MINIREVIEW

Metabolic Engineering of Microorganisms for the Production of Higher Alcohols

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ABSTRACT Due to the increasing concerns about limited fossil resources and environmental problems, there has been much interest in developing biofuels from renewable biomass. Ethanol is currently used as a major biofuel, as it can be easily produced by existing fermentation technology, but it is not the best biofuel due to its low energy density, high vapor pressure, hygroscopy, and incompatibility with current infrastructure. Higher alcohols, including 1-propanol, 1-butanol, isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol, which possess fuel properties more similar to those of petroleum-based fuel, have attracted particular interest as alternatives to ethanol. Since microorganisms isolated from nature do not allow production of these alcohols at high enough efficiencies, metabolic engineering has been employed to enhance their production. Here, we review recent advances in metabolic engineering of microorganisms for the production of higher alcohols.

Increasing concerns on climate change and inevitable depletion of fossil resources are urging us to develop fuels and energy that are independent of fossil resources. Microbial production of biofuels from renewable biomass has been considered one of the solutions (1). Currently, ethanol is a major biofuel produced worldwide, mainly because it can be produced by fermentation technology that has been available for a long time. However, ethanol is not such a great biofuel due to its inferior fuel characteristics, such as low energy density, high vapor pressure, hygroscopy, and incompatibility with current infrastructure. Therefore, there has recently been much interest in producing advanced biofuels possessing fuel characteristics similar to those of petroleum-derived fuels, such as hydrocarbons and higher alcohols. In this paper, we review recent advances in the production of higher alcohols, with a focus on metabolic engineering strategies employed for the development of microbial strains efficiently producing them.

METABOLIC ENGINEERING STRATEGIES FOR THE PRODUCTION OF PRIMARY ALCOHOLS

Primary higher alcohols can be synthesized via either the fatty acid or amino acid pathway in microorganisms. In general, the use of fatty acid metabolism is advantageous for the production of linear-chain alcohols, while that of amino acid metabolism is suitable for branched-chain alcohols. In this section, we review the recent studies for the production of 1-butanol and extend the discussion to higher fatty alcohols. Finally, the strategies employed for the production of branched-chain alcohols are reviewed. Strategies for the production of higher alcohols as well as the metabolic pathways and key enzymes involved are shown in Fig. 1 and 2. Also, the results of recent studies on higher-alcohol production by various microorganisms are summarized in Table 1.

1-Butanol. 1-Butanol can be best produced by the fermentation of clostridia (Fig. 1A) (see references 2–4 for the details on clostridial butanol fermentation). Due to difficulties in genetic manipulation and complex metabolic regulations in clostridia, metabolic engineering of clostridia has been rather difficult and has advanced only recently. The metabolic engineering strategies have been focused mainly on achieving an objective of increasing 1-butanol production and selectivity through the reduced formation of acetate, butyrate, and acetone. However, several previous studies suggested that elimination or reduction of the acetone flux is not sufficient to increase 1-butanol production; this resulted in accumulation of acids and reduced production of 1-butanol (5–7).

Jang et al. (8) successfully engineered *Clostridium acetobutylicum* ATCC 824 for the production of 1-butanol to high titer with high selectivity by reinforcing the direct 1-butanol biosynthetic pathway (hot channel). First, four genes (*pta*, *ack*, *ptb*, and *buk*) involved in production of short-chain fatty acid were individually or combinatorially knocked out. Among them, the *pta*/*buk* double knockout mutant produced the highest titer (16 g/liter) of 1-butanol. 1-Butanol production was further reinforced by the overexpression of a variant *adhE1* gene, which was engineered to utilize NADPH as a cofactor (8). The resulting strain produced over 18.9 g/liter of 1-butanol in batch fermentation. Also, 585.3 g of butanol was produced from 1,861.9 g of glucose by fed-batch culture of this engineered strain with *in situ* recovery. Hou et al. (9) also reported enhanced 1-butanol production in *C. acetobutylicum*. The *adc* gene was replaced with the glutathione-encoding genes from *Escherichia coli*, which was previously suggested to increase 1-butanol production in *C. acetobutylicum* (10). Instead of deleting the acid production pathway, the whole 1-butanol-forming pathway (from *thl* to *adhE1* genes) was amplified to produce 14.9 and 3.3 g/liter of 1-butanol and ethanol, respectively (9).

C. acetobutylicum has a pSOL1 megaplasmid harboring genes involved in solventogenesis, and the loss of this plasmid results in so-called strain degeneration incapable of producing solvents (11). Several clostridial species produce high titers of butyric acid. It was

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TABLE 1 Summary of microbial production of higher alcohols

