MICROBIAL PRODUCTION OF 1-BUTANOL – RECENT ADVANCES AND FUTURE PROSPECTS
(Review)

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ABSTRACT

Butanol is an alcohol that can be obtained by fermentation of organic feedstocks sugars. Biobutanol is soon expected to surpass ethanol as a renewable fuel due to the intensive scientific efforts aimed at substrates broadening, process parameters improvement and genetic manipulations of butanol producing bacteria. This review focuses on the biotechnological synthesis of 1-butanol by natural isolates (Clostridium acetobutylicum, C. beijerinkii) and recombinant producers (E. coli, C. cellulovorans, C. cellulolyticum, C. thermocellum, S. cerevisiae, etc.). Concerning the economical butanol fermentation, the contemporary utilization of cheap renewable and non-food substrates (lignocellulosic materials, glycerol, syngas, and algae) is described. Different strategies for advanced 1-butanol production are considered. Along with media optimizations, immobilization, and co-culturing, the exploration of the strains' tolerance to butanol, and their genetic improvement is widely applied. The recombinants obtained in case of naturally producing strains application as hosts possess enhanced metabolic flux, shifted towards higher butanol titer, yield, and productivity. Another approach refers to the insertion of “butanol pathway” genes in a non-solvent producing host, which is resistant to high butanol concentrations. The present review analyses and summarizes the problems and the future prospects of the development of economically feasible bio-processes for butanol production.

Keywords: butanol, ABE fermentation, Clostridium, fuel.

INTRODUCTION

The increasing demand for alternative fuels employment due to natural resources depletion has forced scientists to explore different strategies in this direction. To date, ethanol is classified as the most proper biofuel due to its simple production through existing fermentation technologies. However, ethanol is not the best choice as a biofuel because of its high vapor pressure and its hygroscopicity. Butanol is probably the most promising among the possible conventional fuel alternatives because of its undoubtable advantages over the other known biofuels and the fact that it can completely replace gasoline with no engine modifications required (Table 1).

Significant efforts are made to achieve an economical microbial production of butanol in view of its advantages over other fuels. It is superior to ethanol in many regards including a high energy content and low vapor pressure as it is less volatile [1]. Butanol’s low hygroscopicity defines its low degree of corrosivity to engines. It can be directly applied with no engines modifications; it blends with gasoline at any ratios and can be easily transported. Table 1 shows the fuel properties of butanol compared to those of ethanol and gasoline. Combined with gasoline, higher alcohols like butanol can contribute to minimization of the global warming effect as smoke gases formation is thus decreased. The alcohols OH-group increases their oxygen content which leads to decreased smoke gases release [2, 3].
Butanol (butyl alcohol) is a five carbon alcohol with four isomeric forms: 1–butanol (n–butanol), 2–butanol, tert–butanol and isobutanol. It is considered that 1–butanol and isobutanol have the properties providing their use as biofuels. Butanol is an important precursor in plastic and polymers production [4]. It is used as a solvent in the production of antibiotics, hormones, vitamins, in the textile industry [5], and as a perfume base in the cosmetics [6].

Butanol can be obtained from fossil fuels by chemical synthesis (as petro – butanol) or from biomass by microbial fermentation (as biobutanol). The chemical synthesis is unprofitable and unfounded because of the over–consumption of petroleum–based products.

As a fermentation metabolite, butanol is mentioned by Pasteur in 1861. Its industrial production on the ground of starch and sugars starts a century ago [7]. Its biological production follows the acetone–butanol–ethanol (ABE) fermentation pathway. The products ratio usually is 3:6:1 [8]. A major disadvantage of ABE fermentation is butanol’s low final concentration (< 20 g L⁻¹) and its low yield (0.28 - 0.33 g g⁻¹) as the other fermentation products are obtained in higher concentrations. The high cost of the substrate is another problem as feed-stocks are usually used. The cost for its separation from the rest of the reaction products is high. Also, butanol is toxic to the microorganism–producers [9].

**MICROORGANISMS - PRODUCERS OF 1-BUTANOL**

Butanol production in nature is extensively performed with the participation of *Clostridium* genus members. In 1916 *C. acetobutylicum* is isolated by Chaim Weizmann who discovers that it produces butanol and ethanol from starch. During WWII the strain is used for acetone synthesis, which is a precursor for cordite production [10]. In 1980, the strain *C. acetobutylicum* that performs ABE fermentation in ratio 7 (acetone) : 2 (butanol) : 1 (ethanol) is isolated [11]. However, *C. acetobutylicum* is Gram-positive spore–forming anaerobic bacterium that can survive just a couple of hours in aerobic atmosphere. This strictly anaerobic physiology impedes its application [10].

