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# Technological Barriers in Biobutanol Production

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***Abstract***

The depletion of fossil fuels coupled with ever-increasing pollution has driven people's attention towards renewable energy sources such as biodiesel and bioalcohols. Among biofuels, biobutanol has emerged as a potential biofuel as a substitute for gasoline. The production of biobutanol comprises different tedious tasks and is hindered by a number of obstacles. Traditionally, biobutanol production by traditional ABE fermentation utilizing *Clostridium acetobutylicum* species suffers from limitations such as low butanol yields, solvent toxicity issues in the solventogenic phase to bacterial cell wall and cost of pretreatment of lignocellulosic biomass. Hence, the present review puts forth the discussion on existing limitations along with feasible solutions such as metabolic engineering approaches and utilization of solvent-tolerant microbial strains for an enhanced biobutanol production.

***Keywords:*** Biobutanol, ABE (acetone-butanol-ethanol) fermentation, oxo-synthesis, aldol condensation strain modification

## 7.1 Introduction

Biofuel is the energy derived from animal material (animal manure, animal fats) and renewable plants (algae, lignin) with the potential of mitigating the upcoming issues of fossil fuel depletion and existing greenhouse gas emissions. Owing to the positive attributes of renewable biofuels,

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overwhelming research is going on towards production technologies of biodiesel, bioethanol and biobutanol. The economics of biofuels production is mostly dependent on the cost of the fermentation substrate. The biomass feedstock cost and biomass availability are the two main decisive factors in establishing a production technology for alternative fuels [1].

Currently, bioethanol and biodiesel are used in the transportation sector. Biobutanol is one of the option, investigated recently and emerging as a potential source of biodiesel and bioethanol. The sources for biobutanol production mainly comprise lignocellulosic waste biomass and non-food agricultural products, which is considered a second-generation biofuel system. Although the biotechnological production of biobutanol is a more complex process than bioethanol, the high energy content associated with the lesser water miscibility, vapour pressure and corrosiveness are the favorable factors towards the ongoing biobutanol production. Moreover, the potential of *Clostridium* bacteria to ferment various substrates such as municipal wastes is also an added advantage to the biobutanol production. Different engineered strains also came into the race of efficient biobutanol production [2].

Different industries are putting maximum effort into biobutanol production on an industrial scale by focusing mainly on novel alternative methods for existing traditional ABE (Acetone, Butanol and Ethanol) fermentation. *Gevo* and *Butamax* are the two leading technology developers of butanol. *Gevo* commenced with the world's first commercial-scale biobutanol production with a capacity of 18 MGPY and targeted to produce 50,000 to 100,000 gallons per month of isobutanol in upcoming years. *Butamax* in conjunction with *Fagen* Inc. set up a large-scale commercial production of biobutanol via retrofit of ethanol plants through the patented *Butamax* technology. India-based Laxmi Organic, Industries, in collaboration with Green Biologics, England, built a commercial-scale plant of 1,000 metric tons of butanol per year and began production from 2010 onwards. The facility would use sugarcane as a feedstock, and a combination of thermophilic organisms and thermostable enzymes to break the biomass down into butanol. Cobalt Biofuels in Mountain View, California, USA, had raised $25 million in equity to continue pursuing its goal of commercializing biobutanol production (http://www.biofuelstp.eu/butanol.html).

## 7.2 Production Technologies of Biobutanol

Butanol could be produced biologically as well as chemically. In chemical processes it is produced through oxo synthesis (through syngas reacts

with propylene) and aldol condensation. In biological process, anaerobic bacteria-based fermentation approach is used to produce acetone and ethanol. The advantages and disadvantages of biological and physicochemical methods for biobutanol production are tabulated in Table 7.1.

