an important role in determining the ratio of C3 + C4 products to C2 products.<sup>44,45</sup> ABE fermentation undergoes an acidogenic phase in the exponential growth phase, followed by a switch to solventogenic phase at the end of the exponential growth phase. Acidogenic phase is accompanied by a higher butyrate concentration than acetate in the fermentation broth. The formation of butyrate resolves an issue of redox equilibrium – the NADH produced during glycolysis is consumed only in butyrate, and not in acetate formation pathway. This results in higher concentration of butanol accumulation than ethanol in the fermentation, since most of the butyrate and acetate is converted to butanol and ethanol in the solventogenic phase, respectively. Development of a fermentation system to produce higher butanol yield and productivity with cost effective way has been practiced by various researchers. The recent developments on the use of various types of biomasses for ABE production is discussed in subsequent sections.

### **3.1. Batch fermentation**

#### **3.1.1. Fermentation with biomass hydrolysate**



Even though batch fermentation processes have several disadvantages such as time consumption during sterilization of bioreactors, major down time, long lag phase, solvent inhibition and low productivity; they are traditional and are still useful in many respects. The use of various cheap feedstocks such as Jerusalem artichokes, cheese whey, apple pomace, algal biomass, sulfite waste liquors, and various hydrolysates for production of biobutanol has been reviewed and described by Jones and Woods.<sup>23</sup>

Continuing our previous work to update on the recent developments on the use of various sustainable feedstocks by various researchers for ABE fermentation is reviewed in Table 2.<sup>46</sup> Jesse et al.<sup>47</sup> performed the fermentation of waste streams such as starch-based packing for peanuts and agricultural wastes as a source of fermentable carbohydrates using *C. beijerinckii* BA101 and produced 21.7 g.l<sup>-1</sup> and 14.8 g.l<sup>-1</sup> ABE, respectively. The use of enzyme hydrolysate of palm oil mill effluent has been reported as a growth medium substitute, a fermentation substrate, and as a source of nitrogen and micronutrients for the production of ABE by Hipolito et al.<sup>48</sup> The agricultural wastes such as wheat bran, rice bran, maize stalk, barley straw etc. were studied by various researchers as sugar sources. Different pretreatments and hydrolysis methods were also studied for respective feedstocks. Qureshi et al.<sup>35-37</sup> studied five different processes to produce ABE from wheat straw (WS) by *Clostridium beijerinckii* P260. They found simultaneous saccharification and fermentation with agitation by gas stripping to be efficient but suggested that fed batch operation with the glucose supplementation can make further improvements in solvent production and yield. Lee et al.<sup>49</sup> used rice bran and defatted rice bran to get the highest butanol production (12.24 g.l<sup>-1</sup>) from the hydrolysate after acid and enzyme treatment with supplementation in production medium. Wheat bran, a by-product from wheat processing industry was shown to be a potential substrate for ABE production. Liu et al.<sup>50</sup> used acid hydrolysed wheat bran and produced 53.1 g.l<sup>-1</sup> monomeric sugars from 100 g.l<sup>-1</sup> of wheat bran. Qureshi et al.<sup>38</sup> studied barley straw hydrolysate (BSH) in four different ways such as i) undiluted/untreated BSH, ii) diluted BSH (20 mL BSH and 20 ml sterile distilled water), iii) diluted BSH with equal amount of wheat straw hydrolysate (WSH: 20 ml BSH and 20 mL WSH), and iv) overlimed BSH. The total solvents produced in various fermentation systems were 7.09, 17.42, 17.18, and 26.64 g.l<sup>-1</sup>, respectively suggesting significance of overliming in reducing the effect of inhibitors. Qureshi et al.<sup>39</sup> further performed similar experiments with corn stover and switchgrass hydrolysates and concluded the importance of dilution and overliming to get comparable ABE solvent production.

The SO<sub>2</sub>-ethanol-water (SEW) spent liquor obtained by fractionation of spruce chips was fermented in batch culture by dilution (4 times) and supplementation with added glucose (35 g.l<sup>-1</sup>) resulted in 8.79 g.l<sup>-1</sup> ABE solvents.<sup>51</sup> Wang and Blaschek,<sup>52</sup> used response surface methodology to optimize pH, sugar concentration and agitation in batch fermentation of maize stalk juice. The optimum condition of pH 6.7, a sugar concentration 42.2 g.l<sup>-1</sup> and an agitation rate 48 rpm were estimated to give a maximum butanol yield of 0.27 g.g<sup>-1</sup> sugar. In yet another study, sugar maple wood was subjected to hot water extraction followed by membrane filtration and hydrolyzed with sulphuric acid. The hydrolysate was further treated with nanofiltration and overliming to remove inhibitors. Overliming treatment significantly improved the butanol concentration from 0.8 to 7 g.l<sup>-1</sup>.<sup>53</sup> Isar et al.<sup>54</sup> showed the potential of *Jatropha* seed cake (JSC) as a substrate for biobutanol production. They reported a butanol titer of 13.2 g.l<sup>-1</sup> in 120 h using acid-treated JSC (7 % w/v) with supplementation of suitable medium components. They further successfully scaled up the process to 15 liter and improved butanol titer to be 18.6 g.l<sup>-1</sup> after 72 h of fermentation.

### 3.1.2. Hydrolysates detoxification

Various compounds such as salts, furfural, hydroxymethyl furfural (HMF), acetic, ferulic, glucuronic, *p*-coumaric acids, and some other phenolic compounds are reported to be formed during a pretreatment and hydrolysis of agricultural biomass. Ezeji et al.<sup>41</sup> reported that, *p*-coumaric and ferulic acids at 0.3 g.l<sup>-1</sup> affected growth and ABE production by *C. beijerinckii* BA101 whereas, furfural and HMF stimulated the growth of the microorganism and ABE production. Qureshi et al.<sup>55</sup> reported similar findings concluding that furfural and HMF formed in the wheat straw hydrolysate (WSH) are enhancing the ABE productivity, specific productivity, and ABE concentration. Among various acid hydrolysis products screened, the inhibition rate of butanol production was 88.1%, 82.4%, 22.1%, 9.8% and 4.6% when 0.5 g.l<sup>-1</sup> formic acid, *p*-coumaric acid, ferulic acid or 1 g.l<sup>-1</sup> furfural and HMF respectively were present in the medium.<sup>56</sup> Cho et al.<sup>57</sup> described the peroxidase-catalyzed detoxification method for *p*-coumaric acid, ferulic acid, 4-hydroxybenzoic acid, vanillic acid, syringaldehyde, and vanillin, and evaluated the inhibitory effects of the detoxified solution on butanol production by *Clostridium beijerinckii*. Qureshi et al.<sup>38,40</sup> and Survase et al.<sup>51</sup> reported overliming and dilution as one of the ways to make hydrolysates fermentable. Mu et al.<sup>58</sup> reported separate fermentation of glucose and xylose obtained by steam explosion method and enzymatic hydrolysis of corn stalk as novel technology for improved ABE production. They also found Ca(OH)<sub>2</sub> treatment as the best detoxification method as compared to NaOH and Na<sub>2</sub>SO<sub>3</sub>. The fermentability of enzymatically hydrolyzed steam-exploded corn stover (SECSH) was improved

using activated charcoal treatment and alkaline peroxide treatment, both mainly affecting the phenolic compounds. The ABE fermentation was found to be inhibited above 1.77 g.l<sup>-1</sup> soluble lignin in the SECSH-based medium whereas fermentation was improved when concentration was less than 0.89 g.l<sup>-1</sup>.<sup>59</sup> Cai et al.<sup>60</sup> reported the use of pervaporation with PDMS membrane for the detoxification of sweet sorghum bagasse (SSB) hydrolysate before the fermentation to remove 94.5% furfural. Guo et al.<sup>61,62</sup> have developed *C. beijerinckii* which was tolerant to inhibitors (total phenolic compounds) when corn fiber hydrolysate was used as a substrate.

### 3.1.3. Immobilization materials in fermentation

The presence of adsorption material and immobilization of solvent producing strain have been reported to be beneficial by many researchers in the literature. The immobilization strategy is claimed to improve the stability and solvent tolerance of microorganisms. There are various reports on different types of immobilization materials ranging from synthetic to biodegradable polymers and fibers. Tripathi et al.<sup>63</sup> suggested agarose-alginate cryogel beads after comparing with the other potential support matrices such as coconut fibers, brick pieces and burnt coal. Efremenko et al.<sup>64</sup> showed that the immobilization of *Clostridium* cells using poly(vinyl alcohol) cryogel changed the ratio of ABE solvents (4:12:1) produced resulting higher butanol titers. Various immobilization matrices were screened in batch fermentation.<sup>63,65,66</sup> Survase et al.<sup>66</sup> recently reported that the addition of support matrix improves the substrate consumption and its conversion to solvents. Coconut fibers and wood pulp were found to be the most promising support matrices. Shamsudin et al.<sup>65</sup> investigated the ABE production by *Clostridium saccharoperbutylacetonicum* N1-4 using different immobilization materials and concluded that the passive immobilization on polyurethane foam increased the production by 215.12%. Napoli et al.<sup>67</sup> screened various carriers including silica gel, Tygon, Teflon, PVC and glass beads for better biofilm formation by observing SEM after 1 week.

### 3.2. Fed-batch fermentation

As compared to batch culture, fed-batch fermentation has some advantages such as the use of highly concentrated substrate, which can reduce hydraulic load and wastewater generated in the process. Li et al.<sup>68</sup> compared different modes of fermentation as batch, fed-batch and continuous with pH-control at 4.5 and claimed that the time needed for passing from acidogenesis to solventogenesis was an intrinsic hindrance to higher butanol productivity. However, the fed-batch mode was not suggested for ABE solvent production. The continuous lactic acid and glucose feeding produced

15.5 g.l<sup>-1</sup> butanol at a rate of 1.76 g.l<sup>-1</sup>.h<sup>-1</sup> using a fed-batch culture with a pH maintained at 5.5.<sup>69</sup> They suggested that different acids such as lactic acid, acetic acid and butyric acid can be used as the substrates for butanol production. Similar findings were reported by Tashiro et al.<sup>70</sup> where they demonstrated enhanced productivity of butanol by feeding butyric acid along with glucose in pH stat fed-batch culture. The maximum butanol concentration of 16 g.l<sup>-1</sup> was achieved with a 72% higher productivity than in a conventional batch process. Qureshi et al.<sup>35</sup> demonstrated that simultaneous hydrolysis of wheat straw and fermentation to butanol is possible if the straw is fed with some extra sugar. They could achieve 100% hydrolysis of WS to monomeric sugars and this also helped in improving the productivity by 16% during the fed-batch experiment of 533h. When Guo et al.<sup>62</sup> used non-detoxified sulphuric acid hydrolysate of corn fiber as substrate in fed-batch fermentation, a total ABE of 12.9 g.l<sup>-1</sup> was produced by *C. beijerinckii* RT66, with yield of 0.35 g.g<sup>-1</sup> sugar and productivity of 0.18 g.l<sup>-1</sup>.h<sup>-1</sup> in 72 h.