*C. acetobutylicum* conducts a two–phase fermentation process. Acetic and butyric acids, as well as H₂ and CO₂ are formed during the first phase. Usually this phase coincides with the bacteria exponential growth, when ATP is formed. Then a solvent formation phase follows when the acids are consumed. Thus acetone, butanol and ethanol are synthesized [12].

As *C. acetobutylicum* produces the highest concentration of butanol among the known microorganisms, it is the most studied bacterial species in relation to optimizing the fermentation process and the butanol final concentration. Tsai et al. study the effect of the media components and pH on butanol fermentation by *C. acetobutylicum*. They add ammonium acetate, acetate buffer and calcium carbonate in order to control the media pH. They find out that the addition of 6 g L⁻¹ of ammonium acetate increases butanol concentration.

<table>
<thead>
<tr>
<th>Property</th>
<th>Butanol</th>
<th>Ethanol</th>
<th>Gasoline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy density (MJ L⁻¹)</td>
<td>29.2</td>
<td>21.2</td>
<td>32.5</td>
</tr>
<tr>
<td>Water solubility (mL 100 mL⁻¹)</td>
<td>9.1</td>
<td>miscible</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>6.4</td>
<td>18 – 22</td>
<td>6.4 – 11.9</td>
</tr>
<tr>
<td>Oxygen content (% mass)</td>
<td>21.6</td>
<td>34.8</td>
<td></td>
</tr>
<tr>
<td>Octane rate</td>
<td>96</td>
<td>112 - 129</td>
<td>91 - 99</td>
</tr>
</tbody>
</table>

**Table 1. Comparison of the fuel properties of butanol, ethanol and gasoline.**
from 2 g L\(^{-1}\) to 3.6 g L\(^{-1}\). The role of the acetate buffer is similar as it increases butanol concentration from 2 g L\(^{-1}\) to 9.8 g L\(^{-1}\). Calcium carbonate affects pH control. It sustains pH above 4.8 when added in a concentration higher than 8 g L\(^{-1}\). According to the authors, the optimal pH for the process is 4.5. The solvent synthesis begins when pH reaches a certain value above which acids are re-assimilated and solvents are produced [14]. On the other hand, the way of pH control conductance is also of importance [15].