Cellulose and hemicelluloses also serve as substrates for biobutanol production with the aid of *Clostridium* sp. and other cellulolytic enzymes. Various pretreatment techniques such as utilization of alkaline peroxidases, steam explosion, hydrothermal techniques, and organic acids could be used to utilize the lignocellulosic substrate effectively. After pretreatment, detoxification is the subsequent step which is done through the utilization of activated charcoal, overliming, electrodialysis, and membrane extraction based detoxification methods. The pretreated and detoxified

**Table 7.1** Advantages and disadvantages of biological and physico-chemical method for the production of biobutanol.

| Method | Advantages | Disadvantages |
|---|---|---|
| Biological method | • Renewable source of fuel (feedstock) | • High feedstock cost significantly increases operating cost<br>• Low butanol titre increase recovery cost. Low titres also increases sugar loading and water usage<br>• Solvent recovery using distillation is energy intensive and expensive<br>• Low butanol yield increases feedstock cost. |
| Physico-chemical | • Requires only one step for producing n-butanol from ethanol.<br>• Relatively high yield | • Catalysts used in the process are costly |

The sequence steps for formation of acetyl-CoA and its utilization for further fermentation intermediates are depicted in Figure 7.1

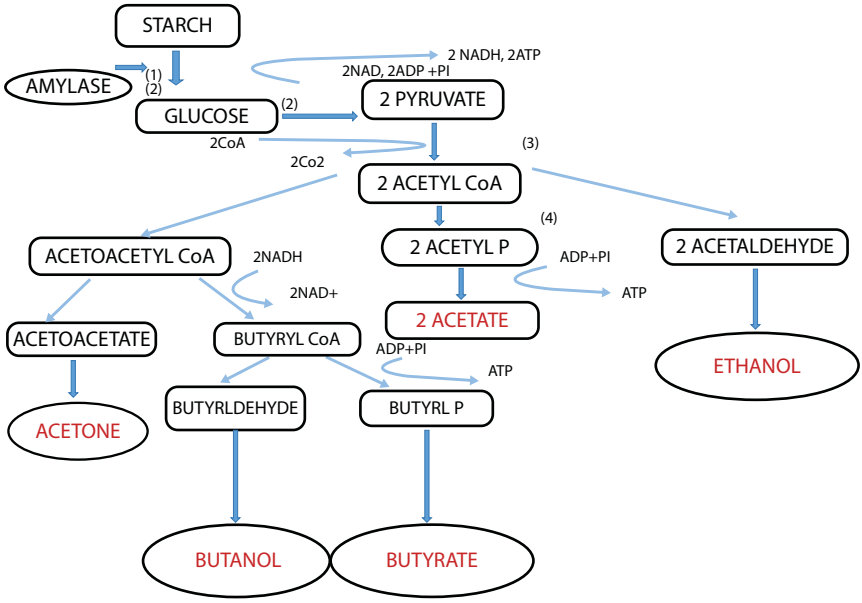


**Figure 7.1** Biobutanol production from starch.

substrate is fermented finally through the utilization of microbial strains which convert polysaccharides to different sugars which are subsequently converted to pyruvate by glycolysis.

The formed pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase complex (PDC), which in turn act as a common originator of all fermentation intermediate [1].

The fermentation process is divided into two phases, namely acidogenesis and solventogenesis. In acidogenesis, glucose in substrate feed stream is converted to butyric acid and acetic acid by the action of *Clostridium tyrobutyricum*. The product streams are then circulated and passed through a series of heat exchangers, where they are sterilized at 250°F and then cooled back to 98.6°F before entering into the next phase of fermentation, solventogenesis [3]. During solventogenesis, the cells enter into the stationary phase where acids are converting to solvents by *Clostridia acetobutylicum*. The product stream obtained is then pumped to a centrifuge where separated solids are sent for Dried Distillers Grain (DDGS) for drying and liquids are sent to the separations process [4]. Butanol must be recovered from the fermentation broth by processes such as adsorption, using immobilization techniques with the help of membrane reactors and gas stripping.

An overview of the biobutanol production from lignocellulosics is shown in Figure 7.2.

