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ORIGINAL RESEARCH PAPER

Metabolic engineering of *Escherichia coli* for 1-butanol biosynthesis through the inverted aerobic fatty acid *β*-oxidation pathway

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Abstract The basic reactions of the clostridial 1-butanol biosynthesis pathway can be regarded to be the inverted reactions of the fatty acid *β*-oxidation pathway. A pathway for the biosynthesis of fuels and chemicals was recently engineered by combining enzymes from both aerobic and anaerobic fatty acid *β*-oxidation as well as enzymes from other metabolic pathways. In the current study, we demonstrate the inversion of the entire aerobic fatty acid *β*-oxidation cycle for 1-butanol biosynthesis. The constructed markerless and plasmidless *Escherichia coli* strain BOX-3 (MG1655 $lacI^Q$ *attB*-$P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$-*adhE*(Glu568Lys) *attB*-$P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$-*atoB* *attB*-$P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$-*fadB* *attB*-$P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$-*fadE*) synthesises 0.3–1 mg 1-butanol/l in the presence of the specific inducer. No 1-butanol production was detected in the absence of the inducer.

Keywords 1-Butanol · *Escherichia coli* · Fatty acid *β*-oxidation

Introduction

Many attempts have been made to construct non-native 1-butanol-producing microorganisms by cloning various heterologous genes in *Escherichia coli* (Atsumi et al. 2008a, 2008b; Inui et al. 2008; Nielsen et al. 2009), *Bacillus subtilis* (Nielsen et al. 2009), *Pseudomonas putida* (Nielsen et al. 2009), *Saccharomyces cerevisiae* (Steen et al. 2008), and *Lactobacillus brevis* (Berezina et al. 2010). While the construction of effective 1-butanol producers is still a challenge, the opportunity to create 1-butanol producers without using foreign genes is also of interest. The reactions of the fatty acid *β*-oxidation pathway, which is native in many organisms, and the main reactions of the clostridial 1-butanol biosynthesis pathway are the redox reaction sequences between metabolites with similar chemical structures (Fig. 1). We hypothesised that the reactions of the fatty acid *β*-oxidation pathway, like most redox reactions, could be reversed. The acyl-CoA derivatives, in particular butyryl-CoA,

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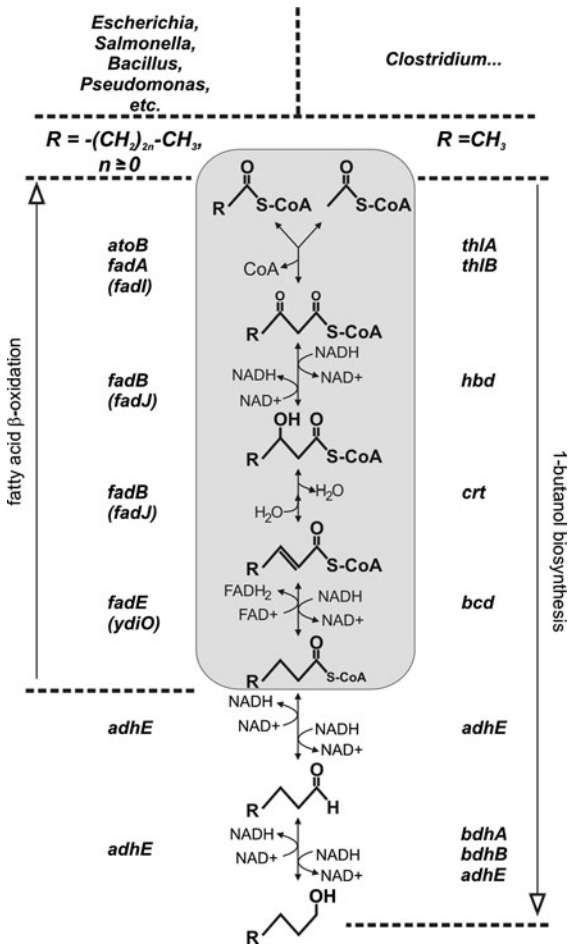

Fig. 1 Comparison of the widespread prokaryotic fatty acid β-oxidation pathway and 1-butanol biosynthesis pathway in *Clostridium acetobutylicum*. Enzymes of *E. coli* and *C. acetobutylicum* are indicated by their gene names: *atoB* acetyl-CoA C-acetyltransferase; *fadA* (*fadI*) acetyl-CoA C-acyltransferase; *fadB* (*fadJ*) 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase; *fadE* (*ydiO*) acyl-CoA dehydrogenase; *thlA*, *thlB* thiolase (acetyl-CoA acetyltransferase); *hbd* 3-hydroxybutyryl-CoA dehydrogenase; *crt* crotonase (3-hydroxybutyryl-CoA dehydratase); *bcd* butyryl-CoA dehydrogenase

formed from acetyl-CoA could be reduced to their corresponding alcohols (in the simplest case, to 1-butanol) by endogenous CoA-dependent alcohol/aldehyde dehydrogenases.

