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Review

Biobutanol Production from Acetone–Butanol–Ethanol Fermentation: Developments and Prospects

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Abstract: With global carbon emissions and environmental issues becoming increasingly prominent, there is an increasing focus on the development of clean energy, and biobutanol has gained widespread attention due to its superior performance. Butanol production by fermentation is affected by various factors, such as raw materials, cultivation environment, and butanol toxicity, which results in lower butanol production and restricts its industrial development. This article elaborates on the research progress of butanol fermentation, including butanol-producing microorganisms, butanol synthesis metabolic pathways, raw materials for ABE fermentation, and butanol fermentation technologies. It also looks forward to the prospects of biobutanol, aiming to provide a theoretical basis for the research direction of butanol fermentation.

Keywords: ABE fermentation; butanol-producing strains; low-cost raw materials; fermentation technologies

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1. Introduction

As an important platform chemical, butanol is widely used in industries such as medicine and chemicals, mainly as a solvent or chemical synthesis raw material. At the same time, butanol can be used as a fuel instead of gasoline or as a fuel additive.

With the highlighting of global carbon emissions and environmental issues, butanol production via fermentation has received widespread attention [1,2]. Butanol fermentation, also known as acetone–butanol–ethanol (ABE) fermentation, refers to the synthesis of butanol by butanol-producing strains using starch crops or sugars as raw materials under strict anaerobic conditions while generating byproducts of acetone and ethanol. ABE fermentation began in the 1850s and has a history of 150 years.

In 1914, Weizmann isolated a strain of butanol-producing clostridia that can synthesize butanol from starch, named *Clostridium acetobutylicum* (*C. acetobutylicum*), and its butanol titer can exceed 10 g/L with stable heredity. To date, *C. acetobutylicum* remains an important species for industrial butanol production, and its discovery laid an important foundation for the industrial development of butanol fermentation.

In the late 1950s, with the rapid development of the petrochemical industry, the cost of synthesizing acetone and butanol using chemical methods became even cheaper, while the high costs of raw materials and subsequent purification of butanol from fermentation resulted in a huge impact on the ABE fermentation industry and many butanol fermentation plants were shut down, leading to a downturn in the butanol fermentation industry [3].

In recent years, with the depletion of petroleum resources and increasingly serious environmental issues, people have gradually realized that although chemically synthesizing butanol from petroleum raw materials is cheaper, it is unsustainable and environmentally destructive. Therefore, using biomass as raw materials and producing butanol through microbial fermentation has once again come to the forefront of history.

2. Butanol-Producing Strain

Clostridium is the main type of bacterium that is capable of producing butanol, including species such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*, *Clostridium saccharobutylicum*, *Clostridium sporogenes*, *Clostridium pasteurianum*, *Clostridium carboxidivorus*, *Clostridium tetanomorphum*, and *Clostridium aurantibutyricum* [3]. All these strains are strict anaerobic bacteria with rod-shaped cell morphology and are Gram-positive bacteria capable of forming endospores. Table 1 shows the butanol fermentation performance of different strains.

Table 1. Butanol fermentation performance of different strains.

Strain	Raw Material	Main Product	Butanol Titer (g/L)	Reference
Clostridium acetobutylicum	Glucose	Butanol, acetone, ethanol	10.4	[4]
Clostridium beijerinckii	Glucose	Butanol, isopropyl alcohol	15.2	[5]
Clostridium saccharoperbutylacetonicum	Glucose	Butanol, acetone, ethanol	16.2	[6]
Clostridium saccharoperbutylicum	Glucose	Butanol, acetone, ethanol	9.7	[7]
Clostridium sporogenes	Glucose	Butanol, ethanol, propyl alcohol, isobutanol, methyl butanol	0.12	[8]
Clostridium perfrigens	Glucose	Butanol, ethanol, propyl alcohol, isobutanol, methyl butanol	0.02	[8]
Clostridium pasteurianum	Glycerinum	Butanol, 1, 3-propylene glycol	6.5	[9]
Clostridium carboxidivorus	CO	Butanol, ethanol	0.37	[10]

Among the butanol-producing microorganisms, *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*, and *Clostridium saccharobutylicum* are commonly used in industry and research with relatively high butanol titers [11]. Common butanol-producing strains can be divided into two categories based on the characteristics of substrate utilization: starch-metabolizing strains and sugar-metabolizing strains. The main difference between these strains is that the former can secrete amylase to hydrolyze starch into monosaccharides for growth and fermentation, while the latter can only use monosaccharides as fermentation substrates. *C. acetobutylicum* ATCC 824 and *C. acetobutylicum* DSM 1731 are typical starch-metabolizing strains, while typical sugar-metabolizing strains include *C. acetobutylicum* NCIMB8052, *C. acetobutylicum* P 262, and *C. beijerinckii* BA101 [12].

Due to the cytotoxicity of butanol, when the butanol titer reaches a certain level, the bacteria gradually form spores, enter a dormant state, and fermentation stops. Therefore, the butanol produced by *Clostridium* is usually below 13 g/L. To solve this problem, researchers have conducted a lot of strain breeding works to improve the butanol tolerance and production of bacteria. Currently, there are many methods available for strain improvement, such as mutation breeding, genome design, evolutionary engineering, and synthetic biology.

Early strain modification mainly involved mutagenesis and domestication, including physical and chemical mutagenesis methods. Annous et al. used chemical mutagenesis with nitrosoguanidine as the mutagen on *C. acetobutylicum* ATCC 824 to obtain a butanol high-yield strain, *C. acetobutylicum* BA105, with a titer of 18 g/L, 1.8 times that of the original strain. The amylase activity of the mutant strain was also significantly improved [13]. Syed et al. combined ultraviolet mutagenesis, methyl methanesulfonate mutagenesis, and nitrosoguanidine mutagenesis to induce mutation in *C.acetobutylicum* PTCC 23, screening for a butanol high-yield strain, *C. acetobutylicum* MEMS7, which produced 18.0 g/L butanol when using molasses as the substrate, a 20% increase over the original strain [14]. Li et al. used low-energy ion beam injection mutagenesis and nitrosoguanidine mutagenesis to induce mutation in *C. beijerinckii* L175 and obtained a butanol high-yield strain, *C. beijerinckii* MUT3, which produced 15.8 g/L butanol, a 46% increase over the original

strain [15]. In 2001, Qureshi et al. used nitrosoguanidine as the mutagen to induce mutation in *C. beijerinckii*, screening for a butanol high-yield strain, *C. beijerinckii* BA101, which produced 19 g/L butanol, a 122.2% increase over the original strain. *C. beijerinckii* BA101 is still an important butanol high-yield strain to date, with stable genetic characteristics [16]. Currently, the highest butanol-producing strain in batch fermentation conditions is *C. acetobutylicum* JB200, which was domesticated under a long-term intermittent addition of butanol, and its butanol titer can reach 20 g/L [17].