Ezeji et al.<sup>71</sup> claimed that in the integrated fed-batch fermentation and product recovery system, solvent productivities were improved to 400% of the control batch fermentation. A total of 500 g of glucose was utilized in 201h to produce 232.8 g solvents with an average solvent yield and productivity of 0.47 g.g<sup>-1</sup> and 1.16 g.l<sup>-1</sup>.h<sup>-1</sup>, respectively. The highly concentrated cassava bagasse hydrolysate containing mainly glucose using the hyper-butanol-producing *C. acetobutylicum* JB200 produced 108.5 g.l<sup>-1</sup> ABE in a fed-batch fermentation with simultaneous butanol recovery by gas stripping in 263 h. The integrated fermentation process is claimed to be attractive because of a stable productivity and high butanol yield for an extended period with periodical nutrients supplementation.<sup>72</sup>

### **3.3. Continuous fermentation**

Continuous fermentation has several advantages over batch and fed-batch fermentations such as minimizing downtime and the lag phase. Additionally, time needed for sterilization and inoculation substantially lowers the productivity. The modes of continuous fermentation includes suspended cell, cell recycling and immobilized cell fermentation. Reactor configurations for continuous production of chemicals can be a continuously stirred tank reactor (CSTR) with or without immobilization material, packed bed reactor (PBR), fluidized bed reactor (FBR), airlift reactor, upflow anaerobic sludge blanket reactor, or any other suitable configuration.<sup>73</sup>

#### **3.3.1. Single stage reactors**

In a chemostat CSTR, feeding of the production medium and product removal are at the same rate to keep broth volume constant. CSTR mainly, include suspended cell fermentations which have been reported for continuous ABE production.<sup>8,29,68,74</sup> The use of saccharified degermed corn in suspended cell continuous fermentation achieved total ABE concentration up to 14.16 g.l<sup>-1</sup> during fermentation run for 504 h.<sup>29</sup> The suspended cell continuous fermentation could not reach high productivities because of non-applicability at higher dilution rates. The operation of such reactors at higher dilution rate caused cell wash out.<sup>8,68,74</sup> To overcome this limitation, CSTRs with an adsorbent material such as porous polyvinyl alcohol<sup>8</sup> and wood pulp<sup>74</sup> to attach the cells on and retain in the reactor are reported in the literature. When cells are retained the limitation  $D < \mu_{max}$  can be circumvented. During a continuous butanol production using porous polyvinyl alcohol immobilized cells; the overall butanol productivity and yield were 0.40 g.l<sup>-1</sup>.h<sup>-1</sup> and 0.44 g.g<sup>-1</sup> of glucose, respectively, which were approximately twice the values in a suspended free-cell continuous reactor.<sup>8</sup> When wood pulp was added to the bioreactor as a cell holding material to prevent the cell wash out; higher dilution rates could be employed. This increased the solvent (isopropanol and butanol) productivity from 0.47 to 5.52 g.l<sup>-1</sup>.h<sup>-1</sup> with the yield of 54 % from glucose.<sup>74</sup> The average butanol concentrations with brick immobilized cells and suspended cells were 8.07 and 4.56 g.l<sup>-1</sup>, respectively. However, the ratio of butanol to the total solvents (ABE) was similar suggesting that the cell immobilization did not alter the metabolic pathway.<sup>75</sup>

### 3.3.2. Single stage reactors with cell recycling

To overcome the low cell density (approximately 2-3 g.l<sup>-1</sup>) in traditional ABE fermentation, continuous fermentation processes with cell recycling and retention have been successfully applied.<sup>76,77</sup> This approach helped to maintain high productivities for long period without cell degeneration.<sup>76</sup> A continuous fermentation of *C. pasteurianum* MBEL-GLY2 (hyper-producing strain) with cell recycling was carried out to get the ABE and the butanol productivity of 8.3 and 7.8 g.l<sup>-1</sup>.h<sup>-1</sup> respectively, at the dilution rate of 0.9 h<sup>-1</sup>.<sup>77</sup> Zheng et al.<sup>78</sup> have reported the continuous butanol production system from glucose and xylose with high cell-density generated by cell recycling. Cell recycling increased cell concentration to 17.4 g.l<sup>-1</sup> which increased butanol productivity to 1.20 g.l<sup>-1</sup>.h<sup>-1</sup> from 0.529 g.l<sup>-1</sup>.h<sup>-1</sup> at a dilution rate of 0.26 h<sup>-1</sup>.

### 3.3.3. Multistage reactors

Multistage reactors were reported in literature to facilitate increased substrate consumption. There are some reports on integrated solvent removal modules to further prevent inhibition by produced

solvents and improve volumetric productivity and yields. Bankar et al.<sup>79,80</sup> optimized continuous ABE fermentation using the two stage chemostat system and two stage column reactors integrated with liquid–liquid extraction module and reported to improve the substrate consumption and total ABE production. An overall solvent production of 25.32 g.l<sup>-1</sup> was observed with volumetric solvent productivity and solvent yield to be 2.5 g.l<sup>-1</sup>.h<sup>-1</sup> and 0.35 g.g<sup>-1</sup>, respectively.<sup>79</sup> The maximum solvent productivity (10.85 g.l<sup>-1</sup>.h<sup>-1</sup>) and the solvent yield (0.38 g.g<sup>-1</sup>) were obtained further at a dilution rate of 1.0 h<sup>-1</sup> in two stage immobilized column reactors integrated with liquid–liquid extraction module.<sup>80</sup> Setlhaku et al.<sup>81</sup> studied an innovative two stage process for ABE fermentation where the first stage was operated in a continuous mode and the second stage as a fed-batch. When gas stripping was coupled to the second stage and as operated intermittently this enabled additional glucose feeding in the second stage to produce 59 g.l<sup>-1</sup> butanol and 73 g.l<sup>-1</sup> of total ABE solvents in the condensate. With *ex-situ* pervaporation using a PDMS membrane at temperature of 37 °C and pressure of 10 mbars, 167 g.l<sup>-1</sup> butanol and 269 g.l<sup>-1</sup> ABE was analysed in the permeate. Corn stover hydrolysate (CSH) and cane molasses as substrates were studied by using four stage continuous reactors. Using cane molasses as substrate, 13.75 g.l<sup>-1</sup> total ABE solvents and productivity of 0.439 g.l<sup>-1</sup>.h<sup>-1</sup> were achieved at a gradient dilution mode of 0.15–0.1 h<sup>-1</sup>. In a continuous fermentation using CSH as substrate, total solvent titer of 11.43 g.l<sup>-1</sup> and productivity of 0.429 g.l<sup>-1</sup>.h<sup>-1</sup> were reached at a dilution rate of 0.15 h<sup>-1</sup>.<sup>82</sup>

#### 3.3.4. Packed bed reactors

Packed bed reactors (PBRs) are packed with suitable immobilization material to form biofilm. They are fed with nutrient-rich production medium usually from the bottom. PBRs suffer from excessive cell growth followed by blockage but the reaction rates are much higher as compared to batch reactors.<sup>73</sup> In the past, various materials such as bonechar,<sup>83</sup> coke,<sup>84</sup> beechwood shavings,<sup>85</sup> chitosan, carrageenan and sodium alginate matrices have been used as the support materials.<sup>86</sup> Recently, more immobilization materials were studied for PBR continuous reactors as listed in Table 3. Qureshi et al.<sup>87</sup> studied packed bed reactor using clay brick as an immobilization material and reported highest productivity of 15.8 g.l<sup>-1</sup>.h<sup>-1</sup> at dilution rate of 2 h<sup>-1</sup>. However, the substrate utilization was only 33% at this dilution rate. Qureshi et al.<sup>88</sup> scaled up the biofilm reactor with high volumetric productivities, the highest being 16.13 g.l<sup>-1</sup>.h<sup>-1</sup> at the dilution rate of 2 h<sup>-1</sup> with reactor operation time of 2302 h. To increase sugar utilization, the reactor effluent was suggested to be recycled by Lienhardt et al.<sup>89</sup> This approach resulted in complete sugar utilization with higher productivity upto

10.2 g.l<sup>-1</sup>.h<sup>-1</sup> at a dilution rate of 1.5 h<sup>-1</sup>. The highest reactor productivity (16.2 g.l<sup>-1</sup>.h<sup>-1</sup>) was reported at a dilution rate of 2.0 h<sup>-1</sup>.

Survase et al.<sup>51,66,90</sup> used SEW spent liquor from spruce chips and sugar mixture identical to wood hydrolysate as a feed on wood pulp immobilized PBR to report the highest productivity of 4.86 g.l<sup>-1</sup>.h<sup>-1</sup>. PBR reactor with Tygon rings as an immobilization material and lactose as a feed was reported by Napoli et al.<sup>67</sup> to get maximum productivity of 5 g.l<sup>-1</sup>.h<sup>-1</sup>. Tygon rings has recently also been used as carriers with un-supplemented cheese whey as renewable feedstock. Under optimized conditions, volumetric butanol productivity, concentration and yield were 2.66 g.l<sup>-1</sup> h<sup>-1</sup>, 4.93 g.l<sup>-1</sup> and yield 0.26 g.g<sup>-1</sup>, respectively.<sup>91</sup>

#### 4. Butanol inhibition

As can be seen from the previous sections, during repeated batch, sub-culturing or continuous culturing of *Clostridia*, butanol formation is known to be inhibitory to the producing microorganisms. This is the most crucial hurdle for butanol fermentation to commercialize the process, as bacterial strains rarely tolerate more than 2 % butanol.<sup>92</sup> Solvent toxicity limits the solvent yield, solvent productivity and leads to low concentration of solvents in the fermentation broth. Higher butanol concentration induces adverse changes in phospholipid and fatty acid composition in cell membrane of *C. acetobutylicum*. This adverse change destroys the unsaturated and saturated fatty acids ratio (U/S) and also decreases the specific interaction of alcohol with individual components of lipids.<sup>93</sup> Specifically, butanol (harmful organic solvent) enters into cytoplasmic membrane and alters the membrane structure. It also disrupts the number of physicochemical characteristics such as preferential solute transport, membrane permeability, maintenance of the proton motive force (or maintenance of intracellular pH), intracellular ATP level, glucose uptake, and conformation and activity of intrinsic membrane proteins. An increment of 20–30% of fluidity in membrane is observed with only 1% butanol exposure.<sup>94,95</sup> The butanol-induced degeneration is more severe than acetone induced degeneration.<sup>96,97</sup>

Researchers all over the world endeavored to reduce the toxicity of butanol during fermentation at molecular level as well as by developing advanced bioprocess. The butanol tolerance to the clostridial microorganisms was facilitated by various techniques as discussed in the subsequent review. Several butanol tolerant strains were developed from classical butanol producer organism (*C. acetobutylicum*) using mutagenesis and by genetic manipulation.<sup>92</sup> One attractive achievement in process development, namely extractive fermentation was also being studied to

reduce the butanol-induced inhibition effect.<sup>79,80</sup> In this approach, production as well as selective removal of butanol was carried out simultaneously to maintain low concentration of butanol in fermentation broth. The common online butanol removal techniques are adsorption, liquid–liquid extraction, perstraction, reverse osmosis, pervaporation, and gas stripping, which could be integrated with ABE fermentation<sup>45</sup> and partly already dealt in this text.