Enzymes of the *E. coli* native fatty acid β-oxidation pathway can operate in a biosynthetic manner (Seregina et al. 2010). However, the corresponding strain TS325 was obtained by both classic genetic methods and mutagenesis stages that did not allow an estimation of the enzymes that formed the inverted β-oxidation pathway. In this study, we present evidence that 1-butanol can be synthesised in *E. coli* cells by reversed action of the aerobic fatty acid β-oxidation pathway enzymes.

Recently, the functional reversal of the fatty acid β-oxidation pathway for the synthesis of advanced chemicals (Dellomonaco et al. 2011) has been reported. However, the engineered pathway included not only separate enzymes of aerobic and anaerobic β-oxidation, but also enzymes of alternate metabolic pathways that have similar actions. Moreover, a significant production of target substances was achieved only by the inactivation of the main alcohol/aldehyde dehydrogenase AdhE and overexpression of key genes from plasmids.

In this study, the constructed markerless and plasmidless BOX-3 strain synthesises 1-butanol as a result of acetyl-CoA to butyryl-CoA conversion that is catalysed by the enzymes of the aerobic fatty acid β-oxidation pathway, which is followed by the reduction of butyryl-CoA to the corresponding alcohol by the main alcohol/aldehyde dehydrogenase AdhE. The BOX-3 strain contains only one chromosomal copy of each gene encoding the enzymes involved in the 1-butanol biosynthesis.

Materials and methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *E. coli* K-12 MG1655 (VKPM B-6195) was used as the parent strain for construction of all mutants described in this study.

All modifications in the *E. coli* MG1655 chromosome were obtained by the method of Datsenko and Wanner (2000). All of the primers that were used are listed in Supplementary Table 1. The modifications were obtained individually and then combined in the chromosome of the resulting strain BOX-3 (MG1655 *lacI*^{Q} *attB*-$P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$-*adhE*(Glu568Lys) *attB*-$P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$-*atoB* *attB*-$P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$-*fadB* *attB*-$P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$-*fadE*) by sequential P1-mediated transductions. Detailed procedures for the strain construction are shown in the Supplementary Information.

Escherichia coli was grown in Luria–Bertani (LB), SOB, SOC and M9 media (Sambrook et al. 1989). Ampicillin (100 μg/ml), chloramphenicol (30 μg/ml)

or kanamycin (50 μg/ml) was added when needed. IPTG, (1 mM) was used to induce the gene expression from the LacI-dependent promoter. Cells were grown aerobically on M9 plates (15 g agar/l) with ethanol (10 g/l) or sodium oleate (1 g/l) as sole carbon source. To improve sodium oleate solubility, Triton X-100 (4 g/l) was used.

Culture conditions

Semi-aerobic conditions

Cells of the BOX-3 strain and the control strain MG *adhE568* were grown overnight in M9 medium containing 2 g glucose/l at 37°C. 1 ml of the overnight cultures was diluted 50 times with 49 ml M9 medium containing 10 g glucose/l. The cultures were grown in 750 ml flasks with ventilation plugs at 37°C on a shaker (250 rpm) for 6 h. Cell suspensions were centrifuged for 15 min at ~3,000×*g* and 4°C. The pellets were resuspended in 7 ml of M9 medium containing 20 g glucose/l and 10 g glycerol/l. The cell cultures were incubated for 24 h in 20 × 200 mm test tubes with ventilation plugs at 37°C on shaker (150 rpm) with or without the presence of 1 mM IPTG.

In addition, cells of the BOX-3 strain and the control strain MG *adhE568* were grown overnight in LB medium at 37°C. 0.5 ml of the overnight cultures were diluted 100 times with the addition of 49.5 ml of LB medium. The cultures were grown in 750 ml flasks with ventilation plugs at 37°C on the shaker (250 rpm) for 3 h. The cell suspensions were centrifuged for 15 min at ~3,000×*g* and 4°C. The pellets were resuspended in 7 ml LB medium containing 20 g glucose/l. The cell cultures were incubated for 24 h in 20 × 200 mm test tubes with ventilation plugs at 37°C on shaker (150 rpm) in the presence of 1 mM IPTG.

Anaerobic conditions

The initial biomass accumulation in M9 and LB media was performed as described above. The cell pellets were resuspended in 15 ml of M9 or LB media containing 20 g glucose/l. The cell cultures were incubated for 24 h in 15 ml test tubes with non-ventilated plugs at 37°C on shaker (150 rpm) with or without 1 mM IPTG.

Analytical techniques

Fermentation samples were centrifuged for 10 min at 15,000×*g*. The supernatants were used for further analysis.

