Advances in butanol production by clostridia

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Increasing oil prices and growing awareness of global warming has brought significant attention to the production of biofuels from biomass. Compared to the traditional biofuel ethanol, butanol has many advantages because of its non-hygroscopicity and low vapour pressure; it can be added to gasoline at any concentration. Therefore, butanol production by fermenting acetone-butanol-ethanol (ABE)-producing clostridia has drawn much attention in recent years. However, despite its many advantages, a typical ABE fermentation process is limited by low product concentrations, low productivity, low yield, and difficulty in controlling culture metabolism. These problems led to a decline in the use of this process in the 20th century. To overcome these problems, till date considerable research on ABE-producing clostridia has been conducted in various fields, including microbial technology, metabolic engineering, and systems biology. This article reviews recent advances in butanol production by ABE-producing clostridia and discusses the possibilities, remaining challenges, and prospects of butanol production.

Keywords biobutanol; acetone-butanol-ethanol-producing clostridia; high productivity; high yield; kinetic model

1. Introduction

Increasing crude oil prices and growing awareness of environmental problems, such as global warming, attributed to the use of fossil fuels, has brought significant attention the production of biofuels from biomass [1]. Because biomass is renewable, abundant, and limitless, and its use is often regarded as carbon neutral, the combustion of biofuels derived from biomass releases fewer greenhouse gases, such as carbon dioxide, than does fossil fuels [2-4]. Biodiesel (fatty acid methyl esters) [5] and bioethanol [6] have attracted considerable attention and have been the focus of interdisciplinary and industrial research for the past several decades. Biodiesel is produced from triglycerides, present in various food oils or waste oils, by transesterification with methanol using acidic [7], alkaline [8], or enzymatic catalysts [9], yielding glycerol as a by-product [5]. Many bioethanol production methods have also been developed by the fermentation of from different types of biomass such as starch, lignocellulose, or agricultural and forest residues by *Zymomonas mobilis* [10], *Saccharomyces cerevisiae* [11, 12], *Pichia stipitis* [13], and *Corynebacterium glutamicum* [14]. Biobutanol, one of the bioalcohols, has also gained considerable attention in recent years. Compared with the traditional biofuel ethanol, butanol has the following advantages [2, 15, 16]:

- —has a higher energy content;
- —can be applied in pure form or blended in any concentration with gasoline or diesel;
- —can be used in any automobile engine without modifications;
- —is non-hygroscopic, and thus, it is easy to preserve and distribute as it does not absorb water;
- —has a lower vapour pressure, making it more safe to use;
- —is less corrosive, and thus, it can be used with the existing infrastructure; and
- —can be converted to valuable chemical compounds such as acrylate, methacrylate esters, glycol ethers, butyl acetate, etc.

These properties suggest that biobutanol could be the next generation biofuels.

Biobutanol is produced by acetone-butanol-ethanol (ABE) fermentation using several genera of bacteria, particularly clostridia, yielding acetone and ethanol [17]. ABE fermentation was one of the first large-scale industrial fermentation processes to be developed. During the early 20th century through World Wars I and II, ABE fermentation was important for the production of butanol and acetone solvents. However, its use has declined since the 1950s owing to increasing costs of the substrate molasses, and the availability of much cheaper feedstocks for chemical solvent synthesis by the petrochemical industry, except in South Africa, the Soviet Union, and China [2]. The 1973 oil crisis led to renewed interest in solvent production by ABE fermentation. In particular, butanol produced by ABE fermentation has been an attractive biofuel alternative because of the advantages described above. In the present century, there have been numerous studies on butanol production in various fields, and many companies have declared their plans for the commercial production of biobutanol using biomass [18].

This review describes the significant reports and knowledge on advances in butanol production in the several fields. We place particular focus on the development of a butanol production system, improvements in butanol production by

molecular breeding, and the establishment and application of metabolic models in the fields of microbial technology, metabolic engineering, and systems biology. We also discuss the remaining challenges and prospects of butanol production.

2. Biochemical and physiological properties of ABE fermentation

2.1 Types and characterization of ABE-producing clostridia

There are several wild strains of ABE-producing bacteria, dominated by clostridia, which are gram-positive, spore-forming obligate anaerobes [15]. ABE-producing clostridia were isolated from various sources, identified by biochemical methods, and were assigned a number of different species names until the 1980s [17]. Recently, ABE-producing clostridia were reclassified into 4 species—*Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*—using the molecular biological methods of 16S rRNA sequencing, DNA fingerprinting, and DNA-DNA hybridization [19-22]. Because these 4 ABE-producing clostridia show no cellulolytic activity and do not directly use cellulosic biomass, their substrate spectrum was broadened by the transfer of genes encoding cellulase from other organisms into ABE-producing clostridia [23-26]. Recently, several cellulolytic ABE-producing clostridia have been isolated, identified, and characterized, and have been found to produce acetone, butanol, and/or ethanol from Avicel [27]. *C. pasteurianum* is also reported to produce ABE by fermentation of glycerol as the sole carbon source [28].