#### **4.1 Strain degeneration in solventogenic *Clostridia***

Strain degeneration is a general challenge experienced in the fermentation industry which includes the loss of capacity to produce solvents when *Clostridia* are concerned to sporulate. Solventogenic *Clostridia* lose their productivity if the cells are maintained in the vegetative state for a prolonged period of time.<sup>98</sup> Strain degeneration is a result of genetic changes and is different from loss of ability to produce solvents under unfavorable cultivation conditions. Degeneration is also different from the loss of the solvent production ability due to a mega-plasmid loss which is a common phenomenon for *C. acetobutylicum*. Thus the maintenance of cells in the vegetative state via re-passaging or continuous cultivation leads to an accumulation of degenerated cells within the culture, while the density of the culture and the rate of metabolism decrease.<sup>99</sup> Compared to the non-degenerated cells, degenerated *C. beijerinckii* and *C. saccharobutylicum* cells are longer and thinner, and they form larger colonies with irregular shapes.<sup>98,100</sup> Kashket and Cao,<sup>101</sup> isolated a degeneration-resistant mutant of *C. acetobutylicum* NCIMB 8052. The longevity of the mutant was three times higher than that of the wild type strain. The disruption of a 486 bp regulatory RNA that is able to form a stable hairpin structure was speculated to be associated with the reduced tendency to degenerate.<sup>102</sup> The development of mutants with a reduced level of degeneration along with an identification of the Spo0A factor as a main regulator of the solvent production is under the progress. Bacteriophage infection is another major problem in ABE fermentation. Among the approaches used to eliminate the negative effect of bacteriophage on *Clostridia* fermentation, the most successful and widespread involves the isolation of phage-resistant mutants or variants.<sup>103</sup>

### **5. Metabolic pathways**

Fermentation process inherently relies on the level of metabolic activities of the organism. Major end products from the metabolic pathway activities of clostridial species (*C. acetobutylicum* and *C. beijerinckii*) are butanol, acetone, ethanol, acetic acid, butyric acid, CO<sub>2</sub>, and H<sub>2</sub>.<sup>45</sup> Metabolic activities of the organism broadly observed in acid producing and solvent producing phases are briefly discussed in subsequent sections. Recently, it has been reported that the *Clostridia* secrete



extracellular enzymes like amylase, glucosidase, glucoamylase, pullulanase, and amylopullulanase to digest complex polysaccharides into simple monosaccharides like glucose, xylose and arabinose.<sup>41,42</sup> However, cellulolytic *Clostridia* were known to be extremely sensitive to oxygen and have low aero-tolerance control. Peroxide repressor (PerR) gene, which is responsible to regulate detoxification genes, was identified as the factor for low aero-tolerance of *C. acetobutylicum*.<sup>104-106</sup> Anaerobes (obligate/facultative) uptake sugars via the phosphoenolpyruvate-dependent phosphotransferase system.<sup>107,108</sup> Generally, hexose sugars are metabolized by the Embden–Meyerhoff pathway (EMP), whereas pentose sugars go through the pentose phosphate pathway (PPP) to produce pyruvate (Fig. 1). Glycolysis utilizes glucose (1mol) and produces pyruvate (2mol), whereas PPP results in the production of CO<sub>2</sub> (6mol). The PPP has several roles including use of nicotinamide adenine dinucleotide (NADH) for anabolism as well as formation of C3 and C7 intermediates. During glycolysis, energy is stored as adenosine triphosphate (ATP), whereas during PPP, energy is stored as NADPH. The most common substrate for the ABE fermentation is starch, which is converted to glucose following acid or enzymatic hydrolysis. Glucose (6 C) is first phosphorylated to glucose-6-phosphate, which is subsequently converted to pyruvate (3 C) via EMP. Other fermentation substrates containing hemicellulose or cellulose (e.g. fibrous biomass) can be converted to xylose and glucose, respectively, after hydrolysis.<sup>109</sup> Glucose follows the metabolic pathway as explained earlier, whereas xylose (existing naturally in the form of D-xylose, an aldose) undergoes the ‘isomerase pathway’ in which the enzyme xylose isomerase convert D-xylose to D-xylulose. Furthermore, D-xylulose undergoes phosphorylation to form D-xylulose-5-phosphate which then dissimilates by means of transketolase and transaldolase through non-oxidative pentose phosphate. This results in the production of glyceraldehydes 3-phosphate and fructose-6-phosphate, which finally enters the EMP pathway for further conversion.<sup>23,110,111</sup> Fermentation of hexose sugar (1mol) results in generation of 2mol of ATP and 2 mol of reduced NADH.<sup>23</sup> ATP produced during the consumption of glucose results in the exponential growth of cells.

The pyruvate formed during glycolysis/EMP pathway is cleaved by pyruvate ferredoxin oxidoreductase in the presence of co-enzyme A (CoA) producing CO<sub>2</sub>, acetyl-CoA, and reduced ferredoxin. Conversion of acetyl-CoA to acetate is achieved by the enzymes phosphate acetyltransferase and acetate kinase, whereas conversion of butyryl-CoA (formed through sequential conversion of acetyl-CoA) to butyrate is catalyzed by the enzymes phosphate butyltransferase and butyl kinase.<sup>6</sup>

### **5.1 Acid production pathways**

*Clostridia* grow exponentially in the first phase of fermentation (acidogenesis phase) along with the formation of acids (acetate and butyrate), leading to a decrease in the pH to 4.5.<sup>111</sup> In acidogenic phase, glycolysis pathway is active to produce pyruvate consuming glucose, which is further converted to Acetyl-CoA (Fig. 1).<sup>111,112</sup> Acetyl-CoA is the main precursor for the synthesis of acetate, butyrate, ethanol, butanol and acetone anaerobically (Fig. 1). Acetate and butyrate are produced in acid producing phase through two analogous steps from acetyl-CoA and butyryl-CoA respectively. Phosphate acetyltransferase and acetate kinase are the enzymes involved in acetate formation, while phosphate butyltransferase and butyrate kinase are active for butyrate synthesis. Four enzymes, namely thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase catalyze the synthesis of butyryl-CoA from acetyl-CoA.<sup>23,113</sup> The acids produced during acidogenic phase can permeate through the cell membrane and are involved in triggering of solventogenic phase.

## **5.2. Solvent production pathways**

The acid production slows down due to low pH during the final stage of acidogenesis. To compensate the unfavorable effect of low pH, organism shifts its metabolic activity from acidogenesis phase to solventogenesis phase. In this phase, acetate and butyrate are consumed as substrates for biosynthesis of acetone and butanol while no growth is observed.<sup>112</sup> However, metabolic activity of this complex phase shift is different for different organisms. For example, conversion of butyrate to butanol is hardly observed in the case of *C. saccharoperbutylacetonicum* N1-4 in the absence of glucose in the culture medium. The highest butanol yield (0.671 mol-butanol/mol-butyrate) from butyrate was observed in presence of culture medium glucose.<sup>114</sup> The acetyl-CoA and butyryl-CoA are the key intermediates in synthesizing ethanol and butanol (Fig. 1). These pathways produce acetaldehyde and butyraldehyde respectively, in the presence of two sets of dehydrogenases. The reduction of butyryl-CoA to butanol is mediated by butyraldehyde dehydrogenase and butanol dehydrogenase.<sup>111,115</sup> Six enzymes (thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase, butyraldehyde dehydrogenase, and butanol dehydrogenase) and corresponding seven genes (*thiL*, *hbd*, *crt*, *bcd*, *etfA*, *etfB*, and *adhe*) were identified for the conversion of acetyl-CoA to butanol (Fig. 1).<sup>115-118</sup> The activity of butanol dehydrogenase in both *C. acetobutylicum* and *C. beijerinckii*, was NADPH dependent instead of NADH.<sup>119</sup> Ethanol could be produced independently under certain adverse medium conditions. However, ethanol formation was observed in small amount in comparison to acetone and butanol. The uptake of acids (butyric and acetic acids) was coupled with the formation of acetone and

butanol resulting from complex mechanism of metabolic pathways (Fig. 1).<sup>23,104,111,120</sup> The switchover from acidogenesis to solventogenesis occurs at reduced pH. Bahl et al.<sup>121</sup> have suggested that the switchover is an adaptive response of the cells to the low pH of the medium, whereas Ballongue et al.<sup>122,123</sup> have suggested that acids produced in the acidogenic phase act as inducers for the biosynthesis of solventogenic enzymes.

## 6. Cocultures

Co-cultures of *Clostridium* strain with enzyme producing microorganisms to simultaneously hydrolyze the cellulose and hemicellulose have been attempted to reduce the cost of pretreatment (hydrolysis) and purification of the hydrolysate. This process is commonly referred as simultaneous saccharification and fermentation (SSF). Soni et al.<sup>124</sup> used mixed cultures of cellulolytic fungi *T. reesei* and *A. wentii* to hydrolyze the agricultural wastes (bagasse, rice straw, and wheat straw). *C. saccharoperbutylacetonicum* and/or *C. acetobutylicum* fermented these wastes to yield 16 g.l<sup>-1</sup> and 17.3 g.l<sup>-1</sup> of butanol, respectively. Petitdemange et al.<sup>125,126</sup> and Fond et al.<sup>127</sup> tried to use mesophilic cocultures of cellulolytic (*C. cellulolyticum* H10) and glycolytic (*C. acetobutylicum*) clostridial strains for direct fermentation of cellulose. However, the major fermentation product was acid (butyric acid), with a small amount of acetone, ethanol, and butanol. Two-stage fermentation process with co-culture of *C. butyricum* and *C. pasteurianum* in the first stage and *C. beijerinckii* and *C. pasteurianum* in the second stage produced 20% more butanol. Consequently, the high level of butyric acid production in the first stage was effectively reduced to butanol in the second stage. Further, continuous immobilized cultures of *C. tyrobutyricum* and *C. acetobutylicum* with corn as a substrate, resulted in the productivity of 4.64 g.l<sup>-1</sup>.h<sup>-1</sup> and yield of 42%. *C. tyrobutyricum* increases the production of hydrogen and butyric acid, whereas *C. acetobutylicum* effectively converts butyric acid to butanol. This in turn eliminates the products such as acetic, lactic, and propionic acids.<sup>128</sup> Qureshi et al.<sup>36</sup> used *Clostridium beijerinckii* P260 to produce ABE from wheat straw hydrolysate in the fed-batch reactor. They found that the simultaneous hydrolysis of wheat straw and fermentation is possible with 100% hydrolysis to simple sugars. Fed-batch fermentation with *C. beijerinckii* P260 was continued to use glucose, xylose, arabinose, galactose and mannose; obtained from the wheat straw. The culture utilized all the sugar components improving the ABE productivity by 16%.