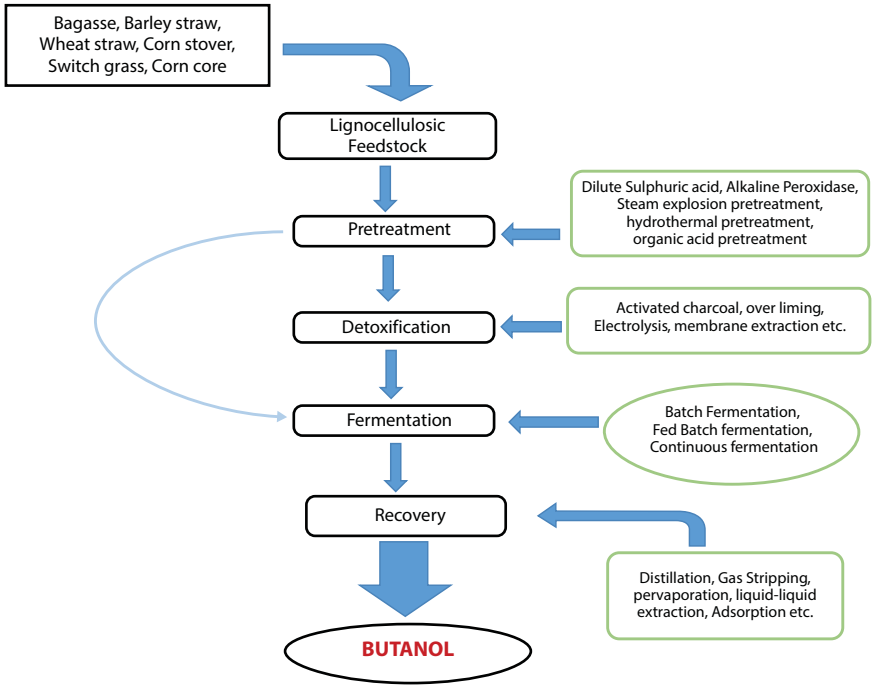


**Figure 7.2** Butanol production from lignocellulosic feedstocks.

## 7.3 Lignocellulosic Materials for Bio-Butanol Production

Lignocellulosic materials used for biobutanol production face many challenges such as cost of the raw material, pretreatment and hydrolysis strategies, low butanol tolerance of fermenting strain which will affect the yield and productivity as well as downstream processing of biobutanol. There exists another barrier also which hinders its production, the inconsistency in biomass availability throughout the year. Lignin, ash, protein and waxes are also present in trace amounts whereas relative proportions of cellulose, hemicellulose, lignin are critical factors in the determination of optimum energy conversion route but other contents can lead to a diminution of theoretical butanol yield [5]. Among different problems encountered during utilization of cellulosic or lignocellulosic material for hydrolysates production, chemical by-products generation results in ceasing of cell growth as well as fermentation. Biologically these lignocellulosic materials are difficult to hydrolyze. Moreover, the significant amount of waste produced during the hydrolytic process also adds to the economy of the process [6].

Recent research has shown that the fermentation of any polysaccharides needs to use an additional nutritional supplement. Lee *et al.* [7], reported the use of different supplements such as $KH_2PO_4$, $K_2HPO_4$, ammonium acetate, para-aminobenzoic acid, thiamin, biotin, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, NaCl, and yeast extract for biobutanol production. Pretreatment is a critical and limiting step which has a predominant effect on overall biobutanol production thus has to be optimized. While using wheat bran as a substrate for biobutanol production by *C. beijerinckii* species, Liu and Qureshi [8], used a combination of pretreatments where wheat bran was treated with sulfuric acid at high temperature followed by neutralization with calcium hydroxide. This procedure raised the cost of the butanol produced considerably, but the solution to this problem found by using a cheap raw material for production which decreased the cost. Combination of pretreatments like 1M HCl with high temperature for 2 hours or enzymatic hydrolysis (using α-amylase and β-amylase) was utilized for cassava flour and resulted in 23.98 and 13.78 $g.L^{-1}$ biobutanol production using enzymatic and acid hydrolysis, respectively [9]. In another study, biobutanol (12.0 g/L) was produced from wheat straw using enzyme mix of cellulose, β-glucosidase and xylanase with the process conditions of pH 5.0, 45°C for 72 h and 80 rpm. To hydrolyze the raw materials, use of other alternative mechanical and physicochemical technologies was also reported and those alternatives include microwave-assisted pretreatment processes, steam explosion, ozonolysis, oxidative delignification and pulsed-electric-field.