Product	Host	Genotype[a] (knockout; overexpression)	Substrate	Medium	Titer	Cultivation	Comment	Reference
1-Propanol	*E. coli* BW25113 F'	Δ*ilvA* Δ*ilvB*; Ll*kivD* Sc*ADH2* Mj*cimA*^{mut} *leuABCD*	Glucose	Defined	2.78 g/liter	Shake flask	1-Butanol, 0.39 g/liter	40
	E. coli W3110	Δ*lacI* Δ*lysA* Δ*metA* Δ*tdhA* Δ*iclR* Δ*ilvIH* Δ*ilvBN* Δ*rpoS* *thrA*^{C1034T} *lysC*^{C1005T} P*thr*::P*tac* P*ppc*::P*trc* *ilvA*^{C1139T,G1341T,C1351G,T1352C}; *thrABC* Mj*cimA* *ackA* *adhE*^{mut}	Glucose	Semidefined	1.5 g/liter	Bioreactor	Aerotolerant AdhE; 20 g/liter initial glucose without feeding	39
	E. coli W3110	Δ*lacI* Δ*lysA* Δ*metA* Δ*tdhA* Δ*iclR* Δ*ilvIH* Δ*ilvBN* Δ*rpoS* *thrA*^{C1034T} *lysC*^{C1005T} P*thr*::P*tac* P*ppc*::P*trc* *ilvA*^{C1139T,G1341T,C1351G,T1352C}; *thrABC* Mj*cimA* *ackA* *adhE*^{mut}	Glycerol	Semidefined	10.3 g/liter	Bioreactor	Yield, 0.259 g/g; aerotolerant AdhE	39
Isopropanol	*E. coli* ATCC 11303	None; *lacI*^{q} Ca*thl* *atoDA* Ca*adc* *adh*$_{B-593}$	Glucose	Semidefined	5 g/liter[b]	Shake baffled flask	Yield, 0.15 g/g; acetone accumulation when glucose is depleted	61
	E. coli ATCC 11303	None; *lacI*^{q} Ca*thl* *atoDA* Ca*adc* *adh*$_{B-593}$	Glucose	Semidefined	143 g/liter	Stirred flask	Yield, 0.23 g/g; with gas stripping; 240 h	63
	E. coli ATCC 11303	None; Ca*thl* *atoDA* Ca*adc* *adh*$_{B-593}$ Tf*bgl*-blc (fused)	Cellobiose	Semidefined	4.1 g/liter	Shake flask	Yield, 0.08 g/g	64
	E. coli JM109	None; Ca*thl* Ca*ctfAB* Ca*adc* *adh*$_{B-593}$	Glucose	Complex	13.6 g/liter	Shake baffled flask	Yield, 0.17 g/g; acetone yield, 0.03 g/g	62
Alcohol mixture (IBE)	*C. acetobutylicum* ATCC 824	Δ*buk*::*ermC*; *adc* *ctfAB* *adh*$_{B-593}$	Glucose	Semidefined	20.4 g/liter	Bioreactor	Yield, 0.30 g/g; isopropanol, 4.4 g/liter; butanol, 14.1 g/liter; with gas stripping	65
	C. acetobutylicum ATCC 824	Δ*buk*::*ermC*; *adc* *ctfAB* *adh*$_{B-593}$	Glucose	Semidefined	35 g/liter	Bioreactor	Yield, 0.26 g/g; isopropanol, 4.1 g/liter; butanol, 25.1 g/liter; with gas stripping	65
	C. acetobutylicum ATCC 824	Δ*buk* ΔCA_C1502; *adc* *ctfAB* *adh*$_{B-593}$	Glucose	Semidefined	20.4 g/liter	Bioreactor	Yield, 0.33 g/g; with gas stripping	66
	C. acetobutylicum Rh8	None; *adh*$_{B-593}$	Glucose	Semidefined	23.9 g/liter	Bioreactor	Random mutagenized strain; yield, 0.31 g/g; isopropanol, 7.6 g/liter; butanol, 15 g/liter	67
	C. acetobutylicum BKM19	Δ*buk*::*ermC*; *adh*$_{B-593}$ *hydG*$_{B-593}$	Glucose	Semidefined	28.5 g/liter	Bioreactor	Obtained in a pilot-scale fermentation; random mutagenized strain; yield, 0.37 g/g; isopropanol, 3.5 g/liter; butanol, 15.4 g/liter; ethanol, 9.6 g/liter	68
1-Butanol	*C. acetobutylicum* ATCC 824	Δ*pta* Δ*buk*; *adhE*^{D485G}	Glucose	Semidefined	18.9 g/liter	Bioreactor	Without *in situ* recovery; yield, 0.29 g/g; acetone, 1.5 g/liter	8
	C. acetobutylicum ATCC 824	Δ*pta* Δ*buk*; *adhE*^{D485G}	Glucose	Semidefined	130 g/liter	Bioreactor	Volumetric productivity, 1.32 g/liter/h; with *in situ* recovery; yield, 0.31 g/g	8
	C. acetobutylicum ATCC 824	Δ*adc*; Ec*gsh* *adhE* *ctfAB* *thl* *hbd* *crt* *bcd*	Glucose	Semidefined	14.9 g/liter	Bioreactor	Yield, 0.34 g/g; 3.3 g/liter of ethanol	9
	C. tyrobutyricum ATCC 25755	Δ*ack*; Ca*adhE2*	Glucose	Semidefined	10 g/liter	Serum bottle; anaerobic	Yield, 0.27 g/g; 5.8 g/liter butyrate; manual pH control by NaOH	16
	C. tyrobutyricum ATCC 25755	Δ*ack*; Ca*adhE2*	Mannitol	Semidefined	16 g/liter	Serum bottle; anaerobic	Yield, 0.31 g/g; 1.0 g/liter butyrate; manual pH control by NaOH	16
	C. tyrobutyricum ATCC 25755	None; Ca*adhE2*	Mannitol	Complex	20.5 g/liter	Bioreactor; anaerobic	Yield, 0.33 g/g; productivity, 0.32 g/liter/h; 1.0 g/liter butyrate; manual pH control by NaOH	17
	E. coli DH1	None; Re*phaAB* Ac*phaJ* Td*ter* Ca*adhE2* *aceEF* *lpd*	Glucose	Complex	3.4 g/liter	Shake flask	Shift to the anaerobic condition after induction	18
	E. coli DH1	None; Re*phaA* Ca*hbd* Ca*crt* Td*ter* Ca*adhE2* *aceEF* *lpd*	Glucose	Complex	4.7 g/liter	Shake flask	Yield, 0.28 g/g; shift to the anaerobic condition after induction	18
	E. coli BW25113/F'	Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pta*; *atoB* Ca*hbd* Ca*crt* Ca*adhE2* Cb*fdh* Td*ter*	Glucose	Complex	15 g/liter	Bioreactor; anaerobic	Yield, 0.36 g/g; without gas stripping	19
	E. coli BW25113/F'	Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pta*; *atoB* Ca*hbd* Ca*crt* Ca*adhE2* Cb*fdh* Td*ter*	Glucose	Complex	30 g/liter	Bioreactor; anaerobic; with gas stripping	Yield, 0.36 g/g; without gas stripping	19
	S. elongatus PCC 7942	None; Scl*nphT7* Ca*hbd* Ca*crt* Td*ter* Cs*bld* Ec*yqhD*	CO_2	Defined	27 mg/liter[b]	Static capped flask	Photosynthesis	22
	S. elongatus PCC 7942	None; Scl*nphT7* Re*phaB* Ac*phaJ* Td*ter* Cs*bld* Ec*yqhD*	CO_2	Defined	29.9 mg/liter	Static capped flask	Photosynthesis	22
	E. coli MG1655	*fadR*$^{-}$ *crp** Δ*arcA* Δ*adhE* Δ*pta* Δ*frdA* Δ*yqhD*; *atoC* *yqeF* *fucO*	Glucose	Defined	14 g/liter	Shake baffled flask		24

(Continued on following page)

TABLE 1 (Continued)

