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REVIEW

Recent advances to improve fermentative butanol production: Genetic engineering and fermentation technology

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Butanol has recently attracted attention as an alternative biofuel because of its various advantages over other biofuels. Many researchers have focused on butanol fermentation with renewable and sustainable resources, especially lignocellulosic materials, which has provided significant progress in butanol fermentation. However, there are still some drawbacks in butanol fermentation in terms of low butanol concentration and productivity, high cost of feedstock and product inhibition, which makes butanol fermentation less competitive than the production of other biofuels. These hurdles are being resolved in several ways. Genetic engineering is now available for improving butanol yield and butanol ratio through overexpression, knock out/down, and insertion of genes encoding key enzymes in the metabolic pathway of butanol fermentation. In addition, there are also many strategies to improve fermentation technology, such as multi-stage continuous fermentation, continuous fermentation integrated with immobilization and cell recycling, and the inclusion of additional organic acids or electron carriers to change metabolic flux. This review focuses on the most recent advances in butanol fermentation especially from the perspectives of genetic engineering and fermentation technology.

[**Key words:** Butanol fermentation; Butanol-producing microorganisms; Lignocellulosic material; Genetic engineering; Fermentation technology]

1-Butanol, also known as *n*-butanol, has a straight-chain structure with a hydroxyl group at the terminal carbon, and it has wide industrial applications. Butanol is used in methacrylate esters and butyl acrylate for latex surface coatings, enamels, and lacquers. Other important derivatives of butanol are butyl glycol ether, butyl acetate, and plasticizers (1). Butanol is also an excellent diluent for brake fluid formulations and a solvent for the production of antibiotics, vitamins, and hormones (2). Another important application of butanol is its use as a direct replacement of gasoline or as a fuel additive. In contrast to ethanol, butanol is a 4-carbon alcohol; therefore, it has double the carbon content of ethanol and contains 25% more energy (3). It also has a low vapour pressure and a gasoline-like octane rating, which allows it to be blended with existing gasoline at much higher proportions than ethanol or used as a pure fuel in some vehicles, without any modification in their engines (2). It is less explosive and corrosive than ethanol, and is less susceptible to separation in the presence of water, which makes it more suitable for distribution through existing pipelines (4). These advantages have allowed butanol to outstrip ethanol as an alternative fuel.

Butanol can be produced either from fossil fuel by chemical synthesis (as petro-butanol) or from biomass by microbial fermentation (as biobutanol). However, many fossil oil-derived raw materials are required during chemical synthesis processes. Therefore, owing to the rapid depletion of fossil oil, butanol production through biological routes, which is called butanol fermentation or acetone-butanol-ethanol (ABE) fermentation (main products acetone, butanol, and ethanol are at a typical ratio of 3:6:1 in the fermentation), has attracted increasing attention (5). Butanol production by microbial fermentation was first reported by Pasteur in 1861, and has been extensively studied in recent years (6–8).

Nevertheless, several drawbacks still impede the application of fermentative butanol as an alternative fuel: (i) relatively high substrate cost arising from the use of edible biomass, (ii) low final butanol concentration (<20 g/L), (iii) low butanol yield (0.28–0.33 g/g), (iv) undesirable butanol selectivity (lower fraction of butanol in the final product), (v) low volumetric butanol productivity (<0.5 g L^{-1} h^{-1}), and (vi) high cost of butanol recovery (conventional distillation is energy-intensive) (9). Therefore, the purpose of this review is to explore recent advances in butanol fermentation, and the improved methods of butanol fermentation will be described from the aspects of genetic engineering and fermentation technology.

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GENERAL ASPECTS OF BUTANOL FERMENTATION

Wild-type and genetically modified butanol-producing microorganisms Butanol-producing strains can mainly be categorized as wild-type butanol-producing strains and genetically modified strains. In nature, butanol production is performed exclusively by members of the genus *Clostridium* (Table 1). In 1916, *Clostridium acetobutylicum* was first isolated by Chaim Weizmann who discovered that it produced acetone, butanol, and ethanol from starch (10). Clostridia are strictly anaerobic, rod-shaped, spore-forming bacteria. However, only a few *Clostridium* species can produce significant amounts of butanol during fermentation under appropriate conditions. Recently, genetically modified strains were also established for butanol production by using non-*Clostridium* genera as listed in Table 1.