## 7. Commercial production and challenges in ABE fermentation

Advanced fuels, (non-ethanol fuels) are high energy and high performance biofuels that include higher molecular weight alcohols (*n*-butanol and isobutanol) as well as infrastructure compatible hydrocarbons produced from either thermochemical (Fischer–Tropsch fuels) or biochemical (fermentation-derived hydrocarbons) pathways. Biobutanol will likely be the first of these biofuels to reach the global markets.<sup>129</sup> One among several partnerships active in this area is the collaboration between BP and DuPont.<sup>130</sup> Several other companies are active in this stream, includes EEI (D. Ramey), Cobalt, Gevo, and Tetravitae.

During 1950s the ABE fermentation process was not competitive to the natural petroleum fuels and hence found to be unattended in the US and Europe. However, some production processes via fermentation continued in China, Russia and South Africa until the early 1980s.<sup>12,13,131</sup> China leads efforts to re-commercialize the ABE fermentation process. Over \$200 million has recently been invested in China to install 0.21 million t pa of solvent capacity with plans to expand it to 1 million t pa. There are six major plants that produce about 30000 t butanol pa from corn starch.<sup>14</sup> Most plants operate in a semi continuous mode where one fermentation cycle can take almost 21 days. Most of the bio-butanol plants are located immediately next to the ethanol plants to reduce utility and operating costs. Co-located operations tend to share effluent treatment facilities based on anaerobic digestion (AD). Biogas, produced from the AD process, can be used to generate power and heat. The recovery of hydrogen from the fermentation exhaust gas (typically 1/10 of mass of butanol produced) gains the additional value to the process. However, some serious problems are associated with industrial ABE production. In general, there is an extensive need for cheaper feedstocks, improved fermentation performance and more sustainable process operations for solvent recovery and water recycle. Feedstocks are known to contribute most to the production cost. The production cost and profitability of the plant is extremely sensitive to the price fluctuation of the feedstock. The fermentation performance can be improved by using chemical mutagenesis, specific genetic manipulation or by combination of both. The technical and related commercial challenges for the conventional ABE fermentation have been extensively reviewed<sup>41,42,45,132</sup> and are summarized in Table 4.

### *7.1. Acid crash in ABE fermentation*

The phenomenon known as “acid crash” is an occasional feature of batch fermentations which are performed without any pH control wherein excess of acid production takes place without a significant switch to the solventogenic phase. It is proposed that the acid crash occurs when the acid concentration in the fermentation broth exceeds the maximum tolerable limit, causing cessation of

glucose uptake and rapid termination of solventogenesis after the switch has occurred.<sup>133</sup> This phenomenon cannot be confused with that of culture degeneration, which is a feature of the strain rather than of particular batch fermentation and which takes place over a longer period of time, particularly in continuous culture, and is normally associated with genetic change. In this case there is a slow drift from a solventogenic to an acidogenic culture (due to accumulation of mutant organisms), rather than switch from an acidogenic to a solventogenic culture.<sup>99</sup> Cho et al.<sup>134</sup> investigated the effect of acetic acid and formic acid on ABE production by solventogenic *Clostridia*. They found that *C. acetobutylicum* and *C. beijerinckii* could tolerate the solvent concentration upto 11.7 g.l<sup>-1</sup>. Moreover, *C. acetobutylicum* was more vulnerable to weak acids such as formic acid than *C. beijerinckii*. Hence, *C. beijerinckii* is preferred against *C. acetobutylicum* for ABE production by some researchers when using lignocellulosic hydrolysate containing acetic and formic acids.

## 8. Mathematical models

Ranjan and Moholkar<sup>6</sup> recently detailed the mathematical models which have been used by various researchers for ABE fermentation. Papoutsakis<sup>135</sup> derived the model equation for butyric acid bacteria fermentation. Chauvatcharin et al.<sup>136</sup> have further applied fermentation model of Papoutsakis for analyzing the culture metabolism of acetone-butanol (AB) fermentation. Besides, the research group at the Technical University of Delft (the Netherlands) has developed a series of models for the continuous ABE fermentation by immobilized *C. beijerinckii*. The salient features of the model were (i) the substrate (glucose) consumption was written in the form of Herbert–Pirt equation; (ii) Monod kinetics was used to explain the relation between the substrate concentration and the specific growth rate, whereas the effect of product inhibition was based on the maximum limit of butanol concentration; and (iii) the substrate consumption rate was defined in terms of dilution rate, concentration of substrate in feed, and reactor voidage. Categorizing the biomass in the reactor in two locations, viz. within immobilization matrix, and as freely suspended cells, the latter was used to determine the rate of substrate consumption. The effectiveness factor can be determined using generalized Thiele modulus.<sup>137,138</sup> Research group at McGill University (Canada) have developed a ‘process kinetics model’ and a ‘physiological state model’ for the AB fermentation. The ‘process kinetics model’ represents the AB process dynamics, while ‘physiological state model’ elucidates the key process parameters and their influence in the control of solvent biosynthesis. The major culture parameters affecting the solvent biosynthesis were permeability of cellular membrane and number of active sugar transport sites.<sup>139-141</sup> Process kinetics

model<sup>139,140</sup> assumes the batch fermentation is glucose limited, with no process limitation by nitrogen source and takes the cellular RNA content as the basis. The physiological model differs from the process kinetics model taking into account both intracellular and extracellular culture conditions and it also contains transport parameters for solvents on cellular level. Further, Jarzebski and coworkers<sup>142,143</sup> have proposed a kinetic and physiological model for continuous AB fermentation with two assumptions: (i) solventogenesis is triggered by attaining a given threshold concentration of intracellular butyrate, (ii) un-dissociated form of butyric acid passes easily through the cell membrane. This model suitably predicts the formation of acids in case of the strong substrate limitations and also the drop in solvent concentration due to increased dilution rate. The model satisfactorily predicts the performance of a continuous as well as a batch fermentation.<sup>144-146</sup> Researchers at Nagoya University (Japan) have developed a general framework for an extractive fermentation, which could be applied to batch, repeated batch, and repeated fed-batch modes.<sup>147</sup> The model comprised a set of differential equations for biomass formation, substrate utilization and products concentration. Furthermore, Shinto et al.<sup>148,149</sup> have developed a kinetic model for ABE fermentation on the basis of metabolic pathway of *C. acetobutylicum* ATCC 824.

The solvents produced in the fermentation are extracted by oleyl alcohol and can be recovered by distillation. However, the distillation of the solvents under vacuum at mild temperature is both practical and reasonable. Hence, Shi et al.<sup>150</sup> have proposed a flash extractive fermentation system to assess its performance with a mathematical model. This model comprises six mass balance equations for the cells, substrate, product in solvent phase, product in aqueous phase, and overall balance. The simulations with the flash extractive model are summarized as: (i) a highly concentrated feed resulted in noticeable improvement in performance; (ii) the solvent distribution rate increases the total productivity but results in the loss of energy utilization efficiency; (iii) two-vessel flash system does not simultaneously satisfy the requirement of product purity and energy efficiency.

## 9. Metabolic engineering

As evident from the previous sections, butanol toxicity to the fermenting microorganisms restricts its concentration in the fermentation broth, resulting in low butanol titer and high production and recovery cost from the dilute solutions. Nevertheless, with the progress on modern molecular biology, several genes involved in the biosynthesis of solvents and acids have been cloned and characterized to study the fermentation physiology. Meanwhile, there is a continuing interest in developing more suitable industrial organisms for butanol production. The *Clostridia* are not

genetically controllable, well-characterized, and fast growing when compared with *E. coli*. and *Saccharomyces cerevisiae*, the current industrial alcohol producers. They are able to tolerate high concentrations of butanol by the same mechanisms by which they tolerate ethanol. Thus, genetic tools are being used to manipulate the metabolism by introducing the genes responsible for butanol production from *C. acetobutylicum* into the *E. coli*<sup>151-153</sup> or yeast.<sup>154</sup> Achievements on genetic engineering of producing strains, reducing butanol toxicity, improving yield, and simplifying downstream processing can contribute to introduce biobutanol fermentation at commercial level.<sup>155</sup>

### **9.1 Genetic engineering**

Genetic modification of *Clostridium* is widely used by inserting some heterologous genes or overexpressing or knocking out/down some relative endogenous genes to improve the butanol production. As discussed earlier, in *Clostridium* sp. hexoses and pentoses are catabolized to produce butanol (Fig. 1).<sup>156-158</sup> Since *C. acetobutylicum* is the first choice *Clostridium* species for industrial biobutanol production, most genetic and biochemical studies focus on *C. acetobutylicum* as a representative species.

Genetic modification in butanol producing bacteria was attempted after recognizing the butanol and acetone producing genes. Metabolic engineering provides the targeted gene for manipulation prior to the genetic engineering. The TargeTron technology has been utilized to disrupt acetoacetate decarboxylase gene (*adc*), which was responsible for acetone production in hyper-butanol producing industrial strain. This enhanced the butanol ratio from 70% to 80.05%, while acetone production was reduced to 0.21 g.l<sup>-1</sup>.<sup>113</sup> Recombinant DNA technology (RDT) is another striking genetic engineering tool in facilitating the solvent producing ability of microbial strains.<sup>159</sup> The use of different host organisms other than clostridial strains for butanol production was encouraged by various researchers because of the genetic complexity of the *Clostridia* and lack of suitable genetic tools. Heterologous organisms (*E. coli* and some other organisms) with butanol producing genes of clostridia have been constructed<sup>45</sup> as discussed in the subsequent sections. Reconstructed microorganism exemplified butanol and propanol titer of approximately 2 g.l<sup>-1</sup> with 1:1 ratio.<sup>160</sup>

### **9.2 Genetic modification of enzymes involved in biosynthetic pathway**

For butyrate formation, phosphate butyryltransferase (PTB) and butyrate kinase (BK) are essential (Fig. 1. Table 5). PTB catalyzes the interconversion of butyryl-CoA and butyryl-P and enables the

conversion of butyryl-CoA to butyrate together with butyrate kinase. This enzyme has eight subunits of equal molecular weight.<sup>161</sup> Butyryl-P is formed with an optimized pH value of 8.1 which is also an optimal for PTB in the physiological path, 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.<sup>162</sup>

In the butanol synthetic pathway, six enzymes, including thiolase, are needed to complete the conversion of acetyl-CoA to butanol (refer Fig. 1; Table 5). Thiolase plays a key role in the production of both acids and solvents, as it efficiently controls the flow of carbon into these pathways. It catalyzes the condensation of two molecules of acetyl-CoA into one acetoacetyl-CoA, which later on is converted to acetone, butyrate or butanol. Regulation of thiolase activity is important for maintaining the C2:C4 acid ratio, which has a profound effect on the energy control of the cell. THL may play an indirect role in acid uptake.<sup>163</sup> 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 butanol dehydrogenase from *C. acetobutylicum* ATCC 824 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  $\alpha$ ,  $\beta$  desaturation of acyl-CoA substrates.<sup>164</sup> The enzymes such as PTB, BK, PTA and AK are more active in acidogenic phase resulting in the production of only acids (butyric and acetic). The activities of these enzymes are known to be decreased with the accumulation of acids. Meanwhile, the enzymes such as CoA, THL and BDH in the solventogenic phase begin to be active and the pH value stabilizes or increases slightly because most of the acids are taken up and converted into solvents (butanol, acetone and ethanol).