Product	Host	Genotype[a] (knockout; overexpression)	Substrate	Medium	Titer	Cultivation	Comment	Reference
Isobutanol	*E. coli* BW25113 F'	ΔadhE ΔldhA ΔfrdBC Δfnr Δpta ΔpflB; BsalsS ilvCD LlkivD ScADH2	Glucose	Semidefined	22 g/liter	Shake flask	Yield, 0.35 g/g	34
	E. coli BW25113 F'	ΔadhE ΔldhA ΔfrdBC Δfnr Δpta ΔpflB; BsalsS ilvCD LlkivD LladhA	Glucose	Semidefined	50.9 g/liter	Bioreactor	With *in situ* gas stripping; volumetric productivity, 0.7 g/liter/h; yield, 0.29 g/g	36
	B. subtilis	Δldh; CgilvCD alsS LlkivD ScADH2	Glucose	Complex	2.62 g/liter	Bioreactor		41
	B. subtilis	Δldh; CgilvCD alsS LlkivD ScADH2	Glucose	Complex	3.83 g/liter	Bioreactor	Auto-inducible 2-ketovalerate synthetic operon	42
	C. cellulolyticum ATCC 35319	None; LlkivD EcyqhD BsalsS EcilvCD	Cellulose (Sigmacell type 50)	Defined	660 mg/liter	Not specified	7–9 days; strong expression of *alsS* might be deleterious	43
	C. glutamicum ATCC 13032	Δpyc ΔldhA; BsalsS LlkivD ilvCD adhA	Glucose	Complex	4.9 g/liter	Shake flask	Yield, 0.09 g/g	44
	C. glutamicum ATCC 13032	ΔaceE Δpqo ΔilvE ΔldhA Δmdh; ilvBNCD EcpntAB LlkivD adhA	Glucose	Semidefined	13 g/liter	Bioreactor	Yield, 0.20 g/g; volumetric productivity, 0.32 g/liter/h; shift to the anaerobic condition	45
	R. eutropha H16	ΔphaCAB ΔilvE ΔbkdAB ΔaceE; adh(con) ilvBHCD LlkivD	Fructose	Defined	270 mg/liter	Shake flask	Coproduced 40 mg/liter of 3-methyl-1-butanol	46
	R. eutropha H16	ΔphaB2C2 ΔphaC1AB1; BsalsS ilvCD LlkivD EcyqhD	CO_2	Defined	90 mg/liter	Bioreactor with electrodes	Coproduced 50 mg/liter of 3-methyl-1-butanol	47
	S. cerevisiae CEN.PK 2-1 C	None; ILV2 ILV5 ILV3	Glucose	Complex	4.12 mg/liter	Shake baffled flask		48
	S. cerevisiae BY4741	lpd1Δ; LlkivD ADH6 ILV2 ILV5c ILV3c ILV2C MAE1	Glucose	Complex	1.62 g/liter	Shake flask	Yield, 0.016 g/g	49
	S. cerevisiae S288C (MAT**a**/α)	his3Δl1/HIS3 leu2Δ0/LEU2 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0; ILV2 ILV3 ADH7 ILV5 LlkivD	Glucose	Defined	635 mg/liter	Shake tube	High-cell-density culture; yield, 6.4 mg/g; mitochondrial expression of ILV, KivD, ADH genes; 2-methyl-1-butanol, 118 mg/liter; 3-methyl-1-butanol, 95 mg/liter	50
2-Methyl-1-butanol	*E. coli* BW25113 F'	ΔmetA Δtdh; StyilvGM ilvCD CgilvA LlkivD ScADH2 thrABC		Defined	1.25 g/liter	Shake baffled flask	Yield, 0.17 g/g; total alcohol, 3 g/liter	37
	S. elongatus PCC 7942	None; LlkivD EcyqhD MjcimA^{mut} leuBCD	CO_2	Defined	177.5 mg/liter	Static flask	Photosynthesis; 12-day culture; isobutanol, 50 mg/liter; 1-propanol, 17.5 mg/liter	70
3-Methyl-1-butanol	*E. coli* BW25113 F'	None; BsalsS ilvCD LlkivD ScADH2 leuA^{G462D} leuBCD	Glucose	Defined	9.5 g/liter	Shake flask	Random mutagenized strain; two-phase culture with oleoyl alcohol; yield, 0.11 g/g; total alcohol, 12.5 g/liter	38
1-Hexanol	*E. coli* BW25113 F'	ΔldhA ΔadhE ΔfrdBC Δpta; atoB RebktB Cahbd Cacrt CaadhE2 Cbfdh Tdter	Glucose	Complex	47 mg/liter	Sealed test tube with shaking	Anaerobic; butanol, 5.1 g/liter	23
3-Methyl-1-pentanol	*E. coli* ATCC 98082	ΔilvE ΔtyrB; ilvGMCD tdcB LlkivD^{V461A,F381L} ScADH6 leuA^{G462D,S139G} leuBCD	Glucose	Semidefined	793.5 mg/liter	Shake flask	Enzyme evolution	52
Fatty alcohols	*E. coli* MG1655	fadR⁻ ΔarcA Δcrp ΔadhE Δpta ΔfrdA ΔfucO ΔyqhD ΔfadD; crp* atoC(con) fadBA yiaY	Glucose	Defined	0.33 g/liter	Shake baffled flask	Yield, 0.08 g/g; mixture of 1-hexanol, 1-octanol, and 1-decanol	24
	S. cerevisiae BY4742 (MATα)	his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 acc1::P_{TEF1}-ACC1 fas1::P_{TEF1}-FAS1 fas2::P_{TEF1}-FAS2; ACC1 FAS1 FAS2 MmFAR1 MalMAE	Glucose and galactose	Defined	98 mg/liter	Shake flask		26
	E. coli DH1	fadE; 'tesA fadD Acacr1	Glucose	Defined	60 mg/liter	Shake baffled flask		27
	E. coli MG1655	ΔaraBAD ΔfadE ΔfadAB ΔackA Δpta; UcfatB fadD Maacr2 (Maqu_2507)	Glucose	Defined	1.65 g/liter	Bioreactor	Yield, 0.134 g/g; mainly C_{12} and C_{14} alcohols	29
	E. coli BL21 (DE3)	ΔfadE; fadD Maacr2 (Maqu_2220) 'tesA	Glucose	Defined	1.73 g/liter	Bioreactor	Yield, 28.3 mg/g	30
	E. coli BL21 (DE3)	None; TPC Mmacar Bssfp Spahr	Glucose	Defined	360 mg/liter	Test tube with shaking		31
	E. coli BL21 (DE3)	ΔfadE; Seaar	Glycerol	Defined	0.75 g/liter	Bioreactor	Yield, 0.02 g/g	33