Table 2 lists the natural and engineered microbial producers of butanol.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>Gene overexpressed</th>
<th>Strain engineering</th>
<th>Titer</th>
<th>Yield</th>
<th>Fermentation reactor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. acetobutylicum</td>
<td>Cassava</td>
<td>pfkA, pykA</td>
<td>Genome shuffling</td>
<td>20.1 g L(^{-1})</td>
<td>0.23 g g(^{-1})</td>
<td>Bioreactor, batch</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>adhE(_{1/2}), Atdh, Apta</td>
<td>-</td>
<td>15 g L(^{-1})</td>
<td>0.36 g g(^{-1})</td>
<td>Bioreactor, batch</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>AtpA, Apta</td>
<td>-</td>
<td>18.9 g L(^{-1})</td>
<td>0.29 g g(^{-1})</td>
<td>Bioreactor, batch</td>
<td>[18]</td>
</tr>
<tr>
<td>C. tyrobutyricum</td>
<td>Sugarcane juice</td>
<td>sscB, sscA, sscK</td>
<td>-</td>
<td>16 g L(^{-1})</td>
<td>0.24 g g(^{-1})</td>
<td>Bioreactor, batch</td>
<td>[19]</td>
</tr>
<tr>
<td>C. pasteurianum</td>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>12 g L(^{-1})</td>
<td>0.34 mol mol(^{-1})</td>
<td>Bioreactor, batch</td>
<td>[20]</td>
</tr>
<tr>
<td>C. carboxidovorans</td>
<td>CO</td>
<td>-</td>
<td>-</td>
<td>2.66 g L(^{-1})</td>
<td>-</td>
<td>Bioreactor, batch</td>
<td>[21]</td>
</tr>
<tr>
<td>C. cellulovorans</td>
<td>Cellulose</td>
<td>adhE2</td>
<td>-</td>
<td>1.42 g L(^{-1})</td>
<td>0.39 g g(^{-1})</td>
<td>Bottle</td>
<td>[22]</td>
</tr>
<tr>
<td>C. beijerinckii BA101</td>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>18.6 g L(^{-1})</td>
<td>0.32 g g(^{-1})</td>
<td>Bioreactor, batch</td>
<td>[23]</td>
</tr>
<tr>
<td>E. coli</td>
<td>Glucose</td>
<td>atoB, bbd, cti, adhE2, fah</td>
<td>AtdhE2, AtdhA, AtdhBC, Apta</td>
<td>15 g L(^{-1})</td>
<td>0.36 g g(^{-1})</td>
<td>Tube</td>
<td>[24]</td>
</tr>
<tr>
<td>E. coli</td>
<td>Glucose</td>
<td>cimA3.7, lenA,BCD, kivD, ADH2</td>
<td>-</td>
<td>0.524 g L(^{-1})</td>
<td>-</td>
<td>Flask</td>
<td>[25]</td>
</tr>
<tr>
<td>E. coli</td>
<td>Glucose</td>
<td>thrA(_{p}^{BC}, sbvA, lenA,BCD, kivD, ADH2</td>
<td>-</td>
<td>1 g L(^{-1})</td>
<td>-</td>
<td>Flask</td>
<td>[26]</td>
</tr>
<tr>
<td>E. coli BuT-8</td>
<td>Glucose</td>
<td>Aadh, Aadh1, AtdhE1, Atdh, Atdh2, AphaA, AtdhE, AtdhA, Apta, AfdA</td>
<td>6.1 g L(^{-1})</td>
<td>0.31 g g(^{-1})</td>
<td>Bioreactor, batch</td>
<td>[27]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>yqeF, fucO</td>
<td>AarcA, AadhE1, Apta, AfdA, AfdBC, AfdA, Afer(Con), crp</td>
<td>2.2 g L(^{-1})</td>
<td>0.28 g g(^{-1})</td>
<td>Flask</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>atoB, bbd, cti, bcd, etfAB, adhE</td>
<td>AadhE1, AadhE2, AtdhBC, AtdhA, Apta</td>
<td>0.552 g L(^{-1})</td>
<td>-</td>
<td>Flask</td>
<td>[29]</td>
</tr>
<tr>
<td>S. cervisiae</td>
<td>Glucose</td>
<td>CoaA, adhE(<em>{2/3}), AADH1.5, AadhE(</em>{2/3})</td>
<td>-</td>
<td>130 mg L(^{-1})</td>
<td>0.071 g g(^{-1})</td>
<td>Batch</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>Agrp1, Agrp2, Amt, Atdh, Adh, AdhB, Afer(Con), crp</td>
<td>-</td>
<td>14.1 g L(^{-1})</td>
<td>-</td>
<td>Flask</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>thl, bbd, cti, bcd, etfAB, adhE2</td>
<td>Aadh, ermR</td>
<td>1.05 g L(^{-1})</td>
<td>0.10 g g(^{-1})</td>
<td>Tube</td>
<td>[32]</td>
</tr>
<tr>
<td>T. saccharolyticum</td>
<td>Xylose</td>
<td>thl, bbd, cti, bcd, etfAB, adhE2</td>
<td>-</td>
<td>2.5 mg L(^{-1})</td>
<td>-</td>
<td>Vial</td>
<td>[33]</td>
</tr>
<tr>
<td>P. putida</td>
<td>Glycerol</td>
<td>thl, bbd, cti, bcd, etfAB, adhE2</td>
<td>-</td>
<td>0.122 g L(^{-1})</td>
<td>-</td>
<td>Flask</td>
<td>[34]</td>
</tr>
<tr>
<td>L. brevis</td>
<td>Glucose</td>
<td>thl, bbd, cti, bcd, etfAB, adhE2</td>
<td>-</td>
<td>0.300 g L(^{-1})</td>
<td>-</td>
<td>Vial</td>
<td>[35]</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Glycerol</td>
<td>thl, bbd, cti, bcd, etfAB, adhE2</td>
<td>-</td>
<td>24 mg L(^{-1})</td>
<td>-</td>
<td>Flask</td>
<td>[36]</td>
</tr>
<tr>
<td>S. elongatus PCC7942</td>
<td>CO(_{2})</td>
<td>atoB, bbd, cti, ter, adhE2</td>
<td>-</td>
<td>15 mg L(^{-1})</td>
<td>-</td>
<td>Tube</td>
<td>[37]</td>
</tr>
<tr>
<td>C. acetobutylicum/B. ceras</td>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>11.2 g L(^{-1})</td>
<td>-</td>
<td>Bioreactor, batch</td>
<td>[38]</td>
</tr>
<tr>
<td>C. beijerinckii/C. tyrobutyricum</td>
<td>Cassava starch</td>
<td>-</td>
<td>-</td>
<td>13.26 g L(^{-1})</td>
<td>0.42 g g(^{-1})</td>
<td>Batch</td>
<td>[39]</td>
</tr>
</tbody>
</table>
SUBSTRATES FOR BUTANOL PRODUCTION

The price of raw materials for fermentations constitutes about ¾ of the total fermentation cost [40]. It is important to use cheap renewable non-food raw materials aiming to decrease the substrate expenses. However, mostly glucose and starch-based substrates are used to date for ABE fermentation [2].