In 2001, with the successful sequencing of the complete genomes of ethanol, acetone, and butanol synthetic pathways and metabolic pathways in the *C. acetobutylicum* TCC824 and *C. beijerinckii* NCIMB8052 strains, researchers aimed to obtain excellent strains with a high yield and high butanol tolerance by studying key genes, key enzymes, and metabolic networks. Jiang et al. knocked out the key enzyme gene acetyl-coenzyme A decarboxylase (*adc*) in the acetone synthesis process of *C. acetobutylicum* EA2018 to reduce the production of the fermentation byproduct acetone, thereby increasing the proportion of butanol in the total solvent. The results showed that acetone production decreased to 0.2 g/L, and the proportion of butanol increased from 70% to 80%, but the butanol titer also decreased from 13.6 g/L to 7.4 g/L, mainly because the acetone generation pathway is coupled with the acid reabsorption pathway, which is associated with the butanol production pathway [18]. Jang et al. selected key enzyme genes in the organic acid synthesis and butanol synthesis metabolic pathways for combined regulation. By knocking out the acetyl phosphate transferase gene (*pta*) and butyrate kinase gene (*buk*) and overexpressing the butanol dehydrogenase gene (*adhE*), the butanol titer increased by 60% to 18.9 g/L [19]. Mann et al. showed that enhancing the expression of the stress protein genes *groESL*, *grpE*, and *htpG* significantly improved butanol tolerance in *Clostridium acetobutylicum*. When the culture medium contained 2% (*v*/*v*) butanol, the original strain died within 2 h, whereas the survival rates of the genetically engineered strains *groESL*, *grpE*, and *htpG* were 45%, 25%, and 56%, respectively. However, the butanol titers of the genetically engineered strains grpE and htpG were only 52% and 68%, respectively, compared to the original strain [20]. Scotcher et al. reported that sporulation formation can affect ABE production. By inhibiting the expression of the SpoIIE gene, which controls sporulation formation, the formation of spores was delayed, and the titers of butanol, acetone, and ethanol increased by 110%, 43%, and 225%, respectively [21]. Cong et al. used metabolic engineering technology and gene editing technology to overexpress, knock out, or inhibit the transcription of key genes in the xylose transport pathway, xylose metabolism pathway, and butanol synthesis pathway and optimized and combined these genes to construct a strain that can efficiently produce butanol from xylose. The highest yield of recombined strain CC101 (ptb-Xyl T) ABE was 0.41 g/g, and when the xylose titer was increased to 55 g/L, the ABE titer increased to 14.6 g/L [22].

Some researchers have attempted to clone the key enzyme genes of the butanol synthesis pathway into nonbutanol-producing bacterial cells such as *E. coli* or yeast through synthetic biology to artificially construct the butanol synthesis pathway. However, studies have shown that although genetically engineered bacteria can synthesize butanol, their titers are low [23,24]. Atsumi et al. expressed six key enzyme genes in the butanol synthesis metabolic pathway in *E. coli* and knocked out several enzyme genes related to byproduct formation in *E. coli*. Butanol titer of 0.35 g/L was observed [25]. Eric et al. constructed a similar butanol synthesis metabolic pathway in *S. cerevisiae* and conducted fermentation using galactose as a substrate. The butanol titer was only 2.5 mg/L [26]. Schadeweg et al. integrated the butanol synthesis metabolic pathway into *S. cerevisiae*, overexpressed key butanol synthesis genes, deactivated competitive pathways, and supplemented pantothenate in a timely manner during the cultivation process, resulting in a butanol titer of 13 g/L [27].

3. ABE Synthetic Metabolic Pathway of *Clostridium*

The ABE synthetic metabolic pathway of *Clostridium acetobutylicum* for the production of butanol is shown in Figure 1 [28]. The major metabolites produced during ABE fermen-

tation include butanol, acetone, and ethanol, accompanied by the generation of CO_2 and H_2. The entire fermentation process is divided into two stages: the acidogenic phase and the solventogenic phase.