[a] For heterologous genes, the abbreviation of the species is followed by the gene name (e.g., ScADH2, the *ADH2* gene from *S. cerevisiae*). The abbreviations of the species are as follows: Ac, *A. calcoaceticus*; Aca, *A. caviae*; Ca, *C. acetobutylicum*; Cb, *Candida boidinii*; Cs, *Clostridium saccharoperbutylacetonicum* N1-4; Ll, *L. lactis*; Ma, *M. aquaeolei*; Mal, *Mortierella alpine*; Mj, *Methanococcus jannaschii*; Mm, *M. musculus*; Mma, *M. marinum*; Sc, *S. cerevisiae*; Scl, *Streptomyces* sp. strain CL190; Se, *Synechococcus elongatus* PCC 7942; Sp, Synechocystis sp. PCC 6803; Td, *Treponema denticola*; Tf, *Thermobifida fusca* YX, Uc, *Umbellularia californica*. Other abbreviations are as follows: *crp**, a cyclic AMP-independent mutant *crp* gene; '*tesA*, a leaderless *tesA* gene; *gene*(con), modified ITS for constitutive expression; adh_{B-593}, the primary/secondary alcohol dehydrogenase gene from *C. beijerinckii* NRRL B-593; $hydG_{B-593}$, a putative gene encoding an electron transfer protein from *C. beijerinckii* NRRL B-593.

[b] These values were estimated from the figures in the original references, as the values were not described in the text.

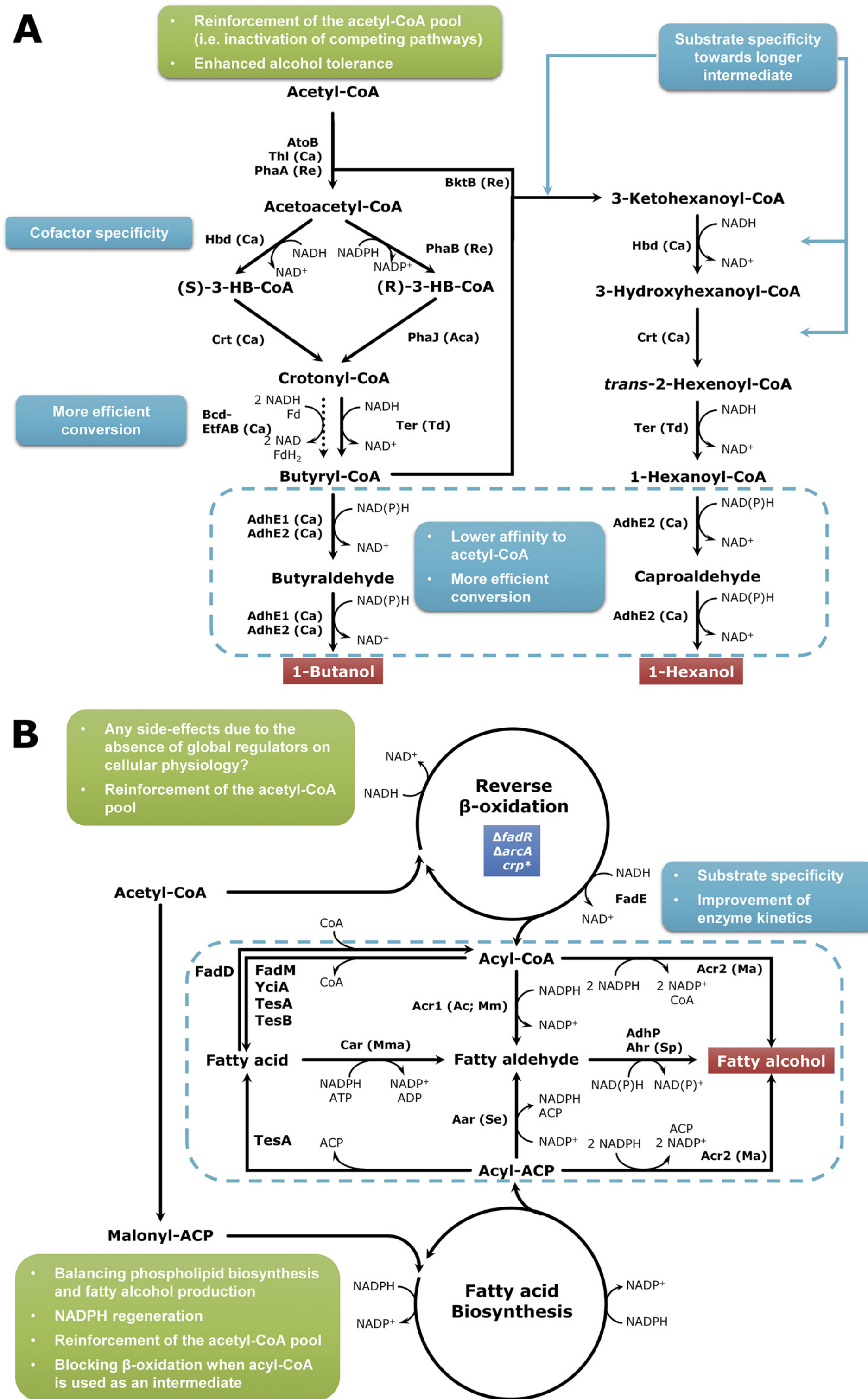

FIG 1 Strategies for the production of linear, primary alcohols. (A) Production of 1-butanol and 1-hexanol through the native or reconstructed clostridial pathway. The dotted arrow adjacent to Bcd-EtfAB indicates the weak activity of the Bcd enzyme in microbes other than clostridia. (B) Production of long-chain primary alcohols. In contrast to short-chain alcohols, long-chain fatty alcohols can be produced via various routes. The blue box in the reverse β-oxidation indicates the essential genetic manipulation to activate this pathway in the presence of glucose. The *crp** gene encodes the mutant catabolite repressor protein for catabolite derepression. Points to be considered for further engineering in enzymatic and cellular levels are indicated in cyan and green boxes, respectively. For each reaction, the names of the corresponding enzymes used in the metabolic engineering studies are shown. The source of the enzyme was noted together with the enzyme, except for *E. coli*. The abbreviations of the species are as follows: Ac, *Acinetobacter calcoaceticus*; Aca, *Aeromonas caviae*; Ca, *Clostridium acetobutylicum*; Ch, *Cuphea hookeriana*; Ma, *Marinobacter aqualeolei*; Mm, *Mus musculus*; Mma, *Mycobacterium marinum*; Re, *Ralstonia eutropha*; Se, *S. elongatus*; Sp, Synechocystis sp. PCC 6803; Td, *Treponema denticola*; Uc, *Umbellularia californica*. See the main text for the abbreviations of the enzymes.