ABE-producing clostridia possess a broad substrate utilization ability; they can use many types of carbon sources such as glucose, sucrose, lactose, xylose, xylan, starch, and glycerol [17, 29, 30]. Furthermore, there are many reports on ABE fermentation by different strains using various biomass substrates such as a hardwood [31], domestic organic waste [32], agricultural waste [33], corn fibre [34], palm oil waste [35], excess sludge [36], whey [37], sago starch [38], etc. These findings may help reduce fermentation substrate costs.

2.2 Metabolic properties of ABE-producing clostridia

ABE hetero-fermentation produces acetate, butyrate, ethanol, and acetone, as well as butanol. The metabolism of ABE-producing clostridia can be divided into the following 2 distinct phases: acidogenesis (acid-production) and solventogenesis (solvent-production) during the exponential and stationary phases of growth [17]. The metabolic pathways of ABE-producing clostridia are summarized in Fig. 1 [17, 39]. Carbon flow proceeds through hexose sugars that are metabolized via the Embden-Meyerhof-Parnas pathway; 1 mol hexose is converted to 2 mol pyruvate, with net production of 2 mol ATP and NADH. Pentose sugars are metabolized via the pentose phosphate pathway, by which they are converted to pentose 5-phosphate and dissimilated by the transketolase-transaldolase sequence, producing fructose 6-phosphate and glyceraldehyde 3-phosphate, which enter the glycolytic pathway [40]. The fermentation of 3 mol pentose yields 5 mol pyruvate, 5 mol ATP and 5 mol NADH. The pyruvate produced from glycolysis is then cleaved in the presence of coenzyme (CoA) to produce carbon dioxide, acetyl-CoA, and reduced ferredoxin. Acetyl-CoA is subsequently condensed, reduced, or dehydrated to yield CoA-derivatives such as acetoacetyl-CoA and butyryl-CoA. The 3 CoA-derivatives are the central intermediates leading to both acid and ABE production [17].

During acidogenesis, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA, respectively; ATP is also produced. Accumulation of these organic acids reduces the culture pH. The organic acids are then re-utilized in solventogenesis, when the culture pH begins to rise. The re-utilization of acetate and butyrate is generally considered to occur via the acetoacetyl-CoA:acetate/butyrate:CoA transferase (CoAT) pathway, and the reverse pathway generates the organic acids [17, 41, 42]. The former pathway is directly coupled to the production of acetoacetate, which is irreversibly decarboxylated to form acetone. In either pathway, acetate and butyrate are converted into acetyl-CoA and butyryl-CoA and then into ethanol and butanol by several reductive reactions without carbon loss.

ABE-producing clostridia generate excess reducing equivalents by glycolysis or the reaction of pyruvate-ferredoxin oxidoreductase [43]. During acidogenesis, hydrogen functions as an acceptor of both excess protons and electrons, increasing hydrogen production. Solventogenesis, reduces hydrogen production, and excess reducing equivalents such as NADH or NADPH are alternatively oxidized to produce butanol or ethanol. The metabolism of ABE fermentation is thus controlled by carbon and electron flows, the fermentation yield depending on the strain and culture conditions, which leaves the main production difficulty in maintaining control of the fermentation state.

2.3 Problems and limitations of ABE fermentation

Butanol production by ABE fermentation could not compete economically with petrochemical synthesis. In order to introduce an economically competitive biological process, 5 major drawbacks in typical batch fermentation must be overcome [17]:

I. High substrate costs (e.g. molasses)
II. Low final butanol concentration (<20 g/L) caused by butanol inhibition

III. Low butanol productivity (<0.5 g/L/h) due to low cell density caused by butanol inhibition
IV. Low yield of butanol due to hetero-fermentation (0.28–0.33 g/g)
V. High cost of butanol recovery from low-concentration yields (distillation was used in the past)

To decrease substrate costs (drawback I), research has focused on the identification of biomass alternatives as abundant and inexpensive substrates for ABE fermentation (section 2.1). To solve the remaining problems (drawbacks II–V), various approaches have been investigated in the fields of microbial technology, metabolic engineering, and systems biology; these findings are described in detail in section 3.