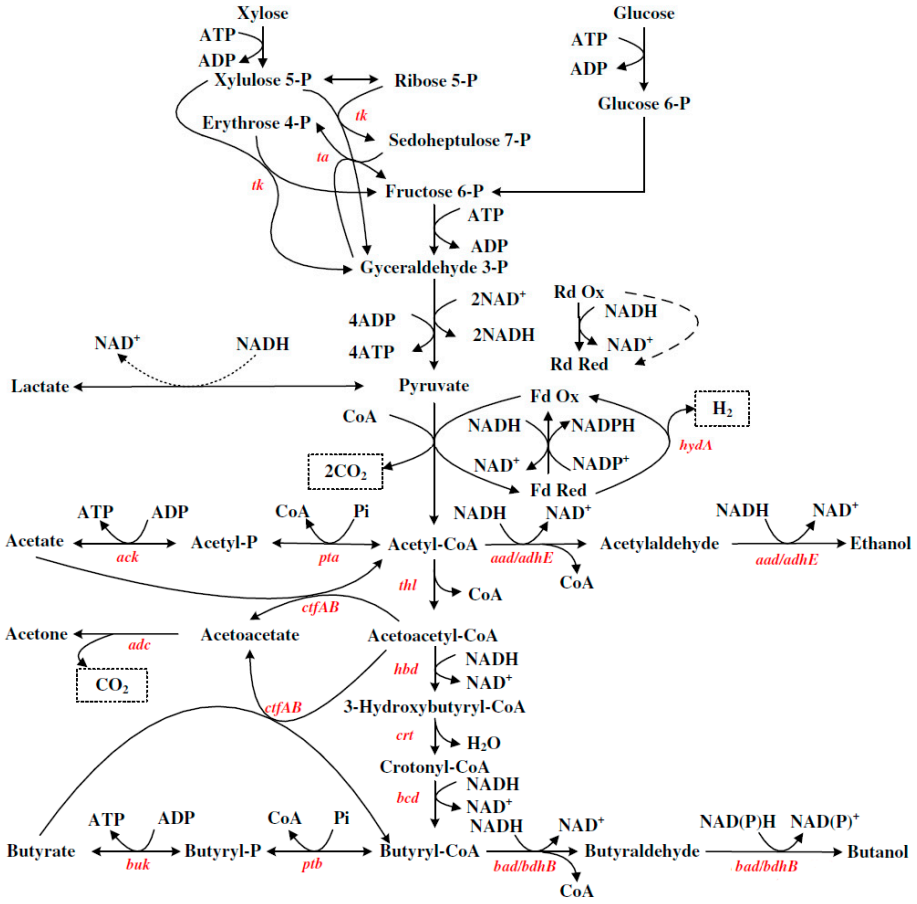

Figure 1. ABE synthesis pathway of *Clostridium acetobutylicum* [28]. (*tk*: transketolase; *ta*: transaldolase; *hydA*: hydrogenase; *aad:* acetaldehyde dehydrogenase; *adhE*: ethanol dehydrogenase; *bad*: butyraldehyde dehydrogenase; *bdhB:* butyraldehyde dehydrogenase; *buk*: butyrate kinase; *pta*: phosphotransacetylase; *ack*: acetate kinase; *ctfAB*: acetate/butyrate:CoA-transferase; *hbd*: 3-hydroxybutyryl-CoA dehydrogenase; *crt*: crotonase; *bcd*: butyryl-CoA dehydrogenase; *adc*: acetoacetate decarboxylase; *ptb*: phosphotransbutyrylase).

Clostridium acetobutylicum generates pyruvate through the Embden–Meyerhof–Parnas (EMP) pathway and then produces acetyl-CoA under the action of ferredoxin oxidoreductase, accompanied by the generation of CO_2 and H_2. The process of generating ABE from acetyl-CoA is divided into the acidogenic phase and the solventogenic phase.

The acidogenic phase is coupled with the logarithmic growth phase of the cell, and ATP is produced during the acidogenic phase, providing energy for cell growth. Phosphate acetyltransferase (*pta*) and acetate kinase (*ack*) control the synthesis of acetic acid, while butyrate kinase (*buk*) and phosphate butyryl transferase (*ptb*) control the synthesis of butyric acid. As acetic acid and butyric acid gradually accumulate, the pH of the fermentation broth gradually decreases. When the pH drops to a certain value (approximately 4.5), bacterial metabolism shifts to the solventogenic phase, and the transition phase between the acidogenic and solventogenic phases is called the transition phase. The metabolic shift of the cells is mainly due to the change in pH in the fermentation broth. The intracellular pH of *Clostridium acetobutylicum* is influenced by the environmental pH, and in general, the intracellular pH is one unit higher than the environmental pH. When the pH of the fermentation broth drops to approximately 4.5, some undissociated acetic acid and butyric

acid enter the cytoplasm and dissociate, disrupting the original proton gradient inside and outside the cell membrane. To resist this adverse environment, the cells convert organic acids into acetyl-CoA and butyryl-CoA while continuing to consume substrates for the synthesis of butanol, acetone, and ethanol [29]. Grimmler et al. found that at least 245 genes showed differential expressions during the transition phase from the acidogenic to the solventogenic phase [30]. Bacterial stress responses, metabolic regulation, and fermentation culture conditions are all factors influencing the transition from the acidogenic to the solventogenic phase [31].

In the solventogenic phase, acetyl-CoA, butyryl-CoA, and acetoacetyl-CoA are the precursors for the synthesis of ethanol, butanol, and acetone, respectively, and are three key intermediate metabolites in the ABE synthesis pathway. Both butanol and ethanol are generated under the catalysis of aldehyde/alcohol dehydrogenases (*aad*, *adhE*), accompanied by the consumption of NAD(P)H, while acetone is produced under the catalysis of acetoacetyl-CoA transferase (*ctfAB*) and acetoacetyl-CoA decarboxylase (*adc*), accompanied by the release of CO_2. The generation of acetone is coupled with the reabsorption of acid, where acetoacetyl-CoA transferase (*ctfAB*) uses acetate or butyrate as CoA acceptors to form acetyl-CoA or butyryl-CoA, which are used for the synthesis of ethanol and butanol. Therefore, inhibiting the synthesis pathway of acetone will also affect the generation of alcohols. As the fermentation enters the late solventogenic phase and the titer of products in the fermentation broth increases, the cells gradually decline, forming spores due to the toxicity of metabolic products (especially butanol) and nutrient depletion. At this point, the fermentation ends [32].

4. Raw Materials for Butanol Fermentation

Traditional raw materials for butanol production mainly include starchy crops, such as corn and wheat. In the 21st century, due to the shortage and price increase in grain resources, exploring nongrain low-cost materials to replace grain-based materials for butanol fermentation has become a research hotspot. Currently, the most researched low-cost materials include nongrain starch materials, lignocellulosic materials, and molasses.