thus thought that 1-butanol can be produced in butyric acid-producing bacteria by introducing aldehyde and alcohol dehydrogenases. However, the overexpression of aldehyde and alcohol dehydrogenases (or bifunctional aldehyde/alcohol dehydrogenases) in degenerate *C. acetobutylicum* variants did not typically result in a higher titer of 1-butanol, as the butyric acid production was still very active (12–15). Recently, production of 1-butanol by engineering one of the best butyric acid producers, *Clostridium tyrobutyricum*, has been reported (16, 17). In one study (16), the *ack* and *ptb* single mutants of *C. tyrobutyricum* were employed for 1-butanol production. When the *adhE2* gene from *C. acetobutylicum* was overexpressed in the *C. tyrobutyricum ack* mutant, about 10 g/liter of 1-butanol was produced. The use of mannitol, a more reduced carbon source than glucose, further increased the 1-butanol titer to 16 g/liter (16). Later, different replicons were examined to overexpress the *C. acetobutylicum adhE2* gene in *C. tyrobutyricum* (17), and the best strain, without any gene knockout, was able to produce 21 g/liter of 1-butanol and 6 g/liter of butyric acid using mannitol as the sole carbon source.

Reconstruction of the clostridial 1-butanol production pathway in other microorganisms has also been reported. However, such engineered microorganisms equipped with clostridial genes did not result in sufficient 1-butanol production, probably due to the poor activity of butyryl coenzyme A (butyryl-CoA) dehydrogenase (Bcd). This bottleneck could be overcome by employing an alternative enzyme, *trans*-enoyl-CoA reductase (Ter) (18, 19). Shen et al. (19) deleted anaerobic fermentation genes in *E. coli*, including fumarate reductase, lactate dehydrogenase, and endogenous aldehyde/alcohol dehydrogenase, and reconstructed a chimeric pathway by overexpressing the *atoB* gene from *E. coli*, the *hbd* (encoding 3-hydroxybutyryl [HB]–CoA dehydrogenase), *crt* (3-hydroxybutyryl–CoA dehydratase), and *adhE2* (bifunctional aldehyde/alcohol dehydrogenase) genes from *C. acetobutylicum*, and the *ter* gene from *Treponema denticola*. The formate production during anaerobic fermentation of engineered *E. coli* was reduced by introducing a fungal formate dehydrogenase, converting formate to CO_2 and NADH (20). The resulting strain produced 1-butanol up to 15 g/liter in batch fermentation, and the yield reached up to 88% of the theoretical maximum.

In the case of redox enzymes, its cofactor preference as well as catalytic activity can affect 1-butanol production. Typically, NADH is the preferential electron donor, but some organisms are capable of efficiently generating NADPH (e.g., photosynthesis in cyanobacteria). In this case, the use of NADPH-dependent enzymes can be beneficial for 1-butanol production. A good alternative of clostridial NADH-dependent Hbd enzyme is the NADPH-dependent PhaB from bacteria producing polyhydroxyalkanoates (Fig. 1A). Unlike Hbd, PhaB produces the *R* form of 3-hydroxybutyryl–CoA instead of the *S* form (21). Since Crt does not accept the *R* form of 3-HB–CoA, it needs to be replaced with the *R*-form-specific dehydratase PhaJ (18). The use of PhaB-PhaJ as well as NADPH-specific aldehyde and alcohol dehydrogenases was successfully employed for the enhanced 1-butanol production from CO_2 in cyanobacteria (22).

Fatty alcohols. Fatty alcohols can be produced from acyl-CoAs. One strategy is to extend the clostridial pathway (Fig. 1A). The enzyme Ter has a broad substrate specificity, and it can be used to produce higher alcohols. Dekishima et al. (23) demonstrated such possibility by producing 1-hexanol in *E. coli*. They confirmed *in vitro* that Ter was able to convert 1-hexenoyl–CoA to 1-hexanoyl–CoA. Unlike the case of the 1-butanol production, the *bktB* gene from *Ralstonia eutropha*, encoding a β-ketothiolase, was additionally overexpressed since the endogenous AtoB could not condense acetyl-CoA and higher acyl-CoA. It was also demonstrated *in vitro* that AdhE2 from *C. acetobutylicum* has an activity toward octanoyl-CoA, even though 1-octanol was not produced. These results suggest that higher alcohols might be produced in the future after improving the substrate specificities of the other enzymes involved in acyl-CoA synthesis.

Another notable strategy is the use of the endogenous β-oxidation pathway in microorganisms (Fig. 1B), which was demonstrated in *E. coli* (24). The key metabolic engineering strategy employed was the elimination of regulatory mechanisms that repress the genes involved in β-oxidation. Through testing of various enzymes involved in initiation (condensation of acetyl- and acyl-CoAs) and termination (conversion of acyl-CoAs into fatty acids or alcohols), the reversal of the β-oxidation cycle combined with endogenous dehydrogenases and thioesterases was established to produce higher alcohols. Even though 1-butanol was preferentially produced (up to 14 g/liter), a mixture of higher alcohols (~C_{10}; 0.33 g/liter) could also be produced by employing a different alcohol dehydrogenase. However, this strategy depended on the derepression of the genes, which made it difficult to fine-tune the pathway to control the chain length and to increase productivity. In a later study, the key enzymes involved in the β-oxidation pathway were characterized *in vitro*, and their various combinations were assembled and examined (25). Even though only fatty acid production was examined, it would be possible to produce higher alcohols by the introduction of aldehyde and alcohol dehydrogenases. In another study, Runguphan and Keasling (26) were able to produce fatty alcohols in *Saccharomyces cerevisiae* by engineering the triacylglyceride (TAG) biosynthetic pathway and blocking the β-oxidation pathway. Introduction of an acyl-CoA reductase from *Mus musculus* into the engineered strain resulted in the production of ca. 100 mg/liter of fatty alcohols.