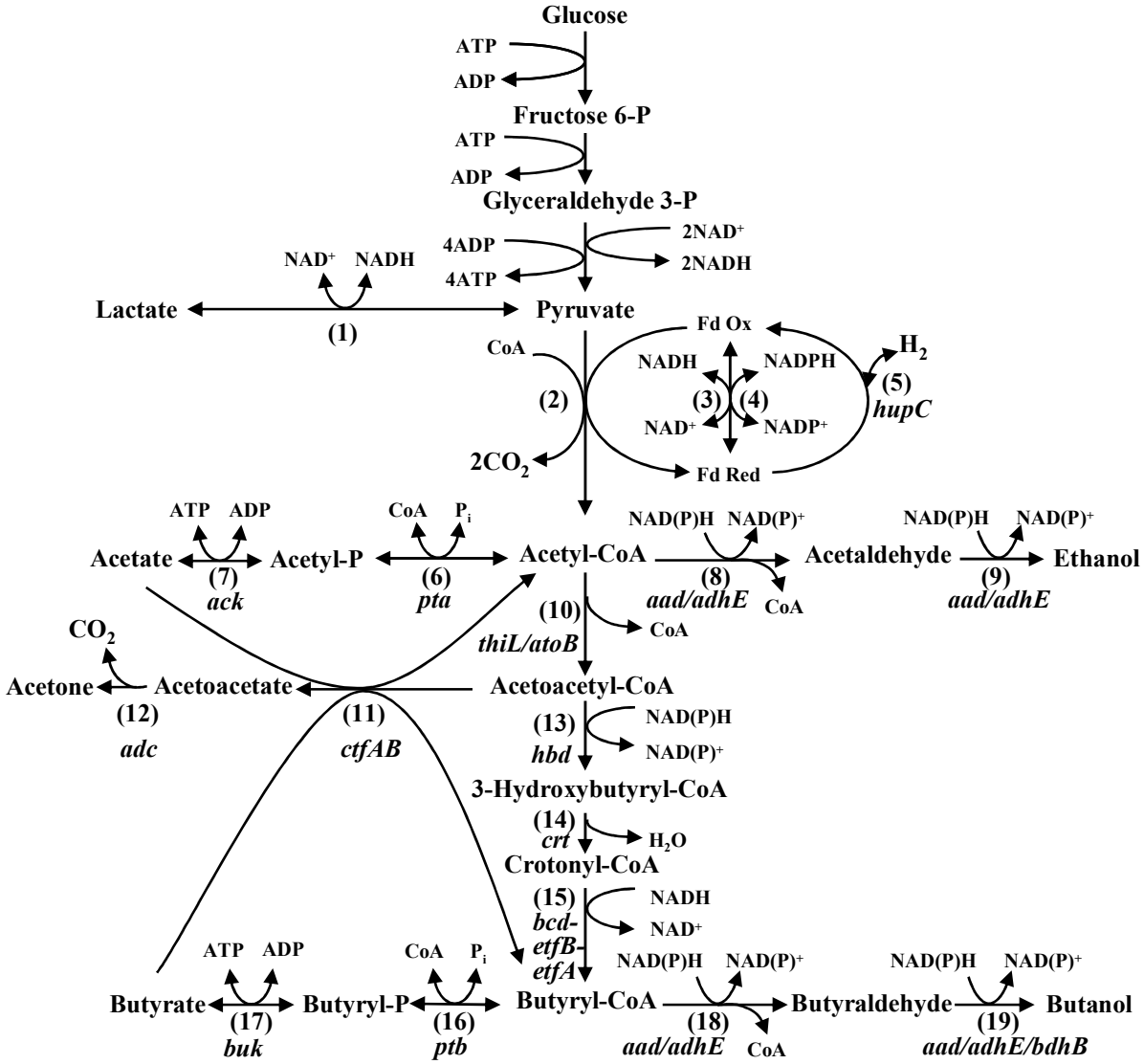

Fig. 1 Metabolic pathways of glucose metabolism in *C. acetobutylicum* [17]. Enzymes are indicated by letters as the followings: (1) lactate dehydrogenase; (2) pyruvate-ferredoxin oxidoreductase; (3) NADH-ferredoxin oxidoreductase; (4) NADPH-ferredoxin oxidoreductase; (5) hydrogenase; (6) phosphate acetyltransferase (phosphotransacetylase); (7) acetate kinase; (8) acetaldehyde dehydrogenase; (9) ethanol dehydrogenase; (10) thiolase (acetyl-CoA acetyltransferase); (11) acetoacetyl-CoA;acetate/butyrate:CoA transferase; (12) acetoacetate decarboxylase; (13) 3-hydroxylbutyryl-CoA dehydrogenase; (14) crotonase; (15) butyryl-CoA dehydrogenase; (16) phosphate butyltransferase (phosphobutyrylase); (17) butyrate kinase; (18) butryraldehyde dehydrogenase; (19) butanol dehydrogenase. The several genes encoding each metabolic enzyme are also indicated with abbreviations in the metabolic map.