Qureshi *et al.* [10], believed that barley straw could be used for butanol production. However, the barley straw showed the presence of few inhibitors which interfered with the production yield of butanol and hence pretreatment step using lime (called as over liming) was carried out to achieve an efficient fermentation. Consequently, this pretreatment step resulted in higher butanol production when compared to the yield with glucose as a substrate. Al-Shorgani *et al.* [11], also reported the formation of inhibitors during the acid pretreatment of cellulosic raw material (rice bran and de-oiled rice bran). Similarly, other studies utilized over liming pretreatment and subsequent extraction of inhibitors with a nonionic polymeric adsorbent. These procedures remarkably improved the biobutanol production and yield. Qureshi *et al.* [6], concluded that the formation of fermentation inhibitors after hydrolysis of cellulosic raw material is substrate and pretreatment dependent. Thus, it is necessary for a specific study to be carried out for each substrate and treatment. Several authors are of the opinion that the industrial feasibility of biobutanol production can only increase if a low-cost substrate can be employed [12, 13]. Diversification of

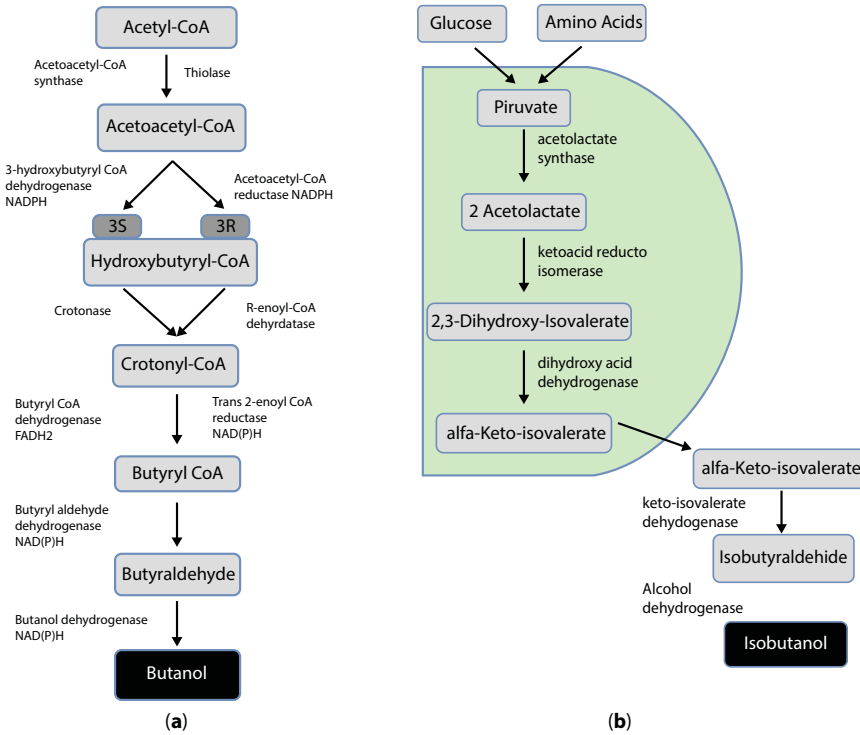


**Figure 7.3** (a) Metabolic representation of CoA-dependent pathway for production butanol, (b) Isobutanol synthesis pathway.

substrates and the use of regional crops such as molasses, starch or cellulose for butanol production is one of the approaches to tackle the high-cost of the fermented substrate.

The metabolic representation of butanol (through CoA-dependent pathway) and isobutanol production is depicted under Figure 7.3.

## 7.4 Natural Producers of Biobutanol

Clostridium sp. is the primary/natural producers involved in the production of biobutanol through the CoA-dependent pathway. The various species utilized for the production include *C. acetobutylicum, C. saccharoperbutylacetonicum, C. beijerinckii, C. saccharoacetobutylicum, C. aurantibutyricum, C. cadaveris, C. sporogenes, C. pasteurianum, and C. tetanomorphum* [14].

In Clostridia sp., acetyl-CoA produced from various carbon sources such as lactose and sugars fermentation through acidogenesis and solventogenesis. Acidogenesis produce acetic and butyric acid whereas solventogenesis produce acetone, butanol and ethanol. To make the process more economical, it is necessary to find a suitable method which shifts the metabolism from acidogenesis to solventogenesis. The primary barrier in the biobutanol production attributed to the adverse effects of the compounds or products obtained after solventogenesis on the microbial cell membrane. Butanol manifests chaotropic effects on the bacterial cell membrane due to which even its concentration as little as 2%, compromises bacterial survival. Therefore it becomes a prime concern to get rid of the toxicity of the solvent products. Deviation of metabolic intermediates from biosynthesis of aliphatic amino acid in yeast is also one of the natural metabolic pathways for biobutanol production. In some yeast species, fusel alcohols are one of the fermentation by-products [15]. In the Ehrlich pathway, keto acids are decarboxylated to produce aldehydes which in turn to alcohols. Since keto acids are the amino-acids precursors such as n-propanol, isobutanol and n-butanol are the precursors of isoleucine, valine and non-valine, respectively. Since isobutanol has a better octane number, therefore, it is preferred over n-butanol for industrial use [16]. The industrial application of isobutanol is hampered by the meager intrinsic production in yeast, but this route of biobutanol production diverts the