GC–MS was used to identify 1-butanol in culture media. The amounts of alcohols in culture media were determined by GC-FID. Concentrations of organic acids, glucose and glycerol in the culture media were measured by HPLC. Detailed procedures for metabolite detection and quantification are provided in the Supplementary information. Cell density was measured from the OD_{600} value; an OD_{600} of 1 = 400 mg dry wt per litre.

Results and discussion

Construction and characteristics of strains

The expression of genes encoding the enzymes of the fatty acid *β*-oxidation pathway in *E. coli* is repressed in the absence of fatty acids by the negative transcriptional regulator of *fad* regulon (the FadR protein) (Fujita et al. 2007). Moreover, the CoA-dependent alcohol/aldehyde dehydrogenase (AdhE), required for 1-butanol biosynthesis, is inactive in *E. coli* cells under aerobic conditions. Therefore, native regulatory regions of *fadE* (encoding acyl-CoA dehydrogenase), *fadB* (encoding 3-hydroxyacyl-CoA dehydrogenase end enoyl-CoA hydratase), *atoB* (encoding acetyl-CoA C-acetyltransferase), and *adhE* (encoding CoA-dependent alcohol/aldehyde dehydrogenase) genes were substituted by the artificial genetic element $P_{trc\text{-}ideal\text{-}4}$-SD*φ*10, which contained a strong LacI-dependent promoter ($P_{trc\text{-}ideal\text{-}4}$) and a Shine-Dalgarno sequence (SD sequence) of the *φ*10 gene from the phage T7. In the case of the *adhE* gene, a point mutation of G→A, leading to a Glu568Lys substitution in the protein product of the gene, was additionally introduced into position 1,702 of its coding region to obtain an aerotolerant mutant (Clark and Cronan 1980; Holland-Staley et al. 2000).

The BOX-3 strain was obtained by combining all modifications in the chromosome of the basic strain MG $lacI^Q$. The engineered *E. coli* strain, MG $lacI^Q$, was constructed by the introduction of a point mutation of CG→TA into the −35 region of P_{lacI} promoter. This mutation is known to cause a $lacI^Q$ genotype that provides an effective repression of LacI-dependent promoters (Glascock and Weickert 1998).

The repression/induction efficiency of the expression of *adhE*, *fadE*, *fadB* and *atoB* genes, regulated by artificial genetic element $P_{trc\text{-}ideal\text{-}4}$-SD$\varphi$10, and the functional activity of the corresponding proteins were verified by aerobically growing strains MG *adhE568* and BOX-3 on M9 plates with ethanol or sodium oleate as a sole carbon source. While the strain MG *adhE568* could aerobically grow on M9 plates that contained ethanol as a sole carbon source and IPTG as specific inducer, strain MG1655 did not grow either in the presence or absence of the inducer. Growth of the BOX-3 strain on M9 plates with sodium oleate as a sole carbon source was observed only in the presence of IPTG, while the growth of the strain MG1655 was independent of the presence of an inducer in the medium. Thus, the expression of the key genes of the fatty acid β-oxidation pathway in BOX-3 occurred only under induction; the corresponding enzymes were functionally active, thereby permitting the utilisation of fatty acids as the sole carbon source for cell growth.

1-Butanol biosynthesis by the BOX-3 strain under semi-aerobic conditions

Cultures of the BOX-3 strain and the control strain MG *adhE568* were grown aerobically in M9 medium containing 10 g glucose/l as a sole carbon source. For 1-butanol fermentation, the biomass was cultivated semi-aerobically for 24 h in M9 medium containing 20 and 10 g glycerol/l, which was used as a source of additional NADH equivalents required for 1-butanol biosynthesis. In the absence of an inducer (IPTG), no 1-butanol was detected in the culture media of either the BOX-3 or MG *adhE568* strain. In the presence of an inducer, 1-butanol was synthesised exclusively by the BOX-3 strain (Table 1A). The 1-butanol was identified in the culture media by GC–MS, and its amount was determined by gas chromatography using flame ionisation detector. As is shown in Table 1A, the amount of ethanol produced by the BOX-3 strain significantly exceeded the amount of 1-butanol. The low specificity of *E. coli* AdhE alcohol/aldehyde dehydrogenase to butyryl-CoA, as compared to acetyl-CoA, is responsible for the high ethanol/butanol ratio (Atsumi et al. 2008a). Moreover, only two NADHs are required to reduce acetyl-CoA to ethanol, while four NADHs are required to form 1-butanol from the same precursor. In this experiment (Table 1A), the fermentation products (such as lactate and succinate) were nearly absent in the culture medium, which indicates that the culture conditions of the strains were rather aerobic and that most of the NADH generated from glycolysis was reoxidized to NAD + through the respiratory selectron transport chain. The ethanol