3. Advances in butanol production in various fields

3.1 Advances in butanol production system in the field of microbial technology

Industrial ABE batch fermentation has a number of limitations, which must be overcome to make butanol fermentation an economically competitive process [17, 44]. It has generally been observed that the cell densities generated by

anaerobic fermentation, including ABE fermentation, are lower than those generated by aerobic fermentation [45]. In addition, butanol produced during cultivation exerts a strong inhibitory effect on cell growth and butanol production; these lead to low cell densities, low final butanol concentrations, low butanol productivity, and the added complication of butanol recovery from the culture broth [17, 46].

Researchers in the field of microbial technology have conducted numerous studies to establish an efficient method of butanol production. In this section, we focus on butanol production in each fermentation mode (batch, fed-batch, and continuous) using growing or living ABE-producing clostridia and with integrated butanol recovery operations.

3.1.1 Improvement of butanol production using growing cells

Enhanced butanol production has been achieved by alternating the addition of substances to drive carbon or electron flow toward butanol production in ABE-producing clostridia in batch, fed-batch, or continuous cultures. Organic acids, such as acetate, butyrate, lactate, and propionate, are used to drive carbon flow. ABE-producing clostridia produce acetic and butyric acids during acidogenesis and re-utilize them during solventogenesis, giving these organic acids great potential as substrates for butanol production. Chen and Blaschek reported that addition of 60–80 mM acetic acid to batch cultures of *C. beijerinckii* NCIMB 8052 and BA 101 (a mutant derived from 8052) not only increased ABE production in these strains from 6.7 g/L and 6.1 g/L to 17.8 g/L and 20.8 g/L, respectively, but also prevented the degeneration of ABE production capacity [47, 48]. Increased ABE production was also observed in batch culture, glucose-fed batch culture, and continuous culture of *C. acetobutylicum* with the addition of butyric acid or acetic acid [49-51]. In batch culture of *C. saccharoperbutylacetonicum* N1-4, acetic acid promoted only acetone production while both acetone and butanol production were enhanced with the addition of butyric acid up to 5.0 g/L [52]. Furthermore, Tashiro et al. investigated a fed-batch culture of the N1-4 strain with pH-stat continuous butyric acid and glucose feeding, and achieved a 1.5-fold greater yield of butanol to glucose (0.55 g/g) and to cells (3.7 g/g) at a feeding ratio of butyric acid to glucose of 1.4, compared with yields in batch culture of 0.32 g/g and 2.4 g/g, respectively. They also succeeded in establishing stable concentrations of butyric acid at approximately 1 g/L and an almost zero concentration of residual glucose in the broth by applying the pH-stat feeding method [52]. Although lactic acid is produced under specific conditions [17, 53], the effect of lactic acid on ABE fermentation using the N1-4 strain was also investigated. Oshiro et al. found that both the concentration and yield of butanol increased in medium containing 20 g/L glucose supplemented with 5 g/L of a racemic mixture of lactic acid [54]. In addition, butanol production associated with lactic acid consumption increased from 6.62 g/L in batch culture without pH control to 12.6 g/L in a pH-controlled fed-batch culture (pH 5.5). Butanol concentrations were elevated up to 15.5 g/L in fed-batch culture by applying a pH-stat continuous lactic acid and glucose feeding method. Addition of 19 mM propionate yielded 19 mM *n*-propanol in *C. acetobutylicum*, a previously unknown product of ABE fermentation [55]. Note that the utilization of the 4 organic acids cited here occurred in the presence of sugars like glucose, because of the requirement for NADH or ATP generated by sugar metabolism [52, 54]. Isotope analysis suggested that while *C. acetobutylicum* converts more than 55% of acetic acid or 84% of butyric acid to butanol [56], 54% of lactic acid utilized by the N1-4 strain should be converted to butanol [54].

Carbon monoxide (CO), hydrogen (H_2) partial pressure, and artificial electron carriers were reported as significant factors driving electron flow in ABE-producing clostridia. CO addition to batch culture of *C. acetobutylicum* yielded increases concentrations of butanol (from 65 to 105 mM) or ethanol (from 5.1 to 13.4 mM) and decreased H_2 production due to CO inhibition of hydrogenase activity [57], consistent with the report by Datta and Zeikus [58]. While both butanol and ethanol production increased with increasing H_2 partial pressure in *C. acetobutylicum* batch culture, and was accompanied by decreased production of acetone and H_2 [59], H_2 escape from the fermentor headspace continued to produce H_2 without butanol and ethanol in *C. saccharoperbutylacetonicum* batch culture [60]. Artificial electron carriers such as methyl viologen and neutral red drastically changed the behaviour of metabolites in both batch and continuous cultures of ABE-producing clostridia. Methyl viologen addition (1 mM) to *C. acetobutylicum* culture not only enhanced the butanol yield from 0.41 to 0.58 mol/mol in batch culture [61], but also increased the specific rates of butanol production from 1.65 to 2.11 mmol/g/h in continuous culture at a dilution rate of 0.05 h^{-1} [62]. Similar results were found with neutral red which, when added to continuous cultures of *C. acetobutylicum*, yielded higher concentrations of butanol and ethanol, together with lower concentrations of H_2, acetic acid, or butyric acid [63]. These studies make clear the significance of both carbon and electron flows in the optimization of butanol production from ABE fermentation in clostridia, for an establishment of highly efficient butanol production system.