Mermelstein et al.<sup>165</sup> constructed a recombinant *C. acetobutylicum* strain with a pFNK6 plasmid, which contains an ace operon including *adc* and *ctfA/B* genes. Compared to the control strain, the final concentrations of acetone, butanol and ethanol were increased by 95%, 37% and 90%, respectively. Harris et al.<sup>166</sup> constructed a strain, PJC4BK, in which *buk* was inactivated. When the pH was  $\geq 5$ , the strain appeared to be a super solvent producer yielding 16.7 g.l<sup>-1</sup> of butanol, 4.4 g.l<sup>-1</sup> of acetone and 2.6 g.l<sup>-1</sup> of ethanol. Butyrate levels were low throughout all



fermentations and never exceeded 20 mM. Tummala et al.<sup>167</sup> downregulated genes coding acetoacetate decarboxylase and coenzyme A transferase with an antisense RNA. Further, they attempted to increase the butanol/acetone ratio through overexpression of the alcohol–aldehyde dehydrogenase gene (*aad*) and downregulation of CoA transferase with an antisense RNA against *ctfB* (the second CoA gene on the polycistronic *aad-ctfA-ctfB*). Jiang et al.<sup>113</sup> disrupted the acetoacetate decarboxylase gene (*adc*) in *C. acetobutylicum* EA 2018 to increase the share of butanol by eliminating the production of other by-products, such as acetone. They found that the butanol/acetone ratio was improved from 70% to 80.05%, and the acetone production was reduced to approximately 0.21 g.l<sup>-1</sup> in the *adc*-disrupted mutant.

### **9.3. Genetic modification to increase the solvent tolerance of the strain**

During the last few years, the volumetric productivity has been greatly increased by implying improved physiological and biological methods. However, when the solvent concentration reaches 20 g.l<sup>-1</sup>, the clostridial cellular metabolism ceases and the butanol production also impedes the microbial growth. This difficulty to achieve more concentrated solvent production with the wild type strain can be overcome by alternative modified microorganisms which tolerate higher amounts of solvent. Hence, developing a tolerant strain is more feasible by genetic modification.<sup>168,169</sup> Alsaker et al.<sup>170</sup> revealed that overexpression of *spo0A* (multifunctional regulator) by DNA microarray technique increased the tolerance and prolonged the metabolism in response to butanol stress. Tomas et al.<sup>171</sup> also constructed an engineered strain with overexpressed *spo0A* which exhibited the butanol production to be higher than with a *spo0A* inactivated strain. Although the solvent tolerance mechanism is still unclear, several genes involved in it are being studied by various researchers. Borden and Papoutsakis<sup>172</sup> built the strain 824(pCAC0003) and strain 824(pCAC1869), by inserting CAC0003 and CAC1869 genes which exhibited 13% and 81% increases in butanol tolerance respectively when compared with the plasmid control strain. A butanol-tolerant mutant strain of *C. beijerinckii* NCIMB 8052 was constructed by using an antisense RNA targeting the *gldA* gene which encodes glycerol dehydrogenase. The overexpression of some non-enzyme proteins such as GroEL, a class I heat-shock protein, can also enhance the solvent tolerance.<sup>169,173</sup> The improvement in the solvent tolerance to increase the final solvent concentration is presumably because of stabilization of solventogenic enzymes.

### **9.4. Genetic modification to control the sporulation**

The solvent accumulation to the level at which it hampers the cell growth may stimulate the bacteria to produce the spores. Sporulation allows the active cells to enter in a dormant state wherein they lose the ability to produce solvents. Hence, the additional efforts on non-sporulation or the delayed sporulation are being continued by various 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*.<sup>174</sup> It controls the beginning of sporulation, the development of competence for DNA uptake and many other stationary phase-associated processes.<sup>174</sup> *abrB*, was described as an inhibitor of sporulation which acts in contrary to the *spo0A*, and acts as both a positive and a negative regulator of cell competence at various stages in the life cycle and regulates the catabolite repression in carbon-limited media. Scotcher et al.<sup>175</sup> constructed *C. acetobutylicum* ATCC 824 (pMSpo) via antisense RNA targeted technology which prolonged the solventogenesis and increased the production of ethanol (225%), acetone (43%) and butanol (110%) when compared with control.

### **9.5. Phase transition regulation from acidogenesis to solventogenesis**

A better understanding of acidogenesis and solventogenesis phases along with the phase transition is important when performing systems-level metabolic engineering. The gene expression analyses of *C. acetobutylicum*<sup>176,177</sup> and *C. beijerinckii*,<sup>150</sup> revealed that during the acidogenic phase, two fold increase in the expression of the *pta* and *ack* genes responsible for acetate formation was observed. Likewise, the expression of *ptb* and *buk* genes responsible for butyrate formation were up-regulated in both the clostridial strains during the acidogenic phase with varied expression levels in *C. acetobutylicum* (1.5- to 2.3-fold)<sup>176</sup> and *C. beijerinckii* (2-fold).<sup>150</sup> A transcriptome analysis for the evaluation of gene expression levels during phase transition in *C. acetobutylicum* with phosphate-limited continuous cultures showed that the expression levels of the *adhE1*, *ctfA-ctfB*, and *adc* genes were induced during the solventogenic phase (pH 4.5) compared with the acidogenic phase (pH 5.8).<sup>178</sup> It was also found that, under phosphate-limited continuous culture conditions, the *adhE1* and *adhE2* genes were antagonistically regulated.<sup>178</sup> However, contrary to previous reports,<sup>176,177</sup> the expression levels of the *thlA*, *hbd*, and *crt* genes were strongly down-regulated during the transition from the acidogenic phase to the solventogenic phase.<sup>178</sup>

### **9.6. Selective butanol production by improved fermentative pathway**

Although butanol is the major product of *Clostridia*, it can also produce acetone and ethanol as associated by-products during the conventional ABE fermentation. To increase the butanol yield

and to reduce the recovery costs, high butanol selectivity is desirable.<sup>113,179,180</sup> *C. acetobutylicum* M5 strain is a mutant of *C. acetobutylicum* ATCC 824 and it cannot produce butanol due to the absence of the megaplasmid pSOL1.<sup>181</sup> *C. acetobutylicum* M5 was metabolically engineered with an overexpression of adhE1 gene under the control of the ptb promoter to restore the selective butanol production without acetone.<sup>115,179</sup> In another study, the butanol selectivity was increased to 0.82 g.g<sup>-1</sup> ABE by disrupting the adc gene encoding acetoacetate decarboxylase in *C. acetobutylicum* and regulating electron flow by the addition of methyl viologen into the culture medium.<sup>113</sup>

### 9.7. Biosynthetic pathway in heterologous organisms

Due to complex and unknown genetic and physiological characteristics of *Clostridia*, it is much more difficult to metabolically engineer the strain as compared to the commonly used industrial microorganisms, such as *E. coli* and *S. cerevisiae*. Thus, there has been considerable effort to introduce the clostridial fermentative pathway into several heterologous organisms, such as *E. coli*, *Bacillus subtilis*, and *S. cerevisiae*, to possibly overcome some of the problems associated with the clostridial butanol production.

Atsumi et al.<sup>153</sup> reported the reconstruction of the functional butanol biosynthetic pathway in *E. coli* by the introduction of the *C. acetobutylicum* thl, hbd, crt, bcd, etfAB and adhE2 genes. Further, they identified the alternatives for thiolase and butyryl-CoA dehydrogenase, and found that the expression of *E. coli* atoB instead of *C. acetobutylicum* thl increased the butanol titer under microaerobic conditions. A further increase in butanol titer was achieved by removing the competing pathways, and finally, a modest maximum of 0.55 g.l<sup>-1</sup> butanol could be produced by cultivating an engineered *E. coli* in a nutritionally rich medium containing 20 g.l<sup>-1</sup> glucose. The potential of *S. cerevisiae*, *B. subtilis*, *Pseudomonas putida*, and *Lactobacillus brevis* as a butanol-producing host were assessed by various researchers.<sup>118,154,182,183</sup> Cloning into *P. putida*, *B. subtilis*<sup>118</sup> and *S. cerevisiae*<sup>154</sup> produced 0.11 g.l<sup>-1</sup>, 0.024 g.l<sup>-1</sup> and 0.0025 g.l<sup>-1</sup> butanol respectively. Transformation of crt, bcd, etfB, etfA, and bcd enabled *L. brevis* to form 0.3 g.l<sup>-1</sup> butanol on glucose media, using its own thiolase, aldehyde and adh genes.<sup>46,182</sup> These results suggested that the introduction of a clostridial fermentative pathway into a heterologous host does not allow efficient butanol production and requires much more metabolic engineering and optimization efforts. More detailed analyses of metabolic fluxes, energy and redox balance, the expression and activity characteristics of enzymes, and interactions between the host metabolism and heterologous butanol pathways need to be performed. Besides, bacteria other than *Clostridia*, are not capable of utilizing

diverse carbon substrates, including both C5 and C6 sugars, for economical butanol production. The two different strategies to enhance the butanol production in heterologous hosts are (a) to enhance the butanol forming flux by fine-tuning the expression levels or introducing an alternative enzymes that catalyzes the same reaction (b) alternative pathways leading to butanol formation or its key intermediates such as L-threonine, 2-ketobutyrate and 2-ketovalerate to form butanol in *E. coli*.<sup>184</sup>

### **9.8 Metabolic engineering for isopropanol/butanol/ethanol biosynthesis**

*Clostridium* metabolic engineering can also be accomplished to produce a mixture of IBE (2-propanol/butanol/ethanol) solvents instead of standard ABE. This mixture of solvents as such can be sold as chemicals or used to replace gasoline in internal combustion engines. Such genetically modified *Clostridium* strains could be used for industrial production of “green” chemicals and biofuels from renewable and sustainable resources such as lignocellulosic biomass. Since, *C. acetobutylicum* does not possess a secondary alcohol dehydrogenase (*adh*); it is unable to produce 2-propanol (isopropanol), from acetone. In order to modify the organism to produce 2-propanol the *adh* gene for 2-propanol dehydrogenase from *C. beijerinckii* NRRL B593 can be inserted into *C. acetobutylicum* DSM792.<sup>185-187</sup> The resulting modified *C. acetobutylicum* DSM792-pADH1 strain produced up to 14.3 g.l<sup>-1</sup> IBE solvents from standard glucose media and 5 g.l<sup>-1</sup> of IBE solvents from hydrolyzed spent liquor<sup>188</sup> from spruce chips. *C. acetobutylicum* PJC4BK (pIPA3-Cm2) lacking the butyrate kinase (*buk*) gene and containing synthetic acetone operon (*adc-ctfA-ctfB*) produced 20.4 g.l<sup>-1</sup> IBE solvents from CGM media supplemented with 80 g.l<sup>-1</sup> glucose.<sup>187</sup>