Different raw materials can be used for butanol production. Butanol producing clostridia are able to utilize a wide spectrum of substrates - hexoses, pentoses, syngas, and glycerol [7]. The ability of engineered cellulitic C. cellulovorans, C. cellulolyticum and C. thermocellum to convert directly cellulose to butanol is reported [41]. These species have cellulosomes for degrading and hydrolyzing crystalline cellulose and hemicelluloses. Higashide et al. introduce C. cellulolyticum to the isobutanol metabolic pathway obtaining 0.66 g L\(^{-1}\) isobutanol from cellulose in 7 - 9 days. C. cellulovorans produces butyric acid as a main product on the ground of different carbon sources like glucose, xylose, cellulose, hemicelluloses, celllobiose. Small amounts of acetic, lactic and formic acids are formed as by-products. Thus, this bacterium is a promising host for butanol production from cellulosic biomass [42]. According to Tamaru et al. the high butyrate/acetate ratio (5:1 - 6:1) suggests the existence of a favorable metabolic pathway from cellulose to butyryl-CoA. Therefore, only one heterologous gene (aad/adhE) is required to obtain butanol from cellulose as a native metabolic pathway exists from cellulose to butyryl-CoA using C. cellulovorans (Fig. 2) [43].

Glycerol is an attractive opportunity for obtaining valuable metabolites as 1,3-propanediol, ethanol and biopolymers. It is synthesized as a by-product in the biodiesel industry. However, the naturally butanol producing microorganisms (C. acetobutlicum, C. beijerinckii, C. saccharobutlicum) can not utilize glycerol as a sole carbon source. This is possible if only a glycerol–glucose mixture can be used [45]. C. pasteurianum is the most studied microorganism capable of utilizing glycerol as a carbon source. CO\(_2\), H\(_2\), butanol, 1,3-propanediol, acetic acid, lactic acid, and butyric acid are its fermentation products. Acetone is not formed. Sarchami et al. optimize the fermentation process using C. pasteurianum DSM 525. They study the effects of the inoculum age, the cellular density, the initial pH and the temperature. Optimal results are obtained in case of an inoculum age of 16 h, a cellular density 0.4 g L\(^{-1}\)CDW, an initial pH value of 6.8 and temperature of 30°C. Thus 0.34 mol butanol/mol glycerol (starting with 50 g L\(^{-1}\) glycerol) are produced [21] with the application of this optimal model. Recently, another strain able to convert glycerol to butanol has been isolated from sediments. C. tetanomorphum GT6 produces 11.5 g L\(^{-1}\) butanol for 72 h [46].

The lignocellulosic biomass is another possible substrate for butanol production. It consists mainly of cellulose, hemicelluloses and lignin. It contains a major part of the pentoses (mainly D-xylose and L-arabinose), which is about 30 % of the total sugar content of the lignocellulosic hydrolysate [47]. Butanol producing clostridia are able to convert pentoses to butanol as a sole carbon source. On the other hand, pentose metabolism is repressed in presence of glucose as it is the preferred carbon source when compared with xylose and arabinose. The metabolic regulation of pentose utilization in clostridia is extensively explored [48]. An over-expression of some of the genes related to the carbon metabolism (ccpA, talA) and the pentose–phosphate pathway (tal, tkl, rpe, rpi) is provoked [49] for pentose utilization acceleration. Thus, a strain capable of simultaneous fermentation of hexoses and pentoses as substrates for solvents production is constructed.

Syngas is also a promising substrate for butanol fermentation. It represents a cheap gas mixture of H\(_2\), CO and CO\(_2\). It can be obtained from different sources like natural gas, coal, and biomass [50]. C. glycolicum [51], C. ljungdahlii [52], C. autoethanogenum [53], and Acetobacterium woodii [54] are acetogenic organisms that are able to utilize it as a carbon and energy source. These bacteria produce solvents and organic acids via Wood–Ljungdahl pathway, where CO or CO\(_2\) are converted to acetic acid. Bruant et al. [55] report the strain C. carboxidovorans that synthesizes 8.91 mmol butanol per CO mol consumed.