Higher alcohols can also be produced via the fatty acid biosynthetic pathway, and various strategies have been reported (Fig. 1B). Fatty acyl-acyl carrier proteins (acyl-ACPs) can be converted into free fatty acids by thioesterase, and the resulting acids can be further converted to fatty acyl-CoAs. Then, acyl-CoA reductase (ACR) and aldehyde reductase (AHR) can convert fatty acyl-CoAs into fatty alcohols (Fig. 1B). Steen et al. (27) first demonstrated this strategy in *E. coli* using the *acr1* gene, encoding ACR, from *Acinetobacter calcoaceticus* BD413. In flask cultures, it was possible to produce up to 60 mg/liter of total fatty alcohols (C_{12} to C_{16}) when combined with the overexpression of the *tesA* and *fadD* genes to enhance the generation of free fatty acids and their conversion into acyl-CoAs, respectively, and deleting the *fadE* gene to block the degradation of acyl-CoAs via the β-oxidation pathway. The chain lengths of fatty alcohols were dependent mainly on the specificity of thioesterase (27). More recently, another type of ACR (Acr2) has been characterized (28). This ACR is able to directly convert fatty acyl-CoA, and acyl-ACP at a lower efficiency, into fatty alcohol. Youngquist et al. (29) found that the acyl-CoA reductase from *Marinobacter aquaeolei* VT8 (Acr2 encoded by Maqu_2507; Fig. 1B) converted acyl-CoA into fatty alcohol more rapidly than Acr1. After the optimization of *fadD* and *acr2* expression, they were able to produce fatty alcohols up to 1.65 g/liter by fermentation with pH and dissolved

oxygen (DO) control (29). Liu et al. (30) used another *acr2* gene (Maqu_2220) from *M. aquaeolei* VT8, which resulted in 2-fold-increased production of fatty alcohols (ca. 650 mg/liter) compared to that obtained by using Maqu_2507 in shaking flask cultures. However, in a bioreactor experiment, fatty alcohols were produced to 1.73 g/liter (30), which is only slightly higher than that obtained by Youngquist et al. (29).

Conversion of acyl-ACPs to acyl-CoAs requires optimal control of both ACP and CoA pools in order to achieve enhanced production of fatty alcohols. To avoid such difficulty, acyl-CoA-independent production of fatty alcohols has been examined. Free fatty acids can be directly converted to fatty aldehydes by carboxylic acid reductase (Car) (Fig. 1B), which directly converts free fatty acids to fatty aldehydes using both NADPH and ATP. Akhtar et al. (31) characterized the kinetic properties of the Car from *Mycobacterium marinum*. When the *car* gene was overexpressed in *E. coli* together with the endogenous *tesA* gene for free fatty acid production, the *Bacillus subtilis sfp* gene (encoding a phosphopantetheinyl transferase) for the activation of Car (32), and also the endogenous *yjgB* gene, the product of which shares homology with the aldehyde reductase from *Synechocystis* sp. PCC 6803, about 360 mg/liter of fatty alcohols could be produced (31). The use of Car might be advantageous in that this enzyme does not depend on the intracellular CoA pool, and the reaction is more exergonic than that of cyanobacterial acyl-ACP reductases (Aar) (Fig. 1B) due to the coupling of ATP hydrolysis. Nonetheless, Liu et al. (33) produced ca. 0.75 g/liter of fatty alcohols in *E. coli*, exceeding the titer achieved by Akhtar et al. (31), by the overexpression of the *Synechococcus elongatus* aldehyde reductase gene alone. In that study, it was demonstrated *in vitro* that endogenous acetaldehyde dehydrogenase (AdhP) was the key enzyme for the reduction of fatty aldehydes to fatty alcohols, although it was downregulated in fatty alcohol-producing strains (33).

Branched-chain alcohols. 2-Ketoacids, which are metabolic intermediates of amino acid metabolism, can be used for the production of various branched-chain alcohols, including isobutanol (34–36), 2-methyl-1-butanol (37), and 3-methyl-1-butanol (38), as well as linear alcohols, including 1-propanol and 1-butanol (39, 40). The metabolic pathways designed for the production of various higher alcohols from the 2-ketoacid pathway are presented in Fig. 2. The key enzyme in this pathway is 2-ketoacid decarboxylase from *Lactococcus lactis* (KDC; encoded by the *kivD* gene), which shows a broad substrate specificity toward various 2-ketoacids (34). This strategy has been employed in a wide range of microorganisms, including *Bacillus subtilis* (41, 42), *Clostridium cellulolyticum* (43), *Corynebacterium glutamicum* (44, 45), *Ralstonia eutropha* (46, 47), and *S. cerevisiae* (48–50), in addition to *E. coli*. Among them, *C. glutamicum* has a great potential for the production of various amino acids and thus is advantageous for producing various alcohols from 2-ketoacids. Blombach et al. (45) have reported high-titer production of isobutanol from glucose by engineered *C. glutamicum*. In this study, an engineered *C. glutamicum* strain capable of producing a high titer of 2-ketoisovalerate (51) was employed as the base strain; in this strain, the *ilvE*, *aceE*, and *pqo* genes encoding transaminase B, pyruvate dehydrogenase subunit E1, and pyruvate:quinone oxidoreductase, respectively, were deleted, and the *ilvBNCD* genes were overexpressed to reinforce the carbon flux toward 2-ketoisovalerate (51). The key engineering strategies for isobutanol production include the inactivation of lactate and malate dehydrogenases, the use of endogenous alcohol dehydrogenase instead of *S. cerevisiae* ADH2, and the expression of the *E. coli* transhydrogenase (45). Interestingly, even though transhydrogenase PntAB from *E. coli* was overexpressed, inactivation of malic enzyme resulted in severe reduction of the production yield, indicating that *C. glutamicum* depends mainly on NADPH generation through the malic enzyme. The final strain was able to produce ca. 13 g/liter of isobutanol (45).