As mentioned in section 2.3, a typical batch culture exhibits volumetric butanol productivity below 0.5 g/L/h due to low cell density and butanol inhibition. Continuous culture is one potential strategy for reducing butanol inhibition by diluting butanol in the broth with fresh medium. In a chemostat culture of N1-4 using a glucose substrate, however, cell wash-out occurred at a dilution rate of 0.26 h^{-1}, which resulted in low ABE productivity (1.85 g/L/h) [64]; similar observations were made in *C. acetobutylicum* [65] and *C. beijerinckii* [66]. Numerous high-density continuous cultures of growing cells were investigated using 2 methods: cell immobilization on various types of carriers and cell-recycling with microfiltration or ultrafiltration modules.

In comparison to free-growing cells, cell immobilization has the advantage of continuous operation with high cell density, cell reutilization, stabilization of metabolic activity in ABE-producing clostridia, and a smaller-scale fermentor.

Table 1 summarizes the studies of high-density continuous cultures of each ABE-producing clostridium by cell immobilization on various types of carriers and substrates such as glucose, lactose, sucrose, and starch. Continuous cultures by cell immobilization prevented cell wash-out from occurring at dilution rates up to 2.0 h^{-1} and yielded improvements in ABE productivity. Among the ABE production systems so far published, Lienhardt's bioreactor with cell immobilization on a brick showed the maximum ABE productivity of 16.2 g/L/h at a dilution rate of 2.0 h^{-1} using *C. beijerinckii* BA101 and glucose [67]. Degeneration was a known problem in typical continuous cultures over long operational periods [68]. High cell density by cell immobilization seemed to provide longer operational stability; in particular, an operational period of more than 60 days degeneration-free was acquired in continuous culture by using bone-char immobilized *C. saccharobutylicum* NCP 262^T [69].

Table 1 Summary of ABE productivity in continuous culture with high density of cell-immobilization.

Strain[a]	Carrier	Substrate[b]	D[c] (h^{-1})	Concentration (g/L)	Productivity (g/L/h)	Reference
C.ace ATCC 824^T	Coke	Glc	0.1	11.2	1.12	[70]
C.ace ATCC 824^T	Sponge	Glc	0.272	15.4	4.2	[71]
C.ace DSM 792	Plastic rings	Lac	0.97	5.19	5.01	[72]
C.ace NRRL-B-643	κ-carrageenan	Glc	0.071	4.91	0.35	[73]
C.ace ATCC 55025	Fibrous matrix	Glc+BA	0.9	5.1[d]	4.6[e]	[74]
C.bei LMD 27.6	Ca Alginate	Lac	0.7	1.43	1.0	[75]
C.bei LMD 27.6	Ca Alginate	Glc	0.333	2.4	0.8	[75]
C.bei NCIMB 8052	Porous polyvinyl alcohol	Glc+BA	0.04	13.4[d]	0.4[e]	[76]
C.bei BA 101	Brick	Glc	2.0	8.1	16.2	[67]
C.bei ATCC 55025	Corn stalk	Glc	1.0	5.1	5.06	[77]
C.sac spoA2	Ca Alginate	Suc	0.196	8.37[d]	1.64[e]	[78]
C.sac NCP 262^T	Bonechar	Lac	1.0	4.1	4.1	[69]
C.sac NCP 262^T	Beads	Starch	0.129	7.32	0.944	[79]

[a]*C.ace*; *C. acetobutylicum*, *C.bei*; *C. beijerinckii*, *C.sac*; *C. saccharobutylicum*. [b]Glc; glucose, Lac; lactose, BA; butyric acid, Suc; sucrose. [c]Dilution rate. [d]Butanol concentration. [e]Butanol productivity.

A few high cell-density continuous cultures methods have been explored using ultrafiltration or microfiltration membrane modules to recycle flow-through cells. Compared to bioreactors with immobilized cells, cell-recycling bioreactors have the advantage of broth homogeneity, which facilitates diffusion in the bioreactor, and the total recycling of microorganisms [80]. Because the cells were not drawn out from the fermentor, cell densities increased to more than 100 g/L under dilution rates of 0.33–0.85 h^{-1}, higher than the critical dilution rate of 0.26 h^{-1} in a typical continuous culture without cell-recycling [64]; this method yielded more than 4.06 g/L/h of ABE productivity (Table 2). Among the cell-recycling bioreactors reported so far, the highest ABE productivity of 11.0 g/L/h was obtained by cell-recycling of the N1-4 strain using a microfiltration module [64]. Two difficulties have arisen during the operation of cell-recycling high cell-density bioreactors: more than 100 h of culture are required to achieve a cell density of 20 g/L [80] and the volume of broth cannot be controlled due to the viscosity of the culture [64]. To solve these critical problems, Tashiro et al. attempted the *in situ* 10-fold concentration of active cells in broth culture, followed by cell bleeding and recycling [64]. This method yielded 20 g/L cell density after only 12 h, and the cell concentration was maintained at approximately 33 g/L throughout the experiment at 0.11–0.16 h^{-1} dilution rates for cell bleeding; the culture was maintained over an operational period of more than 207 h with an overall ABE productivity of 7.55 g/L/h.