4.1. Starch-Based Raw Materials

Cassava is a crop rich in starch and is mainly grown in subtropical and tropical regions. In China, Vietnam, and Thailand, cassava is mainly used for feed production and starch preparation and can grow in infertile land without competing with grain crops [33]. Thang et al. used cassava starch as a substrate for the fermentation of *Clostridium saccharoperbutylacetonicum* N1–4, and a butanol titer of 16.9 g/L was produced with a yield of 0.33 g/g glucose, which is equivalent to the fermentation level of glucose 5. Similarly, Gu et al. used *C. acetobutylicum* EA2018 to synthesize butanol using cassava flour as a substrate. The butanol yield was significantly lower than that using corn as a substrate, but adding 30 mmol/L ammonium acetate to the cassava medium improved the ABE yield, with a butanol titer of 13.0 g/L and a total ABE titer of 19.4 g/L, which is basically equivalent to the fermentation level of corn [34]. Li et al. used atmospheric and room temperature plasma (ARTP) mutagenesis technology to mutate *C. acetobutylicum* PW12 and obtained a mutant strain *C. acetobutylicum* ART18, which could synthesize butanol using gelatinized cassava flour as a substrate, with a butanol titer of 16.3 g/L and a butanol productivity of 0.19 g/(L·h) [35]. Li et al. found that adding 2.5 g/L yeast powder to cassava medium could effectively promote the transformation of *C. acetobutylicum* ATCC824 from the acid-producing phase to the solvent-producing phase, increasing the butanol titer by 15% compared to the control group, reaching 13.6 g/L with a yield of 0.31 g/(g starch) [36]. Cassava has become an ideal raw material for butanol fermentation, and recent research on biobutanol production using cassava is summarized in Table 2.

Table 2. Studies on butanol production from cassava.

Strain	Pretreatment	Butanol Titer (g/L)	ABE Titer (g/L)	Butanol Productivity (g/L·h)	ABE Productivity (g/L·h)	Reference
C.acetobutylicum SE25	Enzymolysis	16.2	--	0.23	--	[37]
C. butylicum TISTR1032	Gelatinization	--	8.9	--	0.12	[38]
C. beijerinckii BA101	Enzymolysis	25.7	37	0.31	0.45	[39]
C. saccharoperbutylace-tonicum N1–4	Gelatinization	16.9	21	0.23	0.29	[6]
C. acetobutylicum PW12	Gelatinization	15.8	23.2	0.19	0.28	[33]
C. species strain BOH3	Gelatinization	17.8	24.2	0.25	0.34	[40]
C. acetobutylicum	Gelatinization	16.1	24.9	0.20	0.31	[35]
Symbiotic system TSH06	Non	13.3	20	0.18	0.27	[41]

In addition, some locally available starchy crops were used as raw materials in some studies. For example, Madihah et al. used coconut sago starch as a raw material for butanol fermentation by *C. acetobutylicum* P262. After optimizing the culture medium and culture conditions, the butanol titer reached 16.0 g/L, and the yield was 0.24 g/g [42].

4.2. Lignocellulose Raw Materials

As a widely available and inexpensive raw material, lignocellulose is also used in ABE fermentation. Liu et al. hydrolyzed wheat bran with dilute acid to ferment *C. beijerinckii* ATCC 55025 with dilute acid hydrolysate. The butanol titer was 8.8 g/L, and the total solvent titer was 11.8 g/L. The productivity and product yield were 0.16 g/L·h and 0.32 g/g, respectively [43]. Lin et al. used response surface methodology to optimize the corn stover hydrolysate culture medium and found that $CaCO_3$ was a key factor for fermentation. Under optimized conditions, *C. acetobutylicum* CICC 8008 produced 6.57 g/L butanol [44]. Steam explosions were used by Li Dongmin et al. to pretreat corn stovers, and then the enzymatic hydrolysate was used for continuous fermentation. Under a dilution rate of 0.075 h^{-1}, the butanol titer was 4.1 g/L, and the butanol productivity was 0.31 g/(L·h) [45].

The Qureshi research group at the US Department of Agriculture is at the forefront of research on the fermentative production of butanol from lignocellulosic biomass. In 2006, Qureshi et al. used *C. acetobutylicum* P260 as the fermentation strain and corn stover hydrolysate as the substrate to produce butanol. The total titer of butanol, ethanol, and acetone was 9.6 g/L, with a total solvent productivity and yield of 0.20 g/L·h and 0.41 g/g, respectively. Under the conditions of coupled fermentation and gas stripping, the total titer of butanol, ethanol, and acetone reached 24.67 g/L, with a total solvent productivity and yield of 0.47 g/L·h and 0.44 g/g, respectively [46]. In 2007, Qureshi et al. treated wheat straw with acid and then enzymatically hydrolyzed it, followed by ABE fermentation using *C. beijerinckii* P260. The results showed that the total solvent titer of ABE reached 25.0 g/L with a productivity and yield of 0.60 g/L·h and 0.42 g/g, respectively [47]. In 2008, Qureshi et al. acid-hydrolyzed corn fiber, detoxified the hydrolysate with ion exchange resin XAD-4, and used *C. beijerinckii* BA101 as the fermentation strain, resulting in a total solvent titer of 9.3 g/L. When enzymatically hydrolyzed corn fiber was used as the fermentation medium, the total solvent titer was 8.6 g/L [48]. In the same year, Qureshi et al. pretreated barley straw with hydrogen peroxide, enzymatically hydrolyzed it, and performed butanol synthesis using *C. beijerinckii* P260. Under batch fermentation conditions, the total solvent titer of ABE reached 21.37 g/L with a productivity and yield of 0.55 g/L·h and 0.30 g/g, respectively [49]. Using *C. beijerinckii* P260 for the simultaneous saccharification and fermentation with dilute acid hydrolysate of wheat straw, the total solvent titer of ABE reached 21.4 g/L [50]. In 2010, Qureshi et al. compared the fermentation performance of *C. beijerinckii* P260 using barley straw, corn stover, and switchgrass hydrolysates as substrates and found that the total solvent titers were 26.6 g/L, 26.3 g/L, and 14.6 g/L,

respectively [51]. In 2021, butanol was produced using sugarcane bagasse medium with high solid content (16% solid content). The ABE productivity was 0.21 g/L/h, and the yield was 0.39 g/g via simultaneous saccharification and fermentation coupled with online recovery [52]. In the same year, corn straw was pretreated with deep eutectic solvent. The cellulose recovery was 99.24%, and the removal rates of xylan and lignin were 57.58% and 61.40%, respectively. After pretreatment with low eutectic solvent, without detoxification and sterilization, 13.65 g/L ABE was produced by *C. beijerinckii* P260, and the yield was 0.38 g/g [53].