Similar to fatty acid biosynthesis, the chain length of 2-ketoacids can be extended by using the *leuABCD* genes; using this pathway, one carbon is added every cycle. As a proof-of-concept example (52), protein engineering was performed on KDC from *L. lactis* and LeuA (2-isopropylmalate synthase) from *E. coli* for the production of 3-methyl-1-pentanol (3MP). Based on the computational prediction of enzyme structures, the binding pockets of KDC and LeuA were modified, and their activities were examined. One combination, KDC V461A/F381L and LeuA G462D/S139G, resulted in a dramatic increase in 3MP production compared to that obtained with the wild-type enzymes (793.5 versus 6.5 mg/liter).

Another notable study is the use of an autolithotrophic bacterium such as *R. eutropha*. However, the low solubility of H_2 in a growth medium acts as a major barrier for the efficient production of alcohols. Thus, Li et al. (47) directly supplied electrons to *R. eutropha* using electrodes, instead of using H_2 as an electron donor. To produce higher alcohols from only CO_2, an engineered *R. eutropha* strain was constructed and examined in a specially designed bioreactor. It was found that NO and O_2^- produced during the electric current flow inhibited cell growth, which was solved by shielding the anode with a ceramic cup, partly blocking their diffusion. Finally, the engineered *R. eutropha* strain produced ca. 90 and 50 mg/liter of isobutanol and 3-methyl-1-butanol, respectively, from only CO_2 and electric current.

There had also been a report on higher-alcohol production by KDC-independent pathways (Fig. 2). Recently, there has been a report on the production of more than 10 g/liter of 1-propanol by engineering the threonine degradation pathway through 2-ketobutyrate to propionate and then to 1-propanol in *E. coli* (39). It was achieved by redirecting the carbon flux toward 2-ketobutyrate by the overexpression of the feedback-resistant threonine dehydratase gene (*ilvA*) and deletion of competing metabolic pathway genes (*ilvI*, *ilvH*, *ilvB*, and *ilvN*) followed by the overexpression of citramalate synthase (*cimA*) and mutant alcohol/aldehyde dehydrogenase (*adhE*^{mut}) genes. In this study, the *E. coli* acetate kinase/propionate kinase II (*ackA*), acetyl-CoA: acetoacetyl-CoA synthase (*atoDA*), and an aerobically functional mutant alcohol/aldehyde dehydrogenase (*adhE*^{mut}) were employed instead of KDCs and ADHs for converting 2-ketobutyrate to 1-propanol. Due to the clear feasibility and advantages of redirecting fluxes to the desired metabolites, the 2-ketoacid pathway will serve as an important platform for the production of biofuels and chemicals.

METABOLIC ENGINEERING STRATEGIES FOR THE PRODUCTION OF SECONDARY ALCOHOLS

Secondary alcohols have chemical properties different from those of primary alcohols due to the position of the hydroxyl group. The secondary alcohols of short-chain length can dissolve polar and nonpolar chemicals and thus are used as solvents in various industrial applications. Secondary alcohols are petrochemically synthe-