Table 2 Summary of ABE productivity in continuous culture with high density of cell-recycling and bleeding.

Strain[a]	Module[b]	Substrate[c]	D_1[d] (h^{-1})	D_2[e] (h^{-1})	Operational period (h)	Concentration (g/L)	Productivity (g/L/h)	Reference
C.sacc N1-4	MF	Glc	0.85	–[f]	48	12.9	11.0	[64]
	MF	Glc	0.76	0.09	60	11.5	9.77	[64]
	MF	Glc	0.71-0.74	0.11-0.16	>207	8.58	7.55	[64]
C.ace ATCC 824^T	UF	Glc	0.33	–[f]	70	9.30-20.5	4.34	[80]
C.ace ATCC 824^T	UF	Glc	0.64	–[f]	–	8.44	5.40	[81]
C.ace ATCC 824^T	UF	Glc	0.435	0.065	50	13.0	6.50	[82]
C.ace DSM 1731	UF	Glc	0.40	–[f]	–	10.3	4.10	[83]
C.sac NCP 262^T	MF	Lac	0.39	0.02	191	9.90	4.06	[84]

[a]*C.sacc*; *C. saccharoperbutylacetonicum, C.ace*; *C. acetobutylicum*, *C.sac*; *C. saccharobutylicum*. [b]MF; Microfiltration, UF; Ultrafiltration. [c]Glc; glucose, Lac; lactose. [d]Dilution rate for permeate. [e]Dilution rate for cell-bleeding. [f]Minus indicates without cell-bleeding.

3.1.2 Advances in butanol production by living cells

Butanol production occurs during stationary phase (solventogenesis) and is not considered to be associated with cell growth; Butanol production should be feasible in living cells (resting or non-growing cells), which are alive and have butanol-producing ability but do not proliferate when under nitrogen-limiting conditions. The use of living cells has several advantages over growing cells: the feasibility of continuous operation with high cell density, the re-utilization of cells, and a decrease in by-products. Continuous butanol production with high-density immobilized living cells was examined by several researchers [85-87]. Living *C. acetobutylicum* ATCC 824^T immobilized to beechwood shavings exhibited the maximum ABE productivity of 1.19 g/L/h at a dilution rate of 0.374 h^{-1} using glucose as a substrate [86]. Note that this value is much lower than those observed using high-density immobilized growing cells [67] and cell recycling [64]. A drastic decrease in ABE productivity over time was also observed, presumably due to the lack of enzyme regeneration under nitrogen-limited conditions [87]. To maintain the activity of living cells, the growth medium is intermittently supplied cells during continuous butanol production [86]. By this method, the operational period could be prolonged to more than 30 days by intermittent dosing of nutrient medium for 15 min every 7 h.

A novel butanol production system in living N1-4 strain was also reported. The metabolic pathways in ABE-producing clostridia are shown in Fig. 1; when hexose (glucose) is used as a carbon source, 2 molecules of carbon are lost as carbon dioxide by pyruvate-ferredoxin oxidoreductase, which yields less than 0.667 mol/mol, the maximum theoretical yield of butanol to carbon source. A yield of 0.671 mol/mol of butanol to butyrate and glucose was accomplished by addition of 0.1 mM methyl viologen as an artificial electron carrier [88]. To our knowledge, this is the only report to exceed the theoretical yield of butanol.

3.1.3 Integration of butanol recovery with butanol production

Butanol production has a strong feedback-inhibitory effect its own production and on cell growth, which leads to low cell densities and low butanol production (final concentration, productivity, and yield) [17]. To eliminate butanol inhibition, any one of several butanol recovery techniques can be applied either in batch-, fed-batch-, or continuous cultures. Methods developed by researchers in the combined fields of microbial technology and separation engineering utilize liquid-liquid extraction [3, 89-91], nitrogen gas-stripping [92-94], membrane pervaporation [95, 96], adsorption [97, 98], and perstraction using polypropylene and biodiesel [99]. Culture techniques with integrated *in-situ* butanol recovery are very advantageous because the butanol concentration throughout the culture may be maintained below the level of butanol inhibition. Ezeji et al. succeeded in drastically improving butanol production from glucose by gas-stripping using *C. beijerinckii* BA 101 in a fed-batch culture compared with that in a typical batch culture: ABE concentration based on the volume in bioreactor, 17.6 g/L to 232.8 g/L; yield of ABE to glucose, 0.39 g/g to 0.47 g/g; ABE productivity, 0.29 g/L/h to 1.16 g/L/h [93]. Note that these fermentation systems should also effectively reduce the cost of butanol recovery from a concentrated butanol solution.