The components in lignocellulose that can be utilized by microorganisms are cellulose and hemicellulose. Cellulose can be degraded into glucose, while hemicellulose can be degraded into xylose. However, both cellulose and hemicellulose cannot be directly utilized by microorganisms and need to be degraded into monosaccharides that can be utilized by microorganisms. The methods for degrading cellulose and hemicellulose include explosion, acid hydrolysis, enzymatic hydrolysis, etc., and these methods can be used in combination or alternation. During the pretreatment of lignocellulose, some substances that inhibit cell growth and fermentation are generated along with the production of monosaccharides. After pretreatment, the hydrolysate needs to be detoxified [54]. Additionally, the monosaccharides in lignocellulose hydrolysate are a mixture of pentose and hexose (mainly glucose and xylose), but the utilization efficiency of xylose by *Clostridium butyricum* is much lower than that of glucose. Moreover, there is generally a glucose repression effect in multiple carbon source fermentation systems, and the utilization efficiency of mixed sugar systems by *Clostridium butyricum* is also an urgent issue to be solved [55]. Therefore, much research work is needed to simplify the complex pretreatment process, improve the tolerance of microorganisms to inhibitors, and improve the utilization efficiency of microorganisms for mixed sugar systems.

4.3. Molasses

Molasses is a byproduct of the sugar industry, and its main components include oligosaccharides such as sucrose, glucose, fructose, amino acids, and various inorganic salt ions. In recent years, it has also been used for ABE fermentation. Shaheen et al. compared the performance of four types of butanol-producing clostridia in molasses fermentation and found that compared with *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*, and *Clostridium saccharobutylicum* could better utilize molasses for fermentation, and the total production of butanol, ethanol, and acetone could reach more than 18 g/L [56]. In 2010, Pei Jianxin et al. used two strains of *C. beijerinckii* gxzp-5 and *C. beijerinckii* gxzp-9 as fermentation strains and used molasses for ABE fermentation. The total solvent titer reached 11.33 g/L and 11.25 g/L, respectively [57]. Qureshi et al. used *C. beijerinckii* BA101 as the fermentation strain and molasses as the substrate for ABE fermentation. In a molasses medium containing 80 g/L sugar, the total solvent yield of ABE was 22.8 g/L [58]. Ni et al. used molasses as the substrate and *C. saccharoperbutylacetonicum* for continuous fermentation. Under the conditions of four-stage continuous fermentation, the total solvent titer reached 13.75 g/L, and the productivity reached 0.44 g/(L·h) [59].

5. Research Progress of Butanol Fermentation Technology

Low product titer and low productivity caused by product inhibition are the main problems in the ABE fermentation process. To eliminate product inhibition, different fermentation processes have been applied to improve the overall performance of ABE fermentation. Common ABE fermentation technologies include batch fermentation, continuous fermentation, fermentation-coupled online separation, and cocultivation.

5.1. Batch Fermentation

Batch fermentation is widely used in the ABE fermentation industry due to its simple operation and low contamination risk during the fermentation process. However, the main disadvantage of batch fermentation is its low productivity and low yield. Gener-

ally, the total solvent titer of batch fermentation does not exceed 20 g/L. Research on batch fermentation mainly focuses on optimizing fermentation conditions and medium composition.

The presence of butyric acid in the ABE fermentation process inhibits cell growth, but when butyric acid is present at a lower titer, it promotes ABE fermentation. Yi et al. added 40 mM butyric acid to the fermentation medium, and the butanol titer of *C. beijerinckii* NCIMB 8052 reached 10.2 g/L, which was 31% higher than that of the control group, and the productivity increased by 133% [60].

Madihah et al. optimized the nitrogen source and carbon/nitrogen ratio in the fermentation medium and found that when the carbon/nitrogen ratio increased from 4.3 to 21.4, the total solvent titer increased from 1.54 g/L to 18.82 g/L. When the nitrogen source was adjusted from yeast powder to a mixed nitrogen source of yeast powder and NH_4NO_3, the total solvent titer further increased to 24.5 g/L [42].

5.2. Continuous Fermentation

Continuous fermentation refers to fresh culture medium being fed in real time at a certain rate during the fermentation process while the fermentation broth is drained from the fermenter at the same rate, maintaining a constant fermentation volume (Figure 2).

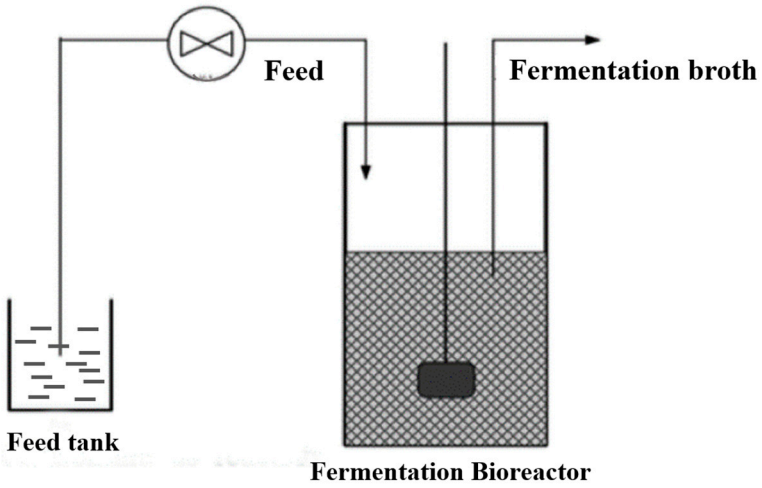

Figure 2. Continuous fermentation device.

Compared with batch fermentation, the advantage of continuous fermentation is that it can effectively improve butanol productivity. This is mainly because by continuously feeding fresh culture medium and flowing out fermentation broth, the titer of solvents in the fermentation broth would be diluted, eliminating product inhibition. Continuous fermentation coupled with cell immobilization, cell cycling, and multistage continuous fermentation are common methods to further promote fermentation productivity.

Cell immobilization and cell recycling techniques can increase the cell density in the fermentation broth, thereby promoting butanol productivity. In continuous butanol fermentation, the commonly used immobilization methods mainly include adsorption, covalent binding, and embedding. Among them, adsorption has mild conditions, is easy to operate, and has little impact on cell activity, so it is widely used. In recent years, various agricultural waste materials have been used as immobilization materials in continuous fermentation processes, including corn straw, wood pulp fiber, loofah sponge, sugarcane bagasse, etc. [61–64].