Although syngas has the advantages to be used as an eventual substrate for solvents production, the fermentation process with the acetogenic bacteria participation has still to be optimized to provide a controlled culture media [56].

The scientists are lately focusing their attention on algae utilization. It is an attractive raw material due to its low cost. It is not a feedstock; it does not require agricultural lands, fertilizers and fresh water for cultivation [57]. Marine algae are brown, red and green and are characterized by their high carbohydrate and low lignin content. The experimental data show that butanol can be produced by algal hydrolysates [58] although the yields
achieved are low. The main disadvantage of the microalgal employment is that the major part of the microorganisms is not able to utilize the alginites. The latter can be rapidly assimilated by Sphingomonas sp. A1 using alginate-lyase enzymes [59]. Wargacki et al. [57] construct an engineered strain E. coli by cloning a 36 kb DNA fragment from the brown microalgae Vibrio splendidus, responsible for alginate metabolism. Wang et al. [60] use Chlorella vulgaris JSC-6 pretreated with 1 % NaOH and 3 % H₂SO₄. The high level of glucose utilization (97.5 %) evidences that the hydrolysate does not contain inhibitors of ABE fermentation. 13.1 g L⁻¹ butanol with a yield of 0.58 mol mol⁻¹ sugar and productivity of 0.66 g L h⁻¹ is reached.

STRATEGIES FOR BUTANOL PRODUCTION ENHANCEMENT

Metal ions impact

The impact of Zn²⁺ on fructose and xylose assimilation as well as on butanol synthesis is investigated. Wu et al. [61] report that the addition of 0.001 g L⁻¹ ZnSO₄.7H₂O to the media including fructose as a sole carbon source results in 12.8 g L⁻¹ butanol with productivity 0.089 g L h⁻¹, compared to the fermentation in absence of Zn²⁺ (4.5 g L⁻¹ butanol and productivity 0.028 g L h⁻¹). Zn²⁺ ions have also a positive impact in experiments with xylose as a carbon source - 8.3 g L⁻¹ butanol is produced with a productivity increased by 31.7 %. The fermentations with fructose/glucose (4:1) and xylose/glucose (1:2) mixtures result in an increase of butanol final concentration and productivity - by 130.2 % and 8.5 % in the first case, while by 203.4 % and 18.4 % in the second one. Zn²⁺ ions affect probably the regulatory mechanisms responsible for sugar transport and metabolism of Clostridium acetobutylicum.

Nitrogen and carbon sources impact

The importance of glucose concentration, butyric acid addition, carbon and nitrogen ratios for ABE fermentation is reported. Al–Shorgani et al. [62] conduct batch-processes with C. acetobutylicum YM1. They reach 13.87 g L⁻¹ butanol at optimal conditions. Glucose initial concentration is 50 g L⁻¹, while the nitrogen sources ratio (tryptone, yeast extract, ammonium acetate) refers to 6:2:3. They find also that the increase of the initial glucose concentration above 50 g L⁻¹ leads to diminished butanol production. Different concentrations of butyric acid (which is a precursor of ABE fermentation) are added to the medium. The highest butanol concentration is obtained in case of butyric acid addition of 87 g L⁻¹.

Immobilization

Aiming to increase the butanol yield, Börner et al. [63] apply a new immobilization technique. Cells of C. acetobutylicum DSM 792 form a macroporous aggregate through cryogelation and simultaneous crosslinking with activated polyethyleneimin (PEI) and polyvinyl alcohol (PVA). The cryogel is a highly porous elastic structure with walls consisting of densely packed crosslinked cells. The immobilization increases the cell’s vitality. Unlike the free cells, the immobilized one demonstrate 2.7 fold increase of butanol concentration and yield - 18.2 g L⁻¹ and 0.41 g g⁻¹, respectively. The immobilized cells have another advantage - they can be used from 3 to 5 times.

Chen et al. [64] fix cells of C. acetobutylicum CG-MCC 5234 on pretreated cotton towel carriers. The substrates used refer to glucose and xylose. The immobilized bacterial cells synthesize 10 g L⁻¹ butanol with a yield of 0.141 g g⁻¹. The butanol obtained is 11.1 g L⁻¹, while its yield is 0.190 g g⁻¹ in case of using a mixed sugar media (30 g L⁻¹ glucose + 30 g L⁻¹ xylose). These values are by 28.3 % higher than those found in free cells processes.