### **9.8. Mutation**

Random mutation has been performed by few researchers to alter the DNA sequence and/or regulation of the genes responsible for ABE production. Lin and Blaschek,<sup>189</sup> developed a SA-1 mutant strain from *C. acetobutylicum* ATCC 824 with serial enrichment of diluted *n*-butanol. The strain produced higher butanol and lower acetone titers with significantly enhanced butanol tolerance (121% higher) over the native strain. Besides, it also showed higher carbohydrate utilization rate and higher  $\alpha$ -amylase activity to prove its potential in utilization of waste cellulosic materials as feedstocks. Another mutated strain of *C. acetobutylicum* (MEMS-7) obtained after treatment with N-methyl-N-nitro-N-nitrosoguanidine and ethyl methane sulphonate and under UV exposure showed better potency in molasses. It was recognized as effective mutant showing 20% higher butanol yield than the parent strain.<sup>190</sup> The most significant achievement with mutation was the development of *C. beijerinckii* BA101 strain which showed highest butanol production capacity

so far reported (19–20 g.l<sup>-1</sup>).<sup>34,36,191,192</sup> To eliminate the tedious and expensive maintenance of anaerobic conditions a successful mutant of *E. coli* (which can grow aerobically) was recognized for the synthesis of 3-methyl-1-butanol (9.5 g.l<sup>-1</sup>).<sup>120,193</sup>

### 9.9. Systems biology

Systems biology is the study of systems of biological components, which may be molecules, cells, organisms or entire species. Understanding the complex cellular systems is essential for the construction of new phenotypes through metabolic engineering or synthetic biology. Traditional molecular biology methods are limited to a few metabolic engineering modifications that could be evaluated simultaneously, and hence it was difficult to get a systems perspective. The chimeric pathway from *C. acetobutylicum* using enzymes *Crt* and *AdhE2* was introduced in n-butanol pathway, from *Ralstonia eutropha* and *Treponema denticola* with *PhaA* and *Hbd* and *Ter* respectively. The overexpression of *aceEF* to increase the acetyl-CoA pool, from the metabolic system was evaluated and resulted in a 1500-fold increase in the 1-butanol titer from the initial 3 mg.l<sup>-1</sup> to 5 g.l<sup>-1</sup>.<sup>194,195</sup> NADH reducing pathways, such as, succinate, lactate, ethanol and acetate pathways were blocked by deletion of *frdBC*, *ldhA*, *adhE* and *pta*, respectively to produce 15 g.l<sup>-1</sup> n-butanol. An implementation of NADH drain in the form of n-butanol pathway composed of enzymes from *C. acetobutylicum* (*Hbd*, *Crt* and *AdhE2*) together with the highly active endogenous acetyl-CoA acyltransferase (*AtoB*) and an irreversible trans-enoyl-CoA reductase (*Ter*) from *T. denticola*.<sup>196</sup>

Although the *S. cerevisiae* seems to be an ideal n-butanol producing host organism, metabolic simulations suggested the lower butanol and propanol yields with it when compared to *E. coli* because of the limited flexibility of the central metabolism.<sup>197-199</sup> Industrial production of n-butanol with yeast targets to increase the acetyl-CoA pool by overexpression of the pyruvate dehydrogenase multienzyme complex (*lpdA*, *aceE*, *aceF*) from *E. coli*.<sup>200</sup>

## 10. Extremophiles in biofuel synthesis

Extremophiles refer to microorganisms, generally prokaryotic, which thrive in environmental conditions considered to be hostile to humans.<sup>201</sup> Temperature, pH salinity, pressure, radiation, light and water content are some of the parameters which contribute towards the extreme conditions. Several advantages are associated with the use of extremophiles in the industrial applications, in particular during the biofuel production. Extremophiles are robust in nature, produce stable

enzymes and are often able to tolerate stringent changes in the environmental conditions, such as pH and temperature. Majority of extremophiles used in biofuel production are from thermophilic sources, which can tolerate the fluctuations in pH, temperature and other environmental change attributes which offer a clear advantage in the development of a commercially viable process.<sup>201-203</sup> Moreover, thermophilic industrial fermentations are less susceptible to microbial contamination and require lower energy inputs as a result of the reduced cooling steps during the fermentation. Besides, the removal of any volatile products, which in turn minimizes the problem of product inhibition, is facilitated.<sup>204</sup>

Knowing the fact that most microorganisms are unable to grow at butanol concentrations above 2%; there are certain species of *Bacillus* and *Pseudomonas* that are able to tolerate butanol concentrations as high as 2.5– 7%.<sup>205-207</sup> *Pseudomonas* achieves high solvent tolerance by removal of solvent using efflux pumps and physico-chemical changes in their membrane lipids.<sup>206,207</sup> The *P. putida* S12 showed moderate tolerance to butanol, while other *P. putida* strains have been evolved to tolerate 6% w/v butanol.<sup>208,209</sup> Hence, the recent engineering of *P. putida* to produce butanol has been tried by few researchers.<sup>118</sup> The UK-based company Green Biologics ([www.greenbiologics.com](http://www.greenbiologics.com)) uses a mixture of thermophiles and thermostable enzymes for the production of butanol from waste biomass which is commercially sold as Butafuel™.<sup>204</sup> Recently, the citramalate synthase (*CimA*) from the extremophile *Methanococcus jannaschii* was found to increase the activity over a temperature range of 30 °C to 70 °C and overexpressed in *E. coli* for the production of both n-propanol and n-butanol.<sup>151,152,210</sup> The citramalate pathway directly converts pyruvate to 2-ketobutyrate bypassing threonine synthesis. Biotechnology company Gevo™ ([www.gevo.com](http://www.gevo.com)) adopted this technology to use *E. coli* for butanol production.

## 11. Solvent recovery techniques

Though many attempts have been made to improve butanol production, the concentrations of butanol in the fermentation broth are not yet satisfactory. The recovery of low concentrations of butanol by traditional distillation is energy intensive and hence economically not feasible.<sup>45</sup> For every 1 t of solvent, approximately 12 t of steam is required. Although the improvements can be made to the conventional distillation processes, the nonconventional methods are required to significantly reduce the energy and associated costs. Therefore, simultaneous fermentation and product removal techniques have been developed to reduce the cost of butanol recovery.<sup>41,96,211-214</sup> Some butanol recovery techniques are discussed in subsequent sections.

### **11.1. Adsorption**

Use of adsorbents that have high affinity for the removal of solvents from the fermentation broth is a feasible technique for *in situ* product recovery. The adsorbents have the advantages of ease of separation, nontoxicity and ease of regeneration/reuse. In this technique, butanol is first adsorbed by adsorbents from the fermentation broth and further desorbed by the heat treatment or displacers to yield concentrated butanol solutions. Activated carbon, silicalite, polymeric adsorbents such as polystyrene cross-linked resins, zeolite, and molecular sieves are few among numerous alcohol selective adsorbents. However, silicalites are most favoured adsorbents which possess zeolite-like structure and hydrophobic properties that can selectively adsorb small organic molecules like C1–C5 alcohols from dilute aqueous solutions.<sup>41,215</sup> Coupling of adsorptive removal of solvents with membrane filtration/centrifugal system overcomes the limitation of biofilm formation by cells from the fermentation broth. The energy requirement for butanol recovery by adsorption-desorption was calculated to be 1948 kcal.kg<sup>-1</sup> butanol, which is significantly smaller than the energy requirement for other techniques such as steam stripping distillation (5789 kcal.kg<sup>-1</sup> butanol), gas stripping (5220 kcal.kg<sup>-1</sup> butanol), and pervaporation (3295 kcal.kg<sup>-1</sup> butanol).

Other adsorbents, such as activated carbon and polymeric resins, suffer from the fouling by cells and other media components with adsorption of butyric and acetic acids, to reduce the solvent yield.<sup>216</sup> The modifications in adsorbent characteristics, such as increasing hydrophobicity and selectivity, are possible means of improving the feasibility of adsorptive recovery of butanol which overcomes the larger scale operation limitations.<sup>216-218</sup>

### **11.2. Liquid–liquid extraction**

The removal of butanol or ABE from fermentation broth by liquid–liquid extraction is an important technique. In this method, the fermentation broth is continuously contacted with a water–immiscible extractant that selectively extracts ABE from the broth, leaving all other components, such as nutrients and cell, in the broth. The choice of extractant is the most crucial aspect of an integrated liquid–liquid extraction fermentation process. The extractant used for the fermentation process should not be toxic to microorganisms and should have high selectivity for the ABE solvents (highest in particular for butanol). It also must have nearly zero partition coefficients for nutrients, substrates, and intermediates of fermentation. Unfortunately, the liquid-liquid extractants with high butanol distribution coefficients are toxic to the culture, while nontoxic liquid extractants have low butanol distribution coefficients. Evans and Wang,<sup>96</sup> used mixed extractants to balance both the

drawbacks. The mixed extractant that contained 20% decanol (high partition coefficient to butanol, but toxic) in oleyl alcohol (low partition coefficient to butanol, but non-toxic) enhanced butanol production by 72% under pH-controlled conditions.<sup>96,79</sup> This mixed extractant further reduced the viscosity when compared with the viscosity of single extractant. Some common toxic solvents used for blending with oleyl alcohol (highly viscous) are kerosene, benzyl benzoate, and decane.<sup>219</sup> The number of researchers<sup>220-222</sup> have reported the removal of butyric acid with extractants. The saturation of extractant with butyric acid prior to contacting with broth or to add supplemental acid directly to the broth, did not affect the butyric acid extraction. In addition, the extractant is also expected to have some advantageous features such as high interfacial tension with water that would assist its easy separation, low viscosity, negligible water solubility, high thermal stability, easy regeneration, biodegradability and high density difference, which would assist counter current operation. However, several problems are associated with liquid–liquid extraction, such as toxicity to the cells, the formation of an emulsion, loss of extraction solvent, and the accumulation of microbial cells at the extractant and fermentation broth interphase. The ABE solvents are recovered from the extractant using distillation.<sup>6</sup>

### ***11.3. Pervaporation***

Pervaporation allows the selective removal of volatile compounds from fermentation broth using a membrane. This membrane is selectively permeable to the solvents in the fermentation broth. Thus, when contacted with the fermentation broth, the ABE solvents in the broth first get solubilized into the membrane, followed by diffusion through the membrane and evaporation on the other side (or permeate side) at low pressure. These vapors can be recovered by either condensation or by using a sweep gas that removes the vapors. In this process, a phase change occurs from liquid to vapor. Concentration and vapor pressure gradient is used to allow one component to preferentially permeate across the membrane.<sup>89</sup> This mechanism by which volatile solvents are removed from the fermentation broth is known as solution–diffusion mechanism. As it is a selective removal process, the desired component requires a heat of vaporization at the feed temperature. The heat required for evaporation of solvents is taken from the broth which reduces the temperature of the broth. The efficacy of pervaporation is measured by two parameters: the selectivity (a measure of the selective removal of volatiles) and flux (the rate at which an organic/volatile passes through the membrane per m<sup>2</sup> membrane area).<sup>41</sup> The solvent selective (asymmetric) membranes available in the market are made up of poly (dimethyl siloxane), poly (methoxy siloxane), polytetrafluoroethylene, and silicone rubber as the skin of the membrane with supports made of polysulfone or polyacrylonitrile



Advantages of pervaporation include low energy consumption, entrainer is not required and low contamination frequency. However, the membrane needs to be regenerated at some point, because a “swelling” effect makes the membrane more permeable but less selective when material passes through the membrane.<sup>45</sup>

Ranjan and Moholkar<sup>6</sup> detailed the features of overall mass transfer through the membrane as (a) The mass flux through the membrane is inversely proportional to the thickness of the membrane. (b) The resistance of mass transfer in liquid phase is determined by the flow rate and viscosity of the medium and (c) The resistance to mass transfer is governed by pressure drop which leads to the reduction in concentration gradient and also the flux. In addition to these, temperature of fermentation broth is also a critical factor governing flux through the membrane. Every 10 °C rise in the temperature of the fermentation broth doubles the membrane flux.