For cell recycling technology, the main focus is on the selection of ultrafiltration membrane materials. Schlote et al. compared the effects of cellulose triacetate, cellulose acetate, and polysulfone as ultrafiltration membranes on ABE fermentation. The results showed that cellulose triacetate had a better effect as an ultrafiltration membrane than the latter two. When the dilution rate was 0.4 h^{-1}, the total solvent titer reached 10.3 g/L, and the

productivity reached 4.1 g/(L·h) [65]. Tashiro et al. used a microporous membrane for cell recycling and carried out continuous fermentation for 207 h, with a total solvent production efficiency of 7.55 g/(L·h) [66]. Currently, the highest solvent productivity achieved via continuous fermentation coupled with cell recycling was 21.1 g/(L·h) and comes from a report by Jang et al., using *C. acetobutylicum* BKM19 as the fermentation strain [67]. Although fermentation coupled with cell recycling technology can maintain high biomass density in the fermentation broth and promote productivity, the main disadvantages are the high price of the ultrafiltration membrane device and the fact that the ultrafiltration membrane is easily polluted and blocked in the fermentation process [28].

The productivity can be Improved by single-stage continuous fermentation technology; however, the product titer is often low, which is not conducive to subsequent separation and purification processes. Multistage continuous fermentation can effectively increase the product titer. According to the report of Mutschlechner et al., a higher dilution rate promotes cell growth and organic acid production in ABE fermentation, while a lower dilution rate is conducive to solvent production. Therefore, the continuous fermentation of butanol is divided into two stages, and different dilution rates are used for each stage of fermentation. The dilution rate of the first stage was 0.12 h^{-1}, and that of the second stage was 0.022 h^{-1}. The total solvent titer reached 15 g/L, and the productivity was 0.27 g/(L·h) [68]. Table 3 shows the research on continuous ABE fermentation.

Table 3. Studies on ABE production via continuous fermentation.

Strain	Dilution Rate (h^{-1})	Raw Material	ABE Titer (g/L)	Yield (g/g)	Productivity (g/(L·h))	Strategy	Reference
C. acetobutylicum ATCC 55025	1.0	Glucose	11.3	0.53	5.6	Cell immobilization	[69]
C. aetobutylicum BCRC 10639	0.05	Glucose	14.3	0.24	--	Cell immobilization	[70]
C. acetobutylicum P-262	0.13	Potato	7.7	0.19	1.0	Cell immobilization	[71]
C. beijerinckii BA 101	2.0	Glucose	7.9	0.38	15.8	Cell immobilization	[72]
C. acetobutylicum DSM 792	1.9	Glucose	7.2	0.40	13.66	Cell immobilization	[62]
C. acetobutylicum DSM 792	1.5	Mix sugar	8.1	0.28	12.14	Cell immobilization	[62]
C. pasteurianum MSEL-GLY2	0.9	Glycerinum	8.6	--	7.8	Cell recycling	[73]
C. saccharoperbuty- lacetonicum N1–4	0.9	Glucose	8.6	--	7.6	Cell recycling	[66]
Symbiotic system TSH06	0.2	Glucose	16.8	--	1.12	Cell immobilization	[41]

5.3. Fermentation Coupled with In Situ Separation

The main product of ABE fermentation, butanol, has strong toxicity to cells. Generally, the titer of butanol in the fermentation broth does not exceed 13 g/L, and the total titer of butanol, acetone, and ethanol does not exceed 20 g/L. The termination of ABE fermentation is not due to the natural death of cells but rather the fermentation product's cytotoxicity. Under the strong inhibitory effect of the fermentation product, fermentation is forced to terminate. On the other hand, the distillation separation of low-titer products will increase energy consumption and cost. To reduce product inhibition and increase product titer and productivity, researchers have attempted to combine various in situ separation methods with butanol fermentation. Common methods include liquid–liquid extraction, gas stripping, adsorption, and pervaporation (Figure 3) [74–77]. Table 4 summarizes several common in situ butanol separation methods.