3.2 Enhancement of butanol production by using molecular breeding in the field of metabolic engineering

Metabolic engineering aims to improve the metabolic capabilities of industrially relevant microorganisms [100]. Over the past decade, in step with progress in molecular biology, studies on ABE fermentation have sought to enhance butanol production in terms of final concentration, productivity, or yield, to improve butanol tolerance, and to broaden substrate specificity. Two classes of organisms have been studied: original ABE-producing clostridia and other microorganisms which have been developed by such genetic manipulations as gene knockout or overexpression. Here we review the reports on butanol production by several metabolically engineered strains.

3.2.1 Investigation of butanol production using ABE-producing clostridia

Metabolic engineering studies have extensively focused on *C. acetobutylicum* ATCC 824^T and its mutant derivatives because several genetic engineering tools have been developed for this strain, such as shuttle vectors, transformation techniques, and knockout/knockdown systems [15]. Studies are generally classified into 3 groups according to the function of the targeted genes and modification aims in *C. acetobutylicum*, *C. beijerinckii*, or *C. saccharoperbutylacetonicum*: (1) metabolic enzymes for the alteration of product concentration, yield, or ratios; (2) enzymes necessary for polysaccharide degradation; (3) other proteins responsible for butanol tolerance or stress.

First, based on the findings of previous studies, several genes encoding metabolic enzymes were selected, and then knocked-out/down (*buk* [101-104], *ptb* [102], *ack* [104], *pta* [101], *adc* [105, 106], and *ctfAB* [105, 107, 108]) and/or overexpressed (*aad*/*adhE* [103, 104, 107, 108] and *thiL* [108]) in *C. acetobutylicum* strains, and knocked-down (*hupC* [109]) in the N1-4 strain. These studies successfully altered the concentration, yield, and ratios of products. In particular, *buk* gene knockout and *aad* gene overexpression increased the butanol concentration from 9.5 g/L to 16.7 g/L, compared with the wild strain [103]. Furthermore, overexpression promoters were suggested to affect ABE production [104, 108]. Second, *celA* and *celD*, encoding cellobiohydrolase and endoglucanase, respectively, were

overexpressed in *C. beijerinckii* NCIMB 8052, after which glucanase activity and slightly increased ABE production from lichenan were detected [110]. Finally, overexpression of the heat shock proteins *groES* and *groEL* was found to improve butanol tolerance in *C. acetobutylicum,* which then achieved more than 17 g/L butanol production, presumably as a result of the stabilization of solventogenic enzymes [111]. Recently, a novel universal gene knockout system was reported for clostridia [112]. This system may enable the targeted knocked-out of genes in *C. beijerinckii* and *C. saccharoperbutylacetonicum.*

3.2.2 Butanol production by engineered non-ABE-producing microorganisms

Solventogenic genes (e.g. *thiL*/*atoB*, *hbd*, *crt*, *bcd-etfB-etfA*, *adhE1*, *adhE2*, and *bdhB*) derived from ABE-producing clostridia or other microorganisms have been transferred for heterologous expression in non ABE-producers such as *Escherichia coli* [113, 114], *Pseudomonas putida* [115], *Bacillus subtilis* [115], *Lactobacillus brevis* [116], and *S. cerevisiae* [117]. Easy and reproducible methods of genetic manipulation have been established in these organisms, making them ideal selections as hosts. Butanol concentrations produced by these engineered microorganisms were as follows: *E. coli*, 1.2 g/L [113] and 0.552 g/L [114]; *P. putida*, 0.112 g/L [115]; *B. subtilis*, 0.024 g/L [115]; *L. brevis*, 0.3 g/L [116]; and *S. cerevisiae,* 0.0025 g/L [117]. Although butanol production was observed in every engineered microorganism, their yield concentrations were much lower than those by wild strains of ABE-producing clostridia (more than 10 g/L). As reported by Inui et al. [113], butyryl-CoA dehydrogenase activity was low or undetectable in the extract of engineered *E. coli*, which is likely the cause of the strain's low butanol production. Butanol tolerance will be a significant factor in future studies of metabolically engineered non-ABE producing microorganisms.