Co-culturing

Another approach to the increase of the concentration of the synthesized butanol refers to the co-culturing of two different butanol producing strains. Free and immobilized cells of C. beijerinckii and C. tyrobutyricum are cultivated [65] in media containing glucose, cassava starch and cane molasses. The highest values are reached in experiments with cassava starch, i.e. 6.66 g L⁻¹ butanol, a yield of 0.18 g g⁻¹, and a productivity of 0.96 g L h⁻¹. According to the authors, this type of co-culturing can be applied in industry. The same strains are used by Zhang et al. [39]. They combine immobilized cells of C. beijerinckii with C. tyrobutyricum. Sugar cane wastes pretreated with diluted H₂SO₄ are the substrates used. Thus, 6.78 g L⁻¹ isopropanol and 12.33 g L⁻¹ butanol are obtained.

The main obstacle in the course of the work with C. acetobutylicum refers to their high sensibility to oxygen. In this regard Wu et al. [66] co-culture C. acetobutylicum and Bacillus cereus strain at microaerophilic conditions. The role of B. cereus is to deplete oxygen and to provide favorable conditions for C. acetobutylicum. It is found that the ratio between the inoculated cultures is of im-
importance. Butanol concentration of 11 g L\(^{-1}\) is reached at the optimal ratio of 5 % \(C.\ acetobutylicum\): 0.5 % \(B.\ cereus\). The total solvents concentration is 18.1 g L\(^{-1}\).

Another disadvantage of working with \(C.\ acetobutylicum\) is connected with the bacteria low tolerance to organic solvents. It is considered that the limitation of 16 g L\(^{-1}\) - 20 g L\(^{-1}\) of total solvents concentration is due to butanol toxicity [67]. It is known [68] that butanol penetrates the cell membrane and destructs the hydrogen bonds between the lipids. This leads to disruption of the cell membrane and hence to cellular death [68].

**Genetic improvement of strains naturally producing 1-butanol**

Genetic engineering is widely applied to strains manipulated for obtaining the desired products. In case of \(C.\ acetobutylicum\), genetic manipulations are focused mainly on increasing butanol titer, yield and productivity. Experiments are concentrated at the genes responsible for acids forming (\(pta,\ ack,\ ptb,\ buk\)) and those concerning solvents production (\(adc,\ adhE,\ ctfAB\)) (Fig 1). This aims at the direct metabolic flow to synthesize butanol and to eliminate acetone and ethanol [18, 69, 70]. High butanol titer (16.7 g L\(^{-1}\)) is achieved by inactivation of the butyrate–kinase gene (\(buk\)) [71]. The inactivation of \(pta\) and \(buk\) with concomitant over-expression of \(adhE\) (encoding aldehyde/alcohol dehydrogenase) contributes to an increase by 60 % of the final butanol titer (18.9 g L\(^{-1}\)) and 154 % of the yield (0.29 g g\(^{-1}\)) [18]. The over-expression of the genes \(aad\) and \(thl\) (encoding acetaldehyde dehydrogenase and thiolase, respectively) leads to a decrease of the accumulation of acetone, acetic and butyric acid and hence, to a considerable increase of alcohols production (mainly ethanol) [72]. Some functional transcription regulators like \(solR,\ spo0A,\ groESL,\ grpE\) and \(htpG\) are examined aiming butanol titer improvement. The inactivation of putative transcriptional repressor gene (\(solR\)) results in 17.8 g L\(^{-1}\) butanol concentration [73].

![Metabolic pathway of Clostridium acetobutylicum for ABE fermentation from glucose](image)