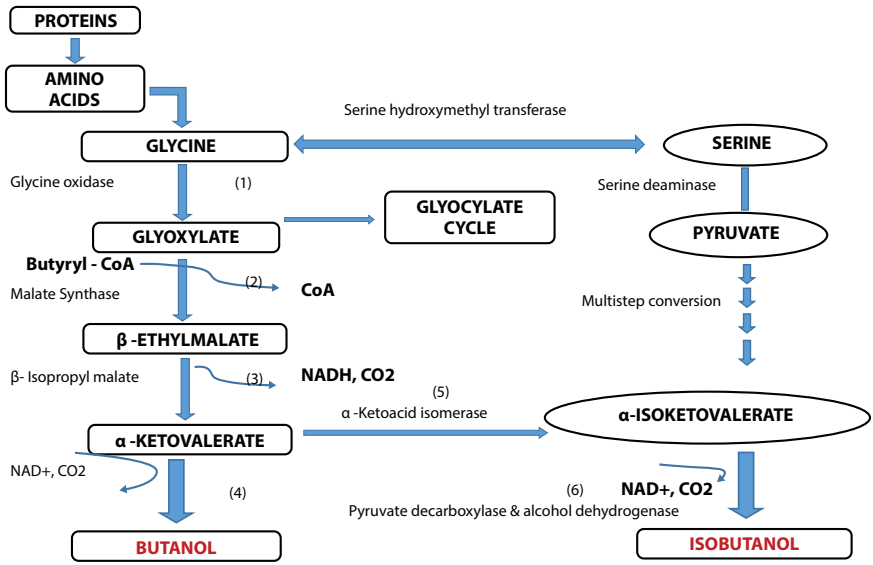


**Figure 7.4** Production of butanol and isobutanol by using glycine as a substrate.

various pathway intermediates towards amino acid biosynthesis which usually is not possible naturally [8].

The steps involved in production of butanol and isobutanol are summarized in Figure 7.4.

## 7.5 Main Obstacles in the Biobutanol Production

The problems associated with biobutanol production were increased cost of feedstock, low butanol titer which further increases butanol recovery and downstream processing, reduced sugar loadings and increased water usage which increases the capital expense. High water usage is not sustainable which further increases the cost of production and it is energy intensive and relatively expensive, low butanol tolerance of the microbes as this much amount of alcohol destroys their cell wall [17].

### 7.5.1 Approaches to Overcome the Obstacles

To overcome the obstacles, metabolic engineering is one of the alternatives that could adapt for modified strains by overexpressing the butanol production genes such as BCS operon related genes and *add*, *bdh*. Overexpression of grosESL gene lead to improved strain tolerance and increase in butanol titer. Recently, Global transcription machinery engineering (gTME) is another promising approach to enhance biobutanol production. If there is any alteration in the transcription factor, there is a scope for gTME to change the metabolic strength and direction. The gTME system has been proved as an efficient solution to improve substrate utilization and product tolerance [18].

## 7.6 Engineered Pathways towards a Better Solventogenic Producer

### 7.6.1 Engineered Pathways in Bacteria

Mutagenesis is gradually becoming a method of choice to improve *C. acetobutylicum*. Several attempts are made in this direction since mutagenesis has helped in improving yield, tolerance to butanol, and sugar source utilization. To increase the efficiency of production, many species of Clostridium have been engineered so that they become capable of utilizing some other carbon sources (liquefied corn flour, glycerol and a mixture

of hydrogen and carbon monoxide) [19]. Acetone has to be removed to improve fuel alcohols in the ABE process which can be done by inactivating *adc* gene which codes for acetoacetate decarboxylase necessary for acetone synthesis [20].