3.3 Establishment and application of metabolic pathway models for ABE fermentation in the field of systems biology

Metabolic pathway modelling of various microorganisms has been developed in the field of systems biology, and is one of the most successful scientific approaches for achieving the aim of metabolic engineering. Metabolic pathway models fall into 2 categories: stoichiometric models and kinetic simulation models [42]. The former have been used for metabolic flux analysis (MFA) [118], a systematic method developed to assess the roles of individual steps in a metabolic pathway network at steady state, and for optimization of cultivation processes [119]. The latter describe the dynamic behaviour of metabolites and provides for the efficient design of bioreactors and operation strategies [120]. To date, the number of studies on kinetic simulation models is fewer than studies of stoichiometric models because of the difficulty in estimating many of the kinetic parameters [42]. We here describe the reports on metabolic pathway models of ABE fermentation and their applications.

3.3.1 Establishment of several models of metabolic pathways in ABE fermentation

Papoutsakis first reported on a stoichiometric model of ABE fermentation [121]. The model was based on stoichiometric equations of each product including cell mass, reducing equivalents such as NADH and Fd Red, and ATP from glucose as a substrate. The validity of the model was tested in several clostridia, including ABE-producing *C. acetobutylicum* and *C. beijerinckii*, and non-ABE producing *C. butyricum*, *C. falsineum*, and *C. lactoacetophilum*.

Kinetic simulation models of the N1-4 strain were established by Shinto et al. in batch cultures over a wide range of initial concentrations of glucose (36.1 to 295 mM) [42] and xylose (40.7 to 292 mM) [40]. They developed the kinetic equations for metabolic reactions, largely based on the Michaelis-Menten equation with considerations for substrate inhibition, butanol product inhibition, butyrate activation, and the cessation of metabolic reactions under energy insufficiency after substrate exhaustion, and estimated the kinetic parameters. Each of the models for glucose and xylose showed high values of the squared correlation coefficient (>0.9) between experimental and calculated metabolite time-courses.

Recently, Lee et al. constructed a genome-scale metabolic network from the annotated genomic sequence of *C. acetobutylicum* ATCC824^T, consisting of 502 reactions and 479 metabolites [122]. This model was validated by comparison with fermentation data, and could compute metabolic and growth performance *in silico*.

As described in the following section, the established models of ABE fermentation could be applied to studies in microbial technology and metabolic engineering, and should help to improve butanol production.

3.3.2 Application of established models for metabolic analysis and prediction of bottlenecks

The established models described above have improved the application of metabolic analysis, prediction of bottleneck pathways, or optimization of cultivation processes. Desai et al. improved a stoichiometric model with non-linear constraints [123], based on the model reported by Papoutsakis [121]. They also performed MFA of *C. acetobutylicum* using the improved model, and then elucidated the importance of the acid-formation pathways in regulating ABE production [41]. To date, MFA by their method has been widely performed in metabolically engineered *C. acetobutylicum* to assess ABE production or to compare metabolic phenomena between strains [103, 111]. The stoichiometric model reported by Papoutsakis was also used for the on-line diagnosis of the physiological state of *C.*

acetobutylicum metabolism in batch culture, integrated with on-line measurement devices [124], which helped determine the best timing for addition of methyl viologen for enhanced butanol production.

The kinetic simulation model developed by Shinto et al. could be applied to predict bottleneck pathways for butanol production by a sensitivity analysis with changing values in kinetic parameters. The sensitivity analysis revealed that a 5% increase the reverse pathway of butyrate production and a 5% decrease in CoA transferase activity contribute to high production of butanol from glucose [42] or xylose [40]. Thus, by using metabolic pathway models, we can successfully create an optimal design for bioreactors and elucidate metabolic networks in detail, and subsequently propose an appropriate genetic manipulation strategy.

4. Conclusions and prospects

In this mini review, we described many studies and findings of the advances in butanol production in different fields, namely, microbial technology, metabolic engineering, and systems biology. There are numerous reports on ABE fermentation processes from the last 30 years, which together accomplished drastic advances in butanol production. Increased butanol concentrations, productivity, and yields were achieved by the establishment of high cell density continuous cultures by cell immobilization [67] or by cell recycling together with cell bleeding [64], fed-batch culture integrated with *in-situ* butanol recovery system [93], and butanol production from butyric acid in living cells [88]. Although the history of metabolic engineering and systems biology is shorter than that of microbial technology, in the last 1 or 2 decades, novel and significant findings have been obtained and useful techniques and powerful tools have been developed, in step with the progress in genetics technology and computer technology.

Nevertheless, industrial biobutanol production currently lags behind production of bioethanol and biodiesel in all but a few regions. To make industrial biobutanol production a reality interdisciplinary studies will be required in the future. For example, only wild strains or their mutants have been used in investigations of process development, and ABE production in metabolically engineered strains have been assessed in typical batch culture. In addition, metabolic pathway models have been developed based on the experimental results of batch culture. The interdisciplinary fusion described here will support the optimization and design of high-performance butanol production systems for metabolically engineered strains that are modified by using metabolic pathway models to obtain desired characteristics and abilities.

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