#### ***11.4. Perstraction***

Perstraction operation essentially combines principles of pervaporation and liquid–liquid extraction in that it is a solvent extraction process combined with membrane extraction. The membrane contactor (preferably in the form of hollow fibers) provides surface area where the two immiscible phases (fermentation broth and the extractant) can exchange butanol. Since there is no direct contact between the two phases this process overcomes several pitfalls of the liquid–liquid extraction process such as solvent toxicity, emulsification, and cell aggregation at liquid–liquid interface. Besides, an independent control over the flow rate of broth and extractant is also advantageous. In this system, butanol diffuses preferentially across the membrane, while other components and fermentation intermediates (acetic and butyric acids) are retained in the aqueous phase.<sup>223</sup> Arrangement of loops of hollow fiber membranes in series for operation in counter current mode gives a true plug flow character to both phases and an effective removal of butanol. Overall mass transfer in this case is determined by the individual mass transfer coefficients on aqueous (fermentation broth) and organic (extractant) side as well as the mass transfer coefficient of the membrane. Although mass transfer coefficient of membrane is difficult to estimate, typical values of  $\sim 10^{-7}$  m.s<sup>-1</sup> can be assumed. The overall resistance to mass transfer is mostly contributed by the membrane itself (~86%). The increased diameter of the hollow fiber membrane, with concurrent rise in the volumetric flow rate reduces the mass transfer resistance which maintains the constant velocities through the fiber.<sup>224</sup> The membrane does, however, present a physical barrier that can limit the rate of butanol extraction.<sup>41</sup>

### ***11.5. Liquid membranes***

A promising solution to large mass transfer resistance offered by dense polymeric membranes is in terms of liquid membranes. This process requires just a small amount of solvent to cover the support membrane. The fluidity of the organic film leads to the higher diffusion coefficient and high fluxes of solvent. However, the stability of the film is the major problem. Matsumara and Kataoka<sup>225</sup> have reported pervaporation through liquid membrane supported with hydrophobic microporous polypropylene flat sheet wherein the stability of the membrane was found to depend on surface tension of feed solution. The principle components of the liquid membranes were oleyl alcohol, di-n-butyl phthalate, and tricresyl phosphate.

### ***11.6. Gas stripping***

The gas stripping technique involves removal of ABE solvents in the gas phase, followed by recovery of solvents through condensation. Gas stripping allows the selective removal of solvents from fermentation broth and uses no membranes which suffer from fouling and clogging. Gas stripping can be applied for *in situ* butanol recovery during the ABE fermentation.<sup>226-228</sup> The production of ABE is accompanied with the generation of gases (CO<sub>2</sub> and H<sub>2</sub>) which are being used to recover butanol during simultaneous fermentation and *in situ* recovery to make the process simpler and more economical.<sup>227,228</sup> The process is directed by vapor–liquid equilibrium. Oxygen-free nitrogen can also be sparged through the fermentation broth and subsequently cooled in a condenser. As the gas is bubbled through the fermentor, it captures the ABE which is further condensed and collected in a receiver vessel. The gas recovered after condensation can be recycled back for further cycles. A separate stripper can be used in some cases to strip off solvents and the stripper effluent can be then recycled back to the reactor.<sup>226</sup> Ezeji et al.<sup>71</sup> investigated the production of butanol in an integrated fed-batch fermentation-gas stripping product-recovery system using *C. beijerinckii* BA101, with H<sub>2</sub> and CO<sub>2</sub> as the carrier gases. They employed a 500 g.l<sup>-1</sup> concentrated glucose solution during fermentation, and achieved 13-fold more solvents (232.8 g.l<sup>-1</sup>) and fourfold higher productivity (1.16 g.l<sup>-1</sup>.h<sup>-1</sup>) in this integrated system than in the non-integrated system. Apart from simplicity of this process, other advantages such as clean volatile products, use of *in situ* gas for stripping and no mass transfer resistance makes it most suitable for application on a large scale.<sup>6</sup>

## 12. Energy consumption and wastewater generation

The energy consumption of the process is vital criteria when choosing the recovery technology for the higher adequacy apart from the simple operation, scalability, and cost. The recovery technologies (gas stripping, flash fermentation, vacuum fermentation, pervaporation) presumed the butanol concentrate to be in aqueous phase, which is compatible with the distillation system. Although the continuous fermentations have the undisputable advantage of enhanced productivity, these fermentation systems, when not accompanied by integrated product recovery, have the worst environmental performance in terms of wastewater footprint.<sup>229</sup> Pervaporation is the most favored technology as compared to other purification techniques according to the distillation duty and wastewater footprint criteria. The increase in butanol concentration from 12.81 to 105.35 g.l<sup>-1</sup> in the product stream with the reduction in the energy consumption of the distillation system of 65.8% (from 24.6 to 8.4 MJ.kg<sup>-1</sup> butanol) was accompanied by a substantial decrease in the wastewater footprint.<sup>230</sup> For pervaporation and gas stripping, the total energies (integrated product recovery technology and distillation) calculated by Qureshi et al.<sup>231</sup> are 13.8 and 21.8 MJ.kg<sup>-1</sup> butanol, respectively. These values support the analysis that pervaporation is very attractive from an energy efficiency viewpoint.<sup>229</sup>

## 13. Economics of biobutanol fermentation

The mass and energy yields determine the absolute, theoretical limits to the process economics. The theoretical mass and energy yield of ABE fermentation are 37% and 94% respectively, calculated on the basis of product ratio and energy combustions in the fermentation.<sup>214</sup> The substrate costs, accounting to approximately 60% of the total production cost, play a major role in the economics of ABE fermentation.<sup>232,233</sup> In order to reduce substrate costs in typical bulk-chemical fermentation processes,<sup>234</sup> the fermentation plant must be able to use a variety of substrates including those of low grade substrates such as lignocellulosic biomass, waste material from food and feed industries and waste from agricultural industries. Qureshi and Blaschek<sup>235</sup> published ABE butanol production costs at \$1.56 gal<sup>-1</sup> based on \$1.80 per bushel corn feedstock cost. If the feedstock is upraised to \$3.35 bushel<sup>-1</sup>, their estimated butanol cost would be \$2.10 gal<sup>-1</sup>. Although the economy of ABE fermentation is more sensitive to the price of the substrate than yield; it is estimated that butanol fermentation process will not be feasible if the yields are less than 25 % w/w.<sup>214</sup> Strain improvement is effective technique to improve the yield; however it has lower influence on the process economics as compared to other factors (mainly cost of substrate and product recovery costs). Interestingly, a capital cost of butanol fermentation was higher and production costs were

less than petrochemical production of butanol.<sup>236</sup> Batch fermentation processes have been found less economical than continuous processes as they demand additional sterilization steps for vessels, pipes and valves. However, the contamination problem in continuous fermentation is a crucial challenge. Product market is another significant parameter in the economics of butanol fermentation. The present sizeable market of ABE fermentation is still fascinating industrialists and researchers and it is expected that after adapting to biobutanol as a liquid fuel, the market demands will be high. By-products including acetone, ethanol, H<sub>2</sub>, and CO<sub>2</sub> can also contribute significantly to the butanol production economics.<sup>235</sup> It should be noted that butanol production itself is a cost-intensive methodology and recovery technology after the fermentation results in higher cost-intensive process. Improvement in butanol tolerance of strain and selective *in situ* solvent removal can enhance the fermentation time, productivity and other economic feasibility aspects of the process<sup>80,237</sup> which can be materialized in the economic feasibility of ABE fermentation.

Investment costs largely affect the economic assessment of ABE fermentation process. The large capital investment before the actual production starts has a huge impact on overall economics of the process. Besides, investment costs directly increase the total costs both as depreciation and for financing (interest and repayments). Hence, major reductions in the investment costs and/or delaying investments especially until the production starts will help to improve the process economics. Aspen Plus simulations have been used to obtain the material and energy balance data, size the specified equipment and to calculate cost structure. It was also used to calculate the fixed (labor and supplies) and variable (raw materials) operating costs of the plant.

The overall production cost data for non-existing or non-commercial biofuel processes (bioethanol, biobutanol) are less readily available and largely detailed with techno-economic models and evaluations. An identical economic assumption reveals that, the product yield on substrate of corn ethanol is almost twice as compared to corn butanol. However acetone, ethanol, and hydrogen co-products in corn butanol process contribute more significantly to the net production cost as compared to the corn ethanol process. This result in an overall production cost of butanol to be \$1.96 gal<sup>-1</sup> compared with \$1.53 gal<sup>-1</sup> of ethanol produced from corn.<sup>238</sup> Besides, the total project investment (TPI) is also doubled for the corn butanol process as compared to the corn ethanol. This is because of the low feedstock yield, and a more complicated separation of biobutanol mixtures and water from the fermentation beer with low solvent concentrations.

Marlatt and Data<sup>236</sup> have reported that with an improved strain tolerance for higher butanol concentrations and an increase in volumetric productivity by about 50%, the production

costs for biobutanol would be similar to the production costs for synthetic butanol. Moreover, Woods<sup>239</sup> found that if the final solvent concentration can be increased by up to 22-28 g.l<sup>-1</sup> and if the fermentation time of the batch fermentation of 40-60 hours can be maintained, the ABE fermentation should be industrially feasible. In order to reach the economic viability, the reductions in both conversion cost-intensity and in recovery cost-intensity were required. The process economics can further be increased with *in situ* product removal techniques.<sup>79</sup> The use of the two-stage continuous solvent-producing cultures with the immobilized biomass or biomass-retention achieves higher solvent productivities with improved substrate consumption and reduced the solvent toxicity problem.<sup>80</sup>

#### 14. Derivative markets for n-butanol

The US consumption of butanol is 740000 metric tons per year, which, at the current price of about \$0.90 per pound, represents a \$1.47B market. The predicted annual growth rate for butanol in the USA is 2.2%. Global consumption of butanol is in the order of 2.9 million metric tons, a \$5.7 billion market, with a predicted annual growth of 4.7%. The market for *n*-butanol is more than 10 times that of the isobutanol, for which the US and global consumption is reported to be 42000 and 509000 metric tons, respectively.<sup>240,241</sup> Recently, Mascari,<sup>242</sup> reviewed the technical description of the range of commercial products accessible from *n*-butanol, derived from renewable sources (biobutanol). Primary derivatives of *n*-butanol in which the oxygen functionality of the molecule is retained (butyraldehyde and butyric acid) and *n*-butanol itself have mainstream applications in the solvent, polymer, fuel oxygenate, and/or specialty chemical markets. The butanol dehydration products (butenes and butadiene) present additional opportunities in the hydrocarbon fuel and synthetic elastomers markets (Figure 2).