**Fig. 1.** Metabolic pathway of \(Clostridium\ acetobutylicum\) for ABE fermentation from glucose [5]. Genes and enzymes: \(ldh\) - lactate dehydrogenase, \(pf or\) - pyruvate - ferredoxin:oxidoreductase, \(ack\) - acetate kinase, \(pta\) - phosphotransacetylase, \(thl\) - thiolase, \(adc\) - acetoacetate decarboxylase, \(ctfAB\) - acetate/butyrate:CoA transferase, \(hbd\) - 3-hydroxybutyryl-CoA, \(crt\) - crotonase, \(bcd\) - butyryl-CoA dehydrogenase, \(buk\) - butyrate kinase, \(ptb\) - phosphotransbutyrylase, 1 - acetaldehyde dehydrogenase, 2 - ethanol dehydrogenase, 3 - butyraldehyde dehydrogenase, 4 - butanol dehydrogenase.
Under certain conditions of ABE fermentation the cells do not switch from acids production to solvents accumulation. This constitutes a common problem. In this regard, Lu [74] achieves an increased re-assimilation of acetic and butyric acid and therefore, higher butanol concentration in presence of \textit{C. beijerinckii} by over-expression of CoA-transferase (\textit{ctfAB}) and aldehyde/alcohol dehydrogenase (\textit{aad}). The over-expression of the genes encoding the enzymes phosphofructokinase (\textit{pfkA}) and pyruvate kinase (\textit{pykA}) in \textit{C. acetobutylicum} 824 contributes to the higher butanol titer. This is due to the increased levels of ATP and NADH in bacterial cells. In contrast to the wild type, higher concentrations of butanol and ethanol are reached - 29.4 % and 85 %, respectively. In a fed–batch process with a glucose substrate 28.02 g L$^{-1}$ of butanol are produced [17].

**Genetic manipulations of non–solvents producing microbial species**

Experiments concerning strains which do not synthesize butanol naturally, but are capable of growing in presence of higher concentrations of solvents are carried out. Genetic engineering approaches manipulating such strains find application as they lend them the ability to produce butanol. Usually genes responsible for solvents accumulation are cloned and genes responsible for acids formation are eliminated. Modified organisms should possess three important qualities: tolerance to butanol, a limited ability to assimilate it (not to metabolize it) and ability to utilize substrates used in the industry (glucose, lactic acid, glycerol) [75]. It is presumed that the tolerance depends on physico–chemical factors like pH and cultivation temperature. However, there are not enough experiments concerning these effects [76].
Knoshaug and Zhang [77] screen 24 different microorganisms for their tolerance to butanol. Some of the yeast strains (*Pichia methanolica, Pachysolen tannophilus, P. guilliermondii*) are found sensitive to butanol concentrations higher than 2 %. *Candida sonorensis, Saccharomyces cerevisiae,* and *Zymomonas mobilis* withstand 2 % butanol. The representatives of *Lactobacillus* genus are more sustainable than yeasts. At 2 % butanol strains of *L. delbrückii* and *L. brevis* demonstrate a relative growth rate of 55 % and 58 %, respectively. These two strains also withstand 2.5 % butanol, but the growth rate is diminished to 30 % and 40 %, correspondingly. The authors explore the temperature effect on *E. coli* sensitivity. They find out that the culture grows faster at 37°C, but is more sensitive to 1 % butanol when compared to that cultivated at 30°C [77]. It is established that *Pseudomonas putida* has the potential to be used for industrial butanol production due to its relatively high tolerance and ability to metabolize cheap substrates. According to Molina-Santiago et al. [78] the strain possesses pump systems used for detoxification. That is to say these systems are the reason for the tolerance to large number of antibiotics and organic solvents of some organisms. Cuenca et al. [75] investigate the tolerance of *Ps. putida* BIRD-1 and find that the strain shows no significant decrease in its vitality up to 2 % butanol presence. Analyzing a mutant library, the authors find 16 genes responsible for butanol tolerance.

An aerobic butanol producing bacteria from soil in Singapore, identified as *Bacillus* sp. is isolated [79]. It produces 10.38 g L\(^{-1}\) butanol in a batch process. The optimization of the fermentation conditions by minimizing the by-products quantities results in 12.3 g L\(^{-1}\) of butanol.

Butanol-tolerant *Enterococcus faecalis* CM4A is isolated from soil polluted with grease [76]. The strain sustains 3.5 % butanol without assimilating or degrading it. A strain *Staphylococcus aureus* is reported [39] to withstand 3 % butanol. It is determined that the addition of 10 g L\(^{-1}\) - 20 g L\(^{-1}\) glucose increases the bacterial tolerance.

Another microorganisms found to be tolerant to butanol belong to genus *Saccharomyces*-yeast. They utilize sugar compounds for biomass accumulation and conversion to ethanol. *Saccharomyces cerevisiae*, among them, is widely spread in bread preparation, wine and beer production [80]. The species was reported to withstand high ethanol concentration [81] and it turns out lately that it is tolerant to butanol too. Zaki et al. [83] examine 90 different *Saccharomyces* strains and detect that some of them (*S. cerevisiae* DBVPG 1788, *S. cerevisiae* DBVPG 6044, *S. cerevisiae* YPS 128) are tolerant to 4 % butanol. It is found out that *S. uvarum* and *S. castelli* do not tolerate more than 3 % butanol. The authors suppose that the tolerance is related to the over-expression of HOR2 gene [82]. Hansson [83] isolates *S. cerevisiae* strains from different types of beer and wine. The most tolerant strain is isolated from beer and is capable to grow in 3 % butanol.