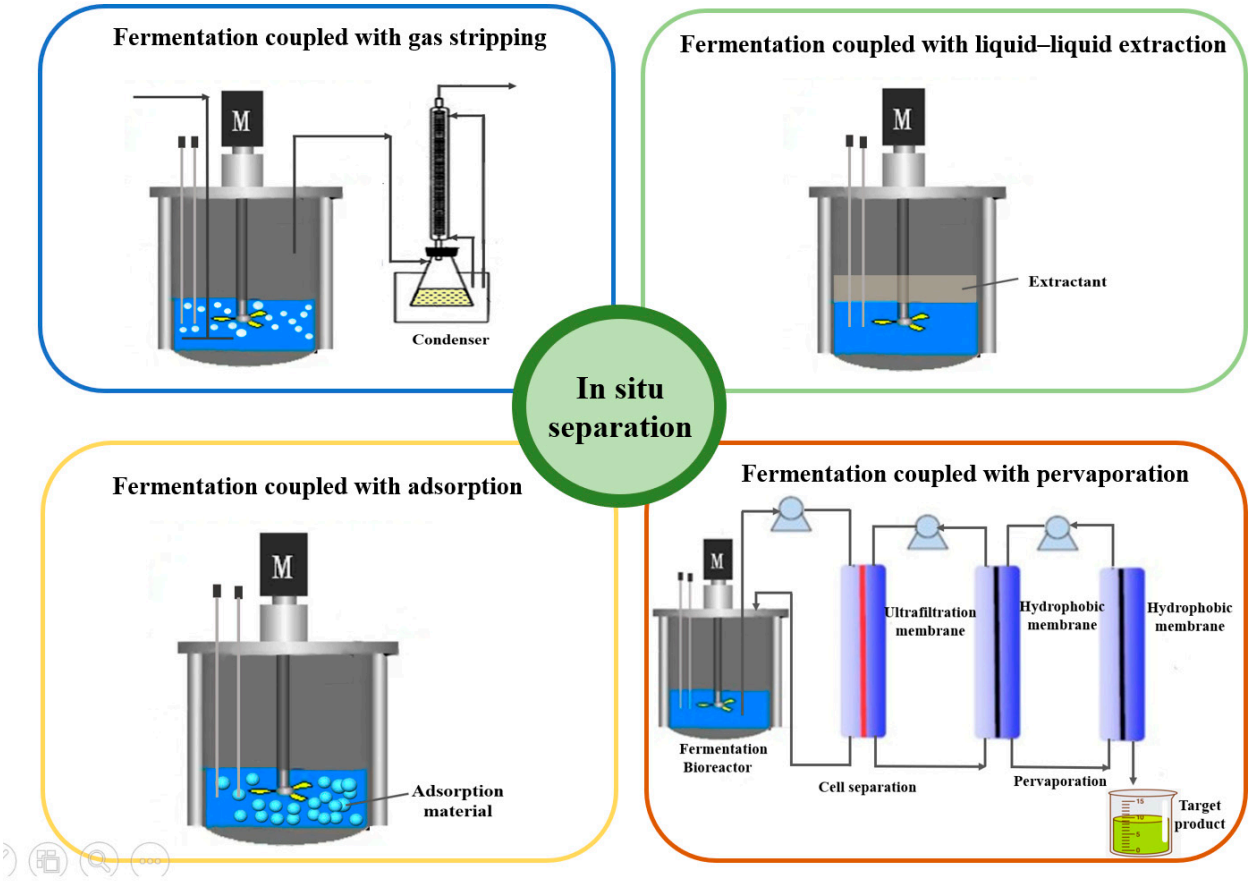

Figure 3. Fermentation coupled with in situ separation.

Table 4. Research on fermentation coupling online Separation.

Separation Method	Titer (g/L)		Products Titer in the Condensate (g/L)		Strategy	Reference
	Butanol	ABE	Butanol	ABE		
Gas stripping	113.2	172.1	150.5	195.9	*C. acetobutylicum* JB200	[78]
Gas stripping	185.7	267.2	624.1	665	Symbiotic system TSH06	[75]
Gas stripping	66.1	106.3	150.6	~200	*C. acetobutylicum* B3	[79]
Liquid–liquid extraction	36.7	--	--	--	*C. acetobutylicum*; Oleyl alcohol; Ionic liquid	[80]
Liquid–liquid extraction	21.4	31.6	--	--	*C. acetobutylicum* ATCC 824; 2-butyl-1-octanol	[81]
Pervaporation	34.5	54.9	169.6	253.3	*C. acetobutylicum* ATCC 55025; Hydrophobic micro-zeolite-mixed matrix membrane	[82]
Pervaporation	18.95	30.83	--	--	*C. beijerinckii* ZL01; PDMS-PVDF composite membrane	[77]
Adsorption	92.6	130.7	--	--	*C. acetobutylicum* B3; Macroporous adsorption resin KA-I	[83]
Adsorption	54.6	--	167.1	190.4	*C. acetobutylicum* JB200; Norit ROW0.8	[84]

5.4. Cocultivation

Cocultivation technology is a fermentation technique that utilizes metabolic pathways between different strains of microorganisms to achieve a specific fermentation or growth objective through synergistic interactions. It is a new fermentation technology that can

achieve similar effects without complex DNA recombination or synthetic biology. Broadly speaking, cocultivation is the symbiotic interaction in nature, in which microorganisms achieve symbiosis by exchanging metabolites, and metabolic interaction is a common interaction in microbial communities [85,86]. In recent years, cocultivation technology has been widely applied in various industries, such as food, agriculture, biodegradation, ethanol, and butanol production [87]. The key to cocultivation technology is to design a stable microbial community, and common strategies include (1) using strains with different carbon source preferences to reduce interspecific competition and (2) establishing cooperative relationships to increase the correlation between strains [88]. Currently, researchers have carried out relevant work based on cocultivation technology in butanol fermentation systems.

5.4.1. Improving Butanol Production

Bergstrom et al. used a mixed system of *C. butylicum* and *C. pasteurianum* for butanol fermentation, and the production increased by 20% compared to *C. butylicum* alone, mainly because *C. pasteurianum* could rapidly synthesize butyric acid and a certain titer of butyric acid could promote butanol production using *C. butylicum* [89]. Similar systems include a mixed system of *C. tyrobutyricum* and *C. beijerinckii*, in which *C. tyrobutyricum* mainly synthesizes butyric acid and *C. beijerinckii* produces butanol [90]. Luo et al. cocultured *C. acetobutylicum* and *S. cerevisiae* and added butyrate to the culture medium. The butanol production increased by 35%, reaching 15.74 g/L. Through the analysis of the interaction between the strains, it was found that the secretion of certain beneficial amino acids, especially lysine, promoted the generation of butanol. At the same time, the cocultivation system promoted the generation of intracellular NADH in *C. acetobutylicum*, thereby promoting butanol synthesis [91].

5.4.2. Synthesis of Butanol from Cellulose-Based Raw Materials

In the process of using lignocellulosic biomass to produce butanol through fermentation, Yu et al. cocultured *C. thermocellum* and *C. acetobutylicum*, where *C. thermocellum* secretes cellulase to degrade cellulose into monosaccharides, while *C. acetobutylicum* produces butanol. In this mixed fermentation system, without the addition of the cellulase, the total acid titer reached 5.9 g/L, while the ABE titer was only 1.4 g/L [92]. Nakayama et al. cocultured *C. thermocellum* and *C. saccharoperbutylacetonicum* N1–4 using microcrystalline cellulose as the raw material, and the butanol titer reached 5.8 g/L with a yield of 0.15 g/g [93]. Salimi et al. cocultured *C. cellulolyticum* and *C. acetobutylicum* and studied their cooperative relationship and found that there may be a substance exchange between the two microorganisms, such as acetone. The results showed that *C. cellulolyticum* could degrade cellulose into monosaccharides, providing substrates for the growth and metabolism of *C. acetobutylicum*. Under this cocultivation system, the butanol titer reached 350 mg/L [94].