#### 15. Conclusions

Since the 19<sup>th</sup> century, many investigators have noted the applicability of biobutanol an advanced biofuel in several industries. Butanol from biomass is a better renewable fuel for replacing gasoline than ethanol. As evident from the foregoing review biobutanol is in the process of its commercialization and studies on the development of further economic process are continuing rapidly. None of the starch and sugar containing crops can make fermentation economically feasible on the basis of the current market needs and scenario. Consequently, cheaper agricultural wastes (lignocellulosic materials) and other industrial wastes may be suitable for the economical ABE fermentation. The feedstock cost comprises a very large fraction of the overall production cost, and

hence selection of feedstock is an important parameter that needs to be taken into consideration for the process economy.

The complex metabolism of *C. acetobutylicum* has been elaborated from different perspectives. Studies regarding solvent, particularly butanol production in *C. acetobutylicum* focused on different aspects such as the development of continuous fermentation technology, improvements in strain tolerance towards butanol, understanding the physiological relationships between acidogenic and solventogenic phases, and genetic modifications. In spite of several attempts in improving the industrial strains by mutation and genetic manipulations, none of the efforts were successful. However, efforts are still needed in identifying better butanol tolerance strains. Development of novel strains may be more challenging as butanol has direct effect on cell membrane ceasing molecular activity of the cell. Despite substantial progress in the development of strains for the production of advanced biofuels there is still need for further improvements to meet the industrial requirements in terms of yield, titer and productivity. Thus, the further engineering is required, not only to improve the yield and productivity, but also other important parameters such as tolerance towards the product of interest and the ability to use complex feedstocks from biomass. Simultaneously, recombinant DNA technology in non-clostridial organisms was unable to improve the yields over native clostridial bacteria. Hence, focus should now be diverted on developing more suitable genetic tools for the better polycistronic expression in host microorganism. 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. The development of process technologies with efficient butanol recovery techniques may be easily achievable and crucial in economics. New recovery processes such as gas stripping and advanced membrane separation may contribute to the economic feasibility in future. Multidirectional endeavors are constrained in achieving the economical usefulness of microbiological butanol synthesis, which will be competitive with petrochemical based butanol. Most of the literature reports on various facets of ABE fermentation are still on laboratory scale, with very few studies on bench or pilot scale. There is an urgent need to assess viability of the process designs on economical pilot scale. One of the biggest challenges in the future will be the scaling-up process in a cost-effective manner.

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### **Figure legends**

**Figure 1** – The metabolic pathway of acetone–butanol–ethanol (ABE) fermentation by *C. acetobutylicum*.<sup>23, 41,116</sup>

**Figure 2** – Derivative markets for butanol.<sup>242</sup>

**Table 1:** Comparison of properties of different alternate liquid fuels.<sup>6</sup>

Fuel	Gasoline	<i>n</i> -butanol	isobutanol	Ethanol	Methanol	Diesel	Biodiesel
Energy density (MJ/l)	32	29	29	19.6	16	39	31 – 33
Vapor pressure (kPa) at 20 °C	0.7 – 207	0.53	1.17	7.58	12.8	less than 0.07	less than 0.07
Vapor pressure of mixture with gasoline (kPa)	53.8 – 103.4	44.1	46.9	138	800	–	–
Air:Fuel ratio	14.6	11.2	–	9.0	6.5	–	–
Heat of vaporization (MJ.kg <sup>-1</sup> )	0.36	0.43	–	0.92	1.16	–	–
Research octane number	91 – 99	96	–	129	129 – 134	–	–
Motor octane number	81 – 89	78	112	102	97 – 104	–	–
Cetane number	–	–	–	54	–	45	49 – 58
Freezing temperature (°C)	less than – 60	– 89.5	– 108	– 114.5	– 97.6	– 30 to – 9.9	7.5 to – 16
Hygroscopicity	Low	Low	Low	High	High	Very low	Very low
Compatibility with existing infrastructure	Yes	Yes	Yes	No	No	Yes	No

**Table 2:** Different feedstocks and hydrolysis methods used along with maximum solvents and productivities achieved.<sup>46</sup>

Hydrolysis method	Substrate	Yield (g.g <sup>-1</sup> ) / Productivity (g.l <sup>-1</sup> .h <sup>-1</sup> )	Total ABE (g.l <sup>-1</sup> )	Reference
None	Waste packing for peanuts	0.37/0.20	21.7	47
	Date fruit	0.41/0.09	6.2	243
Dilute sulfuric acid + enzyme				
	Wheat straw	0.60 / 0.42	25	244
	Wheat straw	0.41/0.31	21.42	36,37
	Barley straw	0.43 /0.39	26.64	38
	Corn stover	0.44 / 0.31	26.27	39
	Switchgrass	0.39 / 0.17	14.61	39
	Rice straw	1.04 <sup>a</sup> /0.017 <sup>a</sup>	3.0 <sup>a</sup>	109
Dilute sulfuric acid				
	Corn fiber	0.39 /0.10	9.3	34
	Wheat bran	0.32 /0.16	11.8	50
	Corn fiber	0.36/0.14	10.1	61
	Corn fiber	0.32/0.14	10.6	62
Enzyme				
	Palm oil mill effluent + sago starch	0.40/0.10	14.38	48
	Cassava bagasse	0.39 / 0.62	33.87	72
	Corn cob residue	0.32/ 0.33	16	245
	Sago pith residue	0.30/ 0.12	8.84	246
Ammonium fiber expansion + enzyme	Dried distillers' grains and soluble (DDGS)	0.34 /0.14	10.4	247
Dilute HCl + enzyme	Rice bran and defatted rice bran	0.31/0.26	12.24	49
HCl	Jatropha seed cake	0.45/ 0.26	18.4	54
Hot water extraction + sulfuric acid	Sugar maple wood	0.22 /0.15	11.0	53
SO <sub>2</sub> -ethanol-water	SO <sub>2</sub> -ethanol-water (SEW) spent wood liquor	0.20 / 0.09	8.79	51

<sup>a</sup> butanol yield and productivity

**Table 3:** Summary of recent continuous fermentation reports for ABE production.<sup>46</sup>

	Yield (g.g <sup>-1</sup> ) / Productivity (g.l <sup>-1</sup> .h <sup>-1</sup> )	Total ABE (g.l <sup>-1</sup> )	References
<b>Single stage reactor with free cell cultivation</b>			
	0.11 /0.37	6.85	75
	0.23/0.89	8.85	68
	0.28 /0.47	5.26	74
	--/0.30	10.12	29
	0.24/0.22 <sup>a</sup>	7.10 <sup>a</sup>	8
	--/1.85	9.27	76
<b>Single stage reactor with cell recycle and bleeding</b>			
	--/8.3	9.2	77
	--/7.55	12.9	76
	--/3.32 <sup>a</sup>	4.26 <sup>a</sup>	78
<b>Immobilized cell reactor</b>			
Brick	0.19 /1.21	11.28	75
Wood pulp	0.29/ 5.52	9.20	74
PVA <sup>b</sup>	0.44/0.40 <sup>a</sup>	13.4 <sup>a</sup>	8
Wood pulp	0.27 /4.86	7.59	51
Wood pulp	0.28 /12.14	8.09	66
Fibrous matrix	0.42/4.6 <sup>a</sup>	5.11 <sup>a</sup>	248
Corn stalk	0.32 /5.06	5.10	249
Brick	0.30 / 2.01	6.29	88
Ceramic D-21 beads	--/ 1.0	7.73	250
Tygon <sup>TM</sup> rings	0.28 /5.0	5.19	67
Tygon <sup>TM</sup> rings	0.26/2.66 <sup>a</sup>	4.93 <sup>a</sup>	91
Brick	0.38 /15.8	7.9	87
Brick	-- / 16.1	8.10	89
<b>Two stage immobilized cell reactor</b>			
None	0.32 /0.84	7.63	74
Sugarcane baggase	0.35/2.47	12.37	79
Wood pulp	0.38/10.85	13.60	80

where, <sup>a</sup> butanol <sup>b</sup> polyvinyl alcohol

Table 4. The challenges and solutions for ABE fermentation<sup>251</sup>

Challenges	Solutions
High feedstock costs increase the operational cost	Transition towards cheaper and sustainable feedstocks such as agricultural wastes and residues
Low butanol titres increase the recovery costs, reduce sugar loadings, increases feedstock costs and increase water usage	Develop improved microbes with improved solvent titres and/or develop <i>in situ</i> product removal techniques to reduce product toxicity effects
Low volumetric solvent productivities increase capital and operating costs	Develop continuous fermentation processes that reduce down time and increase volumetric productivity
Conventional distillation technique for solvent recovery is energy intensive and relatively expensive	Develop low energy methods for solvent recovery and purification. Recovery can also be improved by improving the solvent titre
High water usage is not sustainable and increases the cost of effluent treatment.	Recycle process water back to the fermentation

Table 5. The list of key enzymes of the butanol synthetic pathway in *C. acetobutylicum*.<sup>116</sup>

Pathway	Key enzyme	ab.	Gene	EC no.	Mr (kDa)	
Lactate synthetic pathway	Phosphate acetyltransferase	PTA	pta	2.3.1.8	36.2	
Butyrate synthetic pathway	Acetate kinase	AK	ack	2.7.2.1	44.3	
	Phosphate butyryltransferase	PTB	ptb	2.3.1.19	264	
Butanol synthetic pathway	Butyrate kinase	BK	buk	2.7.2.7	85	
	Acetyl-CoA acetyltransferase	THL	thiL	2.3.1.9	41	
	$\beta$ -hydroxybutyryl-CoA dehydrogenase	BHBD	hbd	1.1.1.35	30.5	
	Enoyl-CoA hydratase (crotonase)	CRT	crt	4.2.1.17	158	
	Butyryl-CoA dehydrogenase	BCD	bcdetfBetfA	1.3.99.2	33	
	Butyraldehyde dehydrogenase	BAD	aad	1.2.1.57	56	
	Butanol dehydrogenase	BDH	bdh AB	1.1.1.1	42	
	Acetone synthetic pathway	Acetoacetate decarboxylase	AADC	adc	4.1.1.4	28
		CoA-transferase	CoAT	CtfA/B	2.8.3.9	93
	Ethanol synthetic pathway	Acetaldehyde dehydrogenase	ALDH	aad	1.2.1.10	96
NAD(P)H alcohol dehydrogenase		ADH	adh	1.1.1.2	44	