Metabolic engineering is another approach which is used to obtain isopropanol from acetone. A mixture of isopropanol, butanol and ethanol (IBE) produced through engineering of *C. acetobutylicum* by overexpressing the dehydrogenase gene of *C. beijerinckii* in *C. acetobutylicum*. The modified strain is capable to produce more than 99% of fuel alcohol with a negligible amount of acetone. Metabolic engineering helps in improving metabolic fluxes of wild strain to improve yield [21]. The metabolic engineered solventogenic *Clostridia* is able to ferment starchy and molasses towards biobutanol production. Gaseous substrates also serve as carbon substrates for engineered acetogenic *Clostridia* strain; hence there is no competence with the nutritional feedstock as substrate. The major genes involved in the butanol synthesis pathway in *C. acetobutylicum* are *thlA*, *hbd*, *crt, bcd, adhE,* and *bdhA*, which codes for the thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase, butanol/butyraldehyde dehydrogenase, and butanol dehydrogenase enzymes, respectively. The *Clostridium* butanol pathway genes were introduced into other fast-growing bacteria such as *E. coli,* the engineered bacteria shown butanol toxicity tolerance and is able to metabolize alternative substrates [22]. *Pseudomonas putida* and *Bacillus subtilis* are another bacterial species which can serve as hosts with butanol toxicity tolerance through efflux pumps. *Lactobacillus brevis*, which has a high tolerance to butanol, and can digest C5 and C6 substrates, has also been used.

Another major hindrance to butanol production is the intrinsic kinetic characteristics and cofactor specify of all enzymes that occur naturally in the bacterial pathway. Synthetic pathways are a solution to this limitation which can be made by combining enzymes from different organisms into a synthetic butanol pathway expressed in *E. coli.* To manifest this, the modified strain of *E. coli* is transfected with the vectors carrying genes of two enzymes: 2-keto-acid decarboxylase of low substrate specificity along with an alcohol dehydrogenase. This manipulation resulted in high yields of isobutanol [23]. The 2-keto-acid pathway of *Corynebacterium glutamicum* has also been engineered, taking advantage of the high amino-acid production characteristic of the bacteria. The pretreatment of lignocellulosic materials is another costly affair which can overcome by using *Clostridium cellulolyticum* which can naturally digest lignocellulose. Gaida *et al*. [24], reported for the first time the metabolic engineering of *C. cellulolyticum* where the bacterium was engineered with the CoA-dependent pathway

to produce *n*-butanol directly from crystalline cellulose using NADH as a cofactor.

When natural producers of butanol are intended to use, then the best strategies are those based on the selection of strains, fermentation conditions and best product recovery techniques that may avoid toxicity or enhance the fuel concentration by avoiding the production of acetone or isopropanol. The introduction of the metabolic pathway for ABE fermentation in other bacteria possesses several advantages such as non-requirement of anaerobic fermentations with fast-growing or butanol toxicity tolerance abilities. A two-fold increase in the concentration of fuel has been observed in comparison to natural strains of *Clostridium acetobutylicum* when the non-natural producer bacteria engineered with the 2-keto-acid pathway. A metabolic approach is a promising area in biobutanol production to attain higher yields [25].

### 7.6.2 Engineered Pathways in Yeast

Although bacterial systems such as *E. coli* have been known to show more efficient systems for biobutanol production, the engineered *Sacchromyces cerevisae* system has also been proved as quite an efficient system to produce biobutanol. Production of biobutanol through yeast systems have some positive attributes such as adaptability in various application sectors and have greater reproducibility. Moreover, the bacterial-based biobutanol system suffered from disadvantages like the requirement of strictly anaerobic conditions, complex downstream processing, narrow pH requirements and infection prone due to phages and viruses. However, these limitations can be overcome by utilizing yeast-based systems especially *S. cerevisae*-based system.