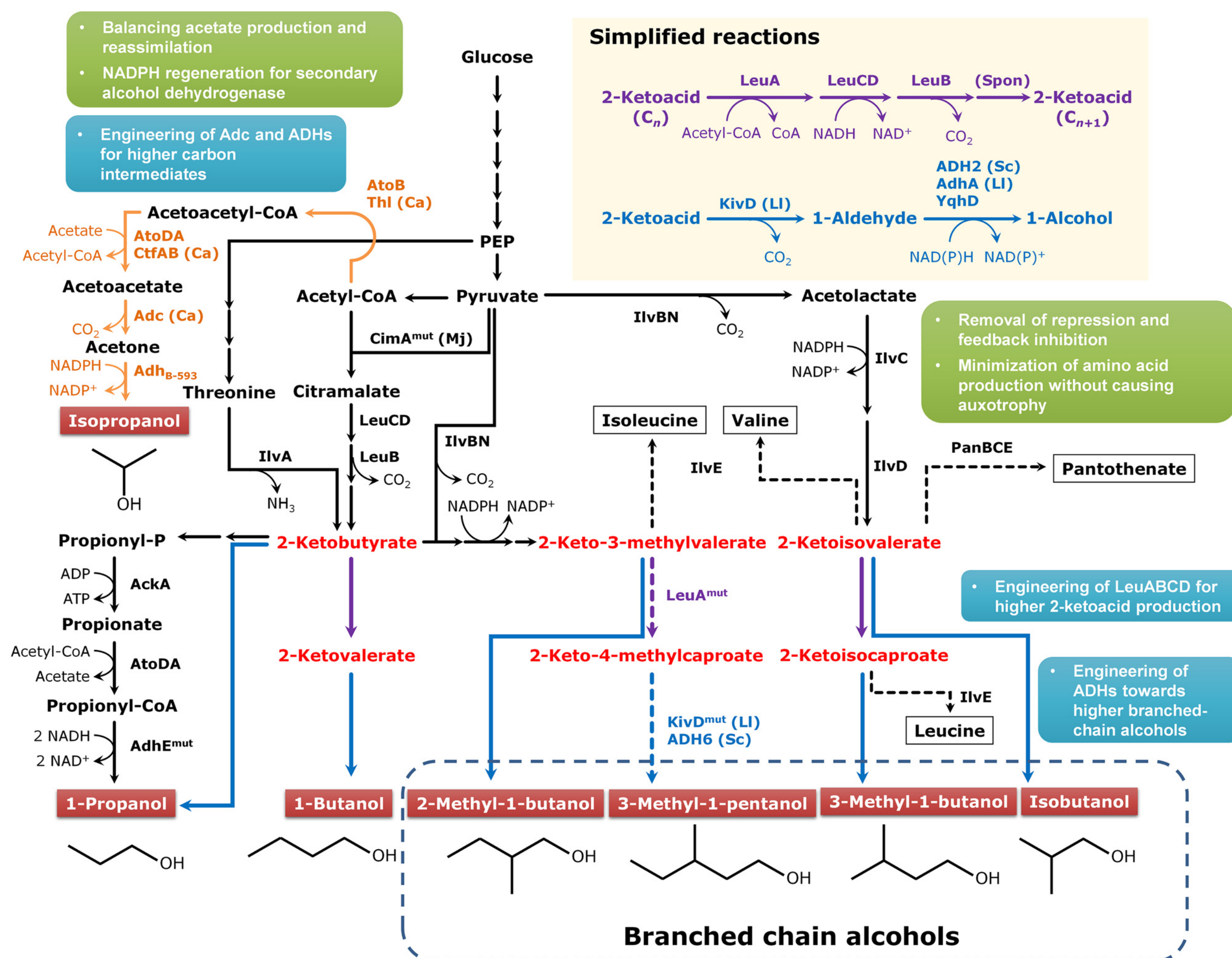

FIG 2 Production of branched-chain and secondary alcohols. Higher alcohols are shown in the red boxes, and the 2-ketoacid precursors are indicated in red text. The reactions in the isopropanol production pathway are shown with orange arrows. As in Fig. 1, points to be considered are indicated in cyan and green boxes. The source of the enzyme is noted together with the enzyme except *E. coli*, and follows that in Fig. 1. Additional abbreviations of the species are Ll, *Lactococcus lactis*; Sc, *Saccharomyces cerevisiae*. The enzymes shown are as follows: AckA, acetate kinase A and propionate kinase II; AdhE^{mut}, aerobically functional alcohol dehydrogenase; IlvA, threonine dehydratase; IlvC, ketol-acid reductoisomerase; IlvD, dihydroxyacid dehydratase; IlvE, branched-chain amino-acid aminotransferase; IlvIH, acetolactate synthase I; IlvBN, acetolactate synthase III complex; KivD, 2-ketoacid decarboxylase; LeuA, 2-isopropylmalate synthase; LeuB, 3-isopropylmalate dehydrogenase; LeuCD, 3-isopropylmalate isomerase complex; YqhD, NADPH-dependent aldehyde reductase; AtoB and Thl, acetyl-CoA acetyltransferase; AtoDA, acetyl-CoA:acetoacetyl-CoA synthase; CtfAB, CoA transferase; Adc, acetoacetate decarboxylase; Adh$_{B-593}$, primary/secondary alcohol dehydrogenase from *C. beijerinckii* B-593.

sized by hydration of alkenes or oxidation of ketones. Biological secondary alcohol production depends on the reduction of ketones by secondary alcohol dehydrogenases (53–58). Acetone is a representative ketone produced as a major metabolite by clostridia, and biosynthesis of 2-butanone (59) and 2-pentanone (60) has only recently been reported. Although only the production of isopropanol will be described in this paper (see Fig. 2), it will be possible to produce other higher secondary alcohols by employing novel biosynthetic routes toward higher ketones and engineering secondary alcohol dehydrogenases.

The first isopropanol production in microorganisms other than clostridia was reported using engineered *E. coli* strains. Hanai et al. (61) produced ca. 5 g/liter of isopropanol by an engineered *E. coli* B strain after overexpressing the *thl* and *adc* genes from *C. acetobutylicum* ATCC 824 and the endogenous *atoDA* genes encoding CoA transferase. Even though their result suggested that the *atoDA* genes were better than *C. acetobutylicum ctfAB* genes, Jojima et al. (62) produced about 13.6 g/liter of isopropanol using the *C. acetobutylicum ctfAB* genes in *E. coli* JM109. The fed-batch fermentation of the final strain developed by Hanai et al. (61) coupled with *in situ* recovery by gas stripping allowed production of 143 g/liter of isopropanol with a yield of 0.23 g/g glucose in 240 h (63). In a recent study, this strain was further engineered to utilize cellobiose, and about 4.1 g/liter of isopropanol was produced from 50 g/liter of cellobiose in shaking-flask cultivation (64). Even though isopropanol could be produced to a high titer by this engineered *E. coli* strain, incomplete conversion of acetone might cause a problem in downstream processes. To solve this problem, the expression levels of heterologous genes and the redox balance, in particular that of NADPH required as the cofactor of the secondary alcohol dehydrogenase, should be optimized.

Isopropanol can be used as a fuel additive since it has a higher

octane rate (118) than 1-butanol. In this context, an interesting idea was generated to convert acetone into isopropanol in the acetone-butanol-ethanol (ABE) fermentation of clostridia; by doing so, isopropanol-butanol-ethanol (IBE) fuel mixture can be produced by one-step fermentation. In a *C. acetobutylicum* PJC4BK *buk*-inactivated strain, Lee et al. (65) overexpressed the primary/secondary alcohol dehydrogenase from *Clostridium beijerinckii* NRRL B-593 (encoded by $adh_{B\text{-}593}$) and endogenous acetone-producing enzymes. The engineered *C. acetobutylicum* PJC4BK strain was able to produce 20.4 g/liter of IBE mixture. Fed-batch fermentation coupled with *in situ* gas stripping allowed production of ca. 35 g/liter of IBE mixture (4.1, 6.3, and 25.1 g/liter of isopropanol, ethanol, and 1-butanol, respectively) from 133 g/liter of glucose. A similar result was obtained by an independent study employing different promoters in gene overexpression (66). In another study, the $adh_{B\text{-}593}$ gene was overexpressed in a 1-butanol-tolerant mutant *C. acetobutylicum* Rh8 strain (67). The resulting strain produced 23.9 g/liter of IBE mixture containing 7.6 and 15 g/liter of isopropanol and 1-butanol, respectively. Jang et al. (68) also reported the use of a *C. acetobutylicum* mutant that overproduces butanol and ethanol. The engineered *C. acetobutylicum* BKM19 strain with the introduced $adh_{B\text{-}593}$ and *hydG* genes was capable of producing butanol and ethanol to higher titers (69). The resulting strain produced 27.9 g/liter of IBE mixture containing 3.6, 14.8, and 9.5 g/liter of isopropanol, 1-butanol, and ethanol, respectively, in pilot-scale batch fermentation. These results suggest that isopropanol with or without other alcohols can be efficiently produced by metabolic engineering of *E. coli* or *C. acetobutylicum*.

CONCLUSION

Limited fossil fuel resources and increasing environmental concerns have been urging us to develop platform technologies for the sustainable and economical production of alternative fuels. In order to develop economically competitive bioprocesses for their production, the metabolic pathways need to be optimally engineered by designing the best pathways to increase the metabolic flux toward the desired product, improving the kinetics and substrate specificities of the enzymes involved, and balancing the cofactors and redox. As described above, several higher alcohols can be efficiently produced by employing metabolically engineered microorganisms. It is expected that more successful examples of microbial higher-alcohol production will appear through the strain development integrated with bioprocess engineering. Metabolic engineering will keep playing a key role in developing such economically competitive bioprocesses.

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