In regard to its tolerance to butanol, *S. cerevisiae* is used as a host where the synthetic metabolic pathway for butanol production from *C. acetobutylicum* is cloned. The authors eliminate the glycerol production pathway and achieve improved butanol production. In addition, the gene encoding trans-enol-CoA reductase is cloned, and after 48 h of fermentation 14.1 mg L\(^{-1}\) butanol is reached [32]. The priority of *S. cerevisiae* as a host for genetic manipulations is that it is a well-characterized organism which is genetically tractable [84].

The ability of carboxydotrophic bacteria to tolerate 1 % - 3 % butanol is reported [85]. These bacteria utilize syngas compounds (CO, H\(_2\) and CO\(_2\)) for butanol production. *B. licheniformis* VP1A, *Pediococcus acidilacti* IMUA 20068 and *Enterococcus faecalis* IMUAU 60169 are found among 11 different strains to be less sensitive to butanol. The advantage of the carboxydotrophic bacteria is that they are capable to tolerate up to 3 % butanol and besides, they synthesize it in small quantities from CO. These properties make them exceptionally appropriate for genetic investigation and manipulations. Fig. 3 shows the Wood-Ljungdahl pathway of CO, CO\(_2\) and H\(_2\) conversion.

*E. coli*, another bacterium used intensively by the genetic engineering, is also manipulated in order to produce butanol. Saini et al. [28] construct a recombinant strain on the ground of clostridia by introducing CoA-dependent synthetic pathway. This strain demonstrates enhanced NADH levels and produces 6.1 g L\(^{-1}\) butanol with a yield of 0.33 g g\(^{-1}\) and a productivity of 0.21 g Lh\(^{-1}\) [28].

*L. brevis* is modified by Berezina et al. [36]. They use plasmid shuttle vector pHYc to clone genes encoding enzymes of the lower part of butanol metabolic pathway of *C. acetobutylicum - crt, bcd, etfA, etfB, hbd* (included in *bcs* operon), and the *thl* gene. However, butanol is found to be produced at very low levels - just 300 mg L\(^{-1}\).

### MAIN PROBLEMS AND FUTURE PERSPECTIVES

The preferred use of biobutanol refers to the production of motor fuels for spark ignition engines by mixing with conventional gasoline. The biobutanol concentration in the fuel can reach up to 30 % v/v, and since the butanol fuel contains oxygen atoms, the stoichiometric air/fuel ratio is smaller than that of gasoline and more fuel could be injected to increase the engine power for the same amount of air induced. The oxygen content is supposed to improve the combustion, therefore lower CO emissions can be expected. That is why biobutanol is currently an exceptionally desired fermentation product, superior to ethanol because of its physical properties and the fact that it can completely replace gasoline.

However, there are several obstacles impeding fermentative butanol production. The work with natural butanol producers is very challenging. Clostridia are not very suitable because of their intolerance to oxygen; they suffer from butanol toxicity, they grow slowly. Moreover, butanol is not synthesized in economically desirable quantities. Thus, there is an interest in producing butanol by using more appropriate organisms. A possible approach to butanol levels improvement refers to the application of methods of genetic engineering and manipulation of the genes providing acid formation and solvents accumulation. Thus, the by-products concentrations can be minimized. Another strategy is finding...
organisms, which do not naturally produce butanol, but are tolerant to it. They can be included in a butanol metabolic pathway.

Considering the global demand of n-butanol, it is expected that it will be worth 9.9 billion USD by 2020. The Asia-Pacific region is the world’s largest market in 2016 with a share of 51.3% in terms of volume. China is the key consumer there. It is also the biggest market of n-butanol. Almost 15.0% demand is met through import from other regions including Russia, Taiwan, Malaysia, the U.S., and Germany although various projects for construction of n-butanol manufacturing facilities are approved. Since the production deficit is still expected to exist in the near future, the production of biobutanol from waste biomass is particularly promising. For instance, the EU funded ButaNexT project (costing €4.6 million) unites the efforts of scientists from 15 European countries in search of alternative lignocellulosic substrates for the production of biobutanol. The use of inexpensive renewable non-food carbon substrates would solve some of the problems worldwide, like global warming and the over-consumption of petroleum-based products.

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