*S. cerevisiae* have the enzyme machinery to synthesize isobutanol by a two-compartment *Ehrlich* pathway. In this pathway, keto-isovalerate, which is an intermediary product of valine synthesis in mitochondria, is catabolised into isobutanol. Genes *ILV2*, *ILV5*, *ILV3*, encode for the enzymes acetolactyl synthase, ketoacid reductasoisomerase, and dihydroxy acid dehydratase respectively, are majorly involved in isobutanol synthesis. Overexpression of these genes leads to enhanced biobutanol production. The above genes mentioned above are present in mitochondria, so this system can replace in cytosol which is done by overexpression of cytosolic form encoded by ILV2, ILV5, ILV3, ARO10 with deletion of mitochondrial ILV2 associated genes. To increase dihydroxyacid dehydratase encoded by ILV3 or to improve the cofactor associated with it, many additional genetic manipulations were researched to enhance other by-products besides

ethanol. With the combination of these strategies, more than 80% of the maximum isobutanol theoretical yield achieved. Codon usage linked with cytosolic pathway than using from associated mitochondrial systems has too proved to be a useful technique. In one of the alternatives, the catabolic pathway of amino acids which resulted from protein hydrolysis was utilized [26]. Many researchers have reported the use of glycine as the substrate for the synthesis of butanol and isobutanol in *S. cerevisiae* through glyoxylate pathway. Various genetic engineering approaches have been utilized to change the metabolic activities in *S. cerevisiae.* These include reconstruction of the 1-butanol biosynthetic pathway through increased flux towards cytosolic acetyl-CoA by means of the transformation with a plasmid expressing the genes (encode for *ADH2* (alcohol dehydrogenase), *ALD6* (acetaldehyde dehydrogenase), *ACS1/ACS2* (acetyl-CoA synthetase), and *ERG10* (acetyl-CoA acetyltransferase) enzymes). An endogenous 1-butanol pathway in *S. cerevisiae* which was dependent on catabolism of threonine, was proposed, discovered, characterized and engineered by Si *et al.* [27]. Various strategies have been introduced to achieve the higher 1-butanol titer in S.cerevisiae which include the overexpression of the Ehrlich pathway enzymes, and single gene deletion adh1delta (which cause a deficiency of alcohol dehydrogenase) was proposed [27].

Matsuda *et al.* [28], reported enhanced biobutanol yield by utilizing *S. cerevisiae*-based metabolic engineering approach. The strains engineered in such a way that they lacked the genes of pyruvate dehydrogenase complex LPD1 to reduce the competition between pyruvate supply for isobutanol and acetyl Co-A biosynthesis in mitochondria. The genes of the enzyme transhydrogenase-like shunts (converts NADH to NADPH) were overexpressed to resolve the cofactor imbalance. Endogenously, α-ketobutyrate is a key intermediate in n-butanol production pathway. Usually, α-ketobutyrate is synthesized from catabolism of threonine and alternately it can be synthesized from acetyl Co-A via Citramalate synthase (Cim A) (Figure 7.5). Through this approach, a maximum theoretical n-butanol yield of 411 mg/g glucose was achieved. The maximum theoretical n-butanol yield reported a value of 411 mg/g glucose through this approach. Further research has been done to improve the n-butanol production by cloning and overexpressing the CimA genes from *Methanococcus jannaschii*, *Leptospira interrogans*, and *Geobacter sulfurreducens* with previously confirmed α-ketobutyrate utilizing genes (mLEU1, mLEU4, mLEU2, and LEU5). The strain *LI* with overexpressed *Cim A* from *L. interrogans*, showed high improved n-butanol titer of 349 mg/L. The yield obtained was far higher than the strain *THRm* solely