Metabolism of wild-type butanol-producing clostridia A typical characteristic of butanol production by clostridia is biphasic fermentation, as shown in Fig. 1, i.e., comprising an acidogenic phase and a solventogenic phase (27). Hexose and pentose sugars are metabolized to pyruvate via different pathways (26). Hexose sugars are degraded to pyruvate by the Embden–Meyerhof–Parnas (EMP) pathway. Through the EMP pathway, 1 mol of hexose yields 2 mol of pyruvate with a net production of 2 mol each of ATP and NADH (28). In contrast, pentose sugars are metabolized via the pentose phosphate (PP)/glycolic pathway. Fructose 6-phosphate and xylulose 5-phosphate are converted to glyceraldehyde 3-phosphate by transaldolase and transketolase, and the glyceraldehyde 3-phosphate then enters the EMP pathway. During this process, the conversion of 3 mol of pentose sugars yields 5 mol of pyruvate, 5 mol of ATP, and 5 mol of NADH (29). After pyruvate formation, the strains enter an exponential growth phase with active hydrogen evolution and acetate and butyrate production, which is called the acidogenic phase. The acetyl-coenzyme A (A-CoA) node serves as an important switch point in the acid- and solvent-producing pathways (30). Acetate is converted from A-CoA by phosphotransacetylase (*pta*) and acetate kinase (*ack*). Phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*) are responsible for butyrate formation. The accumulation of acids leads to a decrease in the external pH in the fermentation broth. Consequently, organic acids are reassimilated into solvents of mainly acetone, butanol, and ethanol at a typical ratio of 3:6:1 (31), which is named as solventogenic phase. Simultaneously, the external pH increases because of acid uptake. The shift to solvent production is an adaptive response of the cells to the low external pH and allows the cells to avoid the inhibitory effects of the acid (32). In the butanol synthetic pathways, 6 enzymes are essential to convert A-CoA to butanol, including thiolase (*thl*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*), butyryl-CoA dehydrogenase (*bcd*), butyraldehyde dehydrogenase, and butanol dehydrogenase (*bdh*). Among these enzymes, thiolase is known to play a key role in both acid and solvent production (33). Acetoacetate, one of the products of CoA-transferase (*ctfAB*) activity, is further converted into acetone and CO_2 by acetoacetate decarboxylase (*adc*) (4). Ethanol is synthesized through the catalysis of A-CoA by CoA-acylating aldehyde dehydrogenase (*aad*) and NADH-dependent alcohol dehydrogenase (*adhE*). In addition to the enzymes, control of electron flow in the EMP pathway is also vital with respect to the regulation of acetate, butyrate, and butanol formation (34). In metabolic pathways, ATP is predominantly generated during the acidogenic phase, whereas high NADPH levels have been proposed to induce the solventogenic phase (35).

Traditional and renewable substrates for butanol fermentation To date, clostridia are known to have advantages of utilizing a large variety of carbon sources such as monosaccharides; oligosaccharides; and polysaccharides, including refined and unrefined starches; and various pentoses and hexoses, as well as glycerol (8,36–38). Traditional substrates such as molasses (39), whey permeate (40), corn (41), cassava (13), potato (42), and Jerusalem artichokes (43) have been studied as substrates for butanol fermentation. However, because of the high cost of above sources of biomass and competition with food supply, there is considerable interest in using renewable substrates, especially lignocellulosic materials, which are cost-effective and sustainable for butanol fermentation. Pretreatment and hydrolysis are crucial steps for utilizing lignocellulosic materials as raw material. Lignocellulosic material is usually subjected to severe conditions for pretreatment such as dilute sulphuric acid, dilute alkali, ammonia, hot water, steam explosion, and alkaline peroxide (44). Some inhibitory components such as formic acid, acetic acid, levulinic acid, furfural, and hydroxymethyl furfural are formed in the process of pretreatment (45). Several detoxification methods that focus on the removal of these inhibitory components have been previously studied, ranging from physical and physicochemical treatments such as overliming,

TABLE 1. Wild-type and genetically modified butanol-producing strains.

Types	Species	Substrate	Main solvent products	$C_{butanol}$ (g/L)	Reference
Wild type	*Clostridium acetobutylicum*	Glucose	Butanol, acetone, ethanol	10.4	11
Wild type	*Clostridium beijerinckii*	Glucose	Butanol, isopropanol	15.21	12
Wild type	*Clostridium saccharoperbutylacetonicum*	Glucose	Butanol, acetone, ethanol	16.2	13
Wild type	*Clostridium saccharoperbutylicum*	Glucose	Butanol, acetone, ethanol	9.7	14
Wild type	*Clostridium sporogenes*	Glucose	Ethanol, butanol, 1-propanol, isobutanol, methyl-butanol	0.119	15
Wild type	*Clostridium perfrigens*	Glucose	Ethanol, butanol, 1-propanol, isobutanol, methyl-butanol	0.019	15
Wild type	*Clostridium pasteurianum*	Glycerol	Butanol, 1,3-propanediol	6.5	16
Wild type	*Clostridium carboxidivorus*	CO	Butanol, ethanol	0.37	17
Wild type	*Clostridium tetanomorphum*	Glucose	Butanol, ethanol	3.5	18
Wild type	*Clostridium aurantibutyricum*	Glucose	Butanol, acetone, isopropanol	3.36	19
Wild type	*Clostridium cadaveris*	Glucose	Butanol	0.829	19
Genetically modified	*Bacillus subtilis*	Glycerol	Butanol, ethanol	0.024	20
Genetically modified	*Clostridium tyrobutyricum*	Glucose	Butanol, ethanol	10.0	21
Genetically modified	*Escherichia coli*	Glucose	Butanol, propanol	2.0	22
Genetically modified	*Lactobacillus brevis*	Glucose	Butanol, ethanol	0.303	23
Genetically modified	*Lactobacillus buchneri*	Glucose	Butanol	0.066	24
Genetically modified	*Lactococcus lactis*	Glucose	Butanol	0.028	24
Genetically modified	*Pseudomonas putida*	Glycerol	Butanol, ethanol	0.12	20
Genetically modified	*Saccharomyces cerevisiae*	Galactose	Butanol	0.0025	25

$C_{butanol}$, butanol concentration.