5.4.3. Enhanced Oxygen Tolerance

Tran et al. attempted to coculture *B. subtilis* WD161 and *C. butylicum* TISTR1032 under nonstrict anaerobic conditions using cassava starch as the substrate for fermentation to produce butanol. Compared with the cultivation of *C. butylicum* TISTR1032 alone, adding *B. subtilis* WD161 significantly improved amylase activity and solvent production. The amylase activity in the fermentation broth increased 9-fold, reaching 17 U/mL, and the total ABE production increased 5.5-fold, reaching 8.9 g/L, with a butanol titer of 5.8 g/L [38]. Wu et al. isolated the symbiotic system TSH06, which can produce butanol under non-strict anaerobic conditions. After isolation and gene identification, it was found that TSH06 was composed of *Clostridium acetobutylicum* and *Bacillus cereus*, and under nonstrict anaerobic conditions, the total solvent titer reached 18.3 g/L [95,96]. Ebrahimi et al. cocultured *C. acetobutylicum* and *Nesterenkonia* sp. strain F, and under nonstrict anaerobic conditions, the butanol titer reached 13.6 g/L [97].

6. Issues and Challenges

Although ABE fermentation has been developed for over 150 years, currently, chemical synthesis is still the primary method for the industrial production of butanol. There are three main reasons for this: (1) low butanol titer and low butanol productivity caused by the cytotoxicity of butanol; (2) high cost of grain raw materials; (3) high requirements for equipment and complicated operations due to strict anaerobic fermentation conditions.

6.1. Toxicity of Butanol to Cells

Butanol contains hydrophobic groups, which can cause cytotoxicity in cells. The hydrophobicity of butanol can destroy the phospholipid structure of the cell membrane, and the destruction of the phospholipid structure can increase the permeability of the cell membrane. The cell membrane is a barrier for the selective passage of substances inside and outside the cell, and the increase in its permeability can destroy the barrier function [98]. When the butanol titer in the fermentation broth reaches a certain titer, it will inhibit cell growth, affect the progress of fermentation, and eventually force fermentation to stop. In general, the titer of butanol in the fermentation broth does not exceed 13 g/L, and the total titer of ABE does not exceed 20 g/L. A low product titer causes high energy consumption and high cost for subsequent separation and purification.

From the perspective of strain modification, after nearly 30 years of effort, the highest butanol production is 20 g/L (*C. acetobutylicum* JB200). In the future, it will remain an important research direction to continue screening for butanol-tolerant bacteria.

From the perspective of fermentation technologies, continuous fermentation and fermentation coupled with in situ separation can effectively improve productivity and achieve a high concentration of products. However, the devices for continuous fermentation and fermentation coupled with in situ separation are more complex and prone to contamination by miscellaneous bacteria. Therefore, more attention should be paid to fermentation process control in continuous fermentation and fermentation coupled with in situ separation.

6.2. High-Cost Raw Materials for Butanol Production

Corn has been the primary raw material for butanol production via fermentation. However, due to factors such as population growth, decreased arable land, and environmental concerns, the price of grain continues to rise. Using grain crops as the raw material for butanol fermentation will inevitably increase production costs and lead to competition for food resources. Studies have shown that when corn is used as the raw material for ABE fermentation, raw material costs account for 70% of the total production cost [29]. Therefore, researchers have been devoted to reducing the cost of producing biobutanol by using low-cost raw materials.

Lignocellulose is one of the world's cheapest and most widely available renewable resources, and using it for butanol fermentation is an important research direction for butanol production from nongrain biomass. However, *Clostridium*, which is used for butanol production, cannot directly utilize lignocellulose. It needs to be pretreated first, and after acid hydrolysis and enzyme hydrolysis, cellulose and hemicellulose are degraded into monosaccharides that microorganisms can utilize (mainly glucose, xylose, and arabinose). The pretreatment process is complex and costly, especially the enzymatic hydrolysis process, which increases the operating cost of the entire fermentation process. In addition, there are many byproducts in the hydrolysate, in addition to monosaccharides, which have a significant inhibitory effect on cell growth and fermentation, limiting the industrial application of lignocellulose for butanol fermentation [99]. Therefore, further research on the cellulose butanol direction is needed to make it feasible for large-scale production.

In addition, low-cost starch materials such as cassava can also be considered. Cassava is widely cultivated in subtropical regions, and its advantages are as follows: (1) it has an extremely high utilization rate of climate resources and can carry out high-intensity photosynthesis and rapidly accumulate biomass; (2) it does not compete with grain for land and has the characteristics of drought resistance and tolerance to barren soil; and (3) it

has a high starch content, with a starch content of 30~35% in fresh cassava tubers and over 70% in dried cassava chips [100].

6.3. High Standard of Equipment

Clostridia, which are used for butanol fermentation, are all strictly anaerobic bacteria. Due to the lack of peroxidases, catalases, or superoxide dismutase in the cell, which can eliminate the toxic product (superoxide anion free radicals) generated under aerobic conditions, these microorganisms are extremely vulnerable to oxygen damage. Strict anaerobic fermentation makes the fermentation process complex and requires a high standard of equipment. Some studies have achieved the butanol production via *Clostridium* under non-strict anaerobic conditions through co-cultivation.

7. Conclusions and Prospects

Biobutanol is an important renewable bioresource, and it holds great promise in replacing petrochemical butanol due to its significant advantages. This paper provides a review of the research progress in butanol production from the perspectives of butanol-producing strains, raw materials for butanol production and butanol fermentation technologies. The paper also summarizes the main challenges currently constraining the industrial development of fermentation-based butanol production. In the future, researchers can make efforts in the following areas:

Alleviating the toxicity of butanol to cells: The toxic effect of butanol on cells can be alleviated by improving the tolerance of bacteria to butanol or maintaining the low butanol titer in fermentation broth. At present, researchers have partially alleviated the cytotoxicity of butanol to cells through genetic engineering, continuous fermentation, fermentation coupled with online separation, coculture, and other methods. In the future, relevant technologies can be further developed to effectively increase butanol production.

Developing low-cost raw materials for butanol production: Lignocellulose is an option as a low-cost raw material, but there is still a need for extensive research on simplifying the complex pretreatment process, improving the tolerance of strains to inhibitors, and enhancing the utilization efficiency of mixed sugar systems. In addition, low-cost starch materials such as cassava can also be considered. Low-cost starch materials are more easily utilized directly by microorganisms.

Simplifying the butanol fermentation process: In the future, researchers can continue to use cocultivation technology or other methods to enable butanol fermentation to be carried out under nonstrict anaerobic conditions, simplify the fermentation process, and reduce production costs.

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