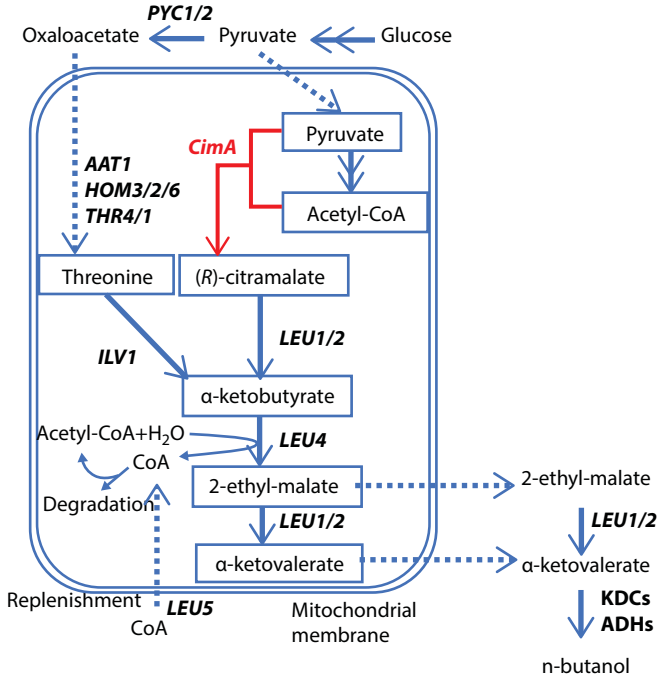


**Figure 7.5** The synergistic pathway for *n*-Butanol production via the endogenous threonine pathway and an introduced CimA mediated pathway (Atsumi *et al.* [29]). Single and double arrows represent single and multiple enzymatic steps, respectively; red arrows represent heterologous pathways; overexpressed genes are marked with red colour. KDCs: keto-acid decarboxylases; ADHs: alcohol dehydrogenases.

overexpressing the threonine pathway. The strain LI, uses two metabolic pathways to synthesize butanol, i.e., the endogenous threonine pathway and the introduced citramalate pathway. This synergistic path leads to a maximum theoretical yield of 411 mg/g glucose n-butanol [30].

## 7.7 *In-Situ* Butanol Recovery Integrated with Batch and Fed-Batch Fermentation

There were many problems and disadvantages associated with the traditional fed-batch system. One of such problems was increased solvent toxicity during the biphasic batch butanol fermentation. *In-situ* recovery process integrated with fed-batch culture is the ideal setup to overcome the

solvent toxicity using silicone and oleyl alcohol as pre-extraction solvents along with the utilization of silicon membrane [31]. The butanol extraction process through diffusion was carried out using silicone membrane which limits the diffusion of acetone and acids that subsequently results in higher biobutanol yield. Gas stripping was another batch process method where a semi-synthetic medium with embedded lactose which was entirely fermented by *C. Acetobutylicum*. The integration of gas stripping setup with the liquid-liquid extraction process results in higher utilization of lactose which led to the enhanced biobutanol production [32].

The lower yields of butanol production through traditional fermentation process were mainly due to the accumulation of butanol in the fermentation broth. Butanol removal from the fermentation broth and its separation was a costlier process. To avoid such problems addition of butyrate as a precursor to the system is the ideal approach to trigger the metabolic pathway towards the butanol production and further promising results were attained through the integration with *in-situ* butanol removal via vacuum membrane distillation [33] which alleviates the butanol toxicity issues. This integration is a very effective method to enhance the butanol yield with higher economic feasibility with easier downstream processing**.** Another approach for enhanced biobutanol titers was opting for adsorbent based fermentation along with renewable carrier [34]. Alkali-treated steam explodes straw showed as a suitable carrier for adsorbent fermentation of biobutanol. The adsorption of ABE solvent on substrate facilitates the increased bacterial concentration alleviation of the end product inhibition with improved biobutanol production features.

## 7.8 Future Prospects

The future of the industrial process for the production of biobutanol can improve by utilization of novel omics-based approaches and sophisticated downstream processes. *Clostridium acetobutylicum* is the most intensively studied solvent-producing species involved in biobutanol production. A thorough investigation of existing metabolic pathway towards biobutanol production helps in pinpointing the responsible genes, and its overexpression in different hosts. Advances in continuous culture technology, integrated fermentation processes, *in situ* product removal and improved downstream processing can also provide new approaches to improve the substrate utilization that also provides a future direction of economic biobutanol production by reducing butanol toxicity and process stream volumes towards the enhanced bioreactor performance.

## 7.9 Conclusions

Biobutanol is also a superior biofuel and in a very long term has been shown to meet the demands for the next-generation biofuels. Production of biofuels is even considered as a useful means to slow down carbon dioxide emissions; this is also a green industry with ecological benefits to humankind, and that could also contribute to decreasing the present concerns over global climate change. Production of biobutanol through Clostridium sp. in higher volumes is a viable strategy to compete with the chemical-based butanol production. Recent advances in bioethanol plants could be cost-effectively retro-fitted for biobutanol production requiring relatively minor changes to fermentation. The lower titers associated with the biobutanol production with the Clostridium sp. can resolve by utilizing the novel engineering, metabolic approaches coupled with integrated recovery processes.

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