Table 1 Metabolite production and substrate consumption by the MG *adhE568* and BOX-3 strains under semi-aerobic conditions

Strain	IPTG	Pyruvate (mM)	Lactate (mM)	Acetate (mM)	Succinate (mM)	Ethanol (mM)	Butanol (μM)	Glucose[a] (mM)	Glycerol[a] (mM)	DCW (g/l)
A (M9 + g glucose/1, + 10 g glycerol/l)										
MG *adhE568*	–	n.d.	n.d.	77.9 ± 1.6	0.9 ± 0.1	1.9 ± 0.1	n.d.	47.6 ± 1.1	7.8 ± 0.5	5.08 ± 0.27
MG *adhE568*	+	n.d.	n.d.	78.8 ± 1.5	1 ± 0.1	3.6 ± 0.3	n.d.	47.2 ± 0.9	9.8 ± 0.5	5.36 ± 0.27
BOX-3	–	n.d.	n.d.	81.9 ± 1.6	1.1 ± 0.1	1.7 ± 0.1	n.d.	46.6 ± 1.1	7.3 ± 0.4	5.72 ± 0.29
BOX-3	+	n.d.	n.d.	74.8 ± 1.6	1.2 ± 0.1	2.9 ± 0.2	4.5 ± 0.8	47.9 ± 1.1	8.8 ± 0.5	6.52 ± 0.31
B (LB + 20 g glucose/l)										
MG *adhE568*	–	n.m.	n.m.	47.0 ± 1.1	n.m.	1.7 ± 0.1	n.d.	n.m.	–	5.92 ± 0.31
MG *adhE568*	+	n.m.	n.m.	52.1 ± 1.2	n.m.	3.0 ± 0.2	n.d.	n.m.	–	5.84 ± 0.29
BOX-3	–	n.m.	n.m.	44.7 ± 1.2	n.m.	1.9 ± 0.1	n.d.	n.m.	–	6.08 ± 0.32
BOX-3	+	n.m.	n.m.	57.3 ± 1.4	n.m.	3.1 ± 0.3	12.1 ± 0.8	n.m.	–	5.96 ± 0.31

Data are the means ± standard deviations from three replications

n.d. Not detected; *n.m.* not measured; "–" not added

[a] Consumed

production by the BOX-3 and MG *adhE568* strains in the absence of an inducer could be most likely due to the action of other enzymes that have similar activities, such as AdhP (EC 1.1.1.1), YqhD (EC 1.1.1.–), MhpF (EC 1.2.1.10) etc.

The ability of the BOX-3 strain to synthesise 1-butanol under semi-aerobic conditions was also shown when the strain was cultured in LB medium with 20 g glucose/l. As in the case of minimal medium, 1-butanol biosynthesis was observed only in the presence of an inducer (Table 1B). The control strain MG *adhE568* was not able to produce 1-butanol either in the presence or the absence of an inducer. The amount of 1-butanol synthesised by the BOX-3 strain in the rich medium was higher the amounts seen in M9 medium, but the amount of ethanol was the same in both the rich and minimal media. Simultaneously, acetic acid accumulation in the rich medium was less. Thus, it is possible that the more effective redistribution of acetyl-CoA between competing reactions of ethanol biosynthesis and acetoacetyl-CoA formation led to a relatively higher 1-butanol biosynthesis by the BOX-3 strain.

1-Butanol biosynthesis by the BOX-3 strain under anaerobic conditions

The ability of BOX-3 to synthesise 1-butanol was also examined under anaerobic condition. The aerobically grown cells of strains BOX-3 and MG *adhE568* were cultured in M9 or LB media supplemented with 20 g glucose/l for 24 h with or without 1 mM IPTG. As in semi-aerobic conditions, the 1-butanol synthesis was observed only in the BOX-3 strain upon induction of expression of *fad* regulon genes and alcohol/aldehyde dehydrogenase, irrespective of culture media (Table 3). Along with MG *adhE568*, the BOX-1 and BOX-2 strains, with altered regulation of an incomplete set of β-oxidation cycle genes, did not produce 1-butanol under any test condition (data not shown). The amounts of 1-butanol anaerobically produced by BOX-3 in rich medium were nearly identical to those synthesised under semi-aerobic conditions. On the other hand, the amount of 1-butanol produced by the BOX-3 strain in M9 media was higher under anaerobic conditions than in semi-aerobic conditions. Under anaerobic conditions, the main source of NADH in the minimal glucose media is via glycolysis, and the reducing equivalents are reoxidized in fermentative reactions. Thus, the redistribution of glycolytic NADH between fermentative pathways, instead of its oxidation through the respiratory electron transport chain, could increase the relative availability of reducing power for 1-butanol biosynthesis, thus causing the higher anaerobic production of 1-butanol by the BOX-3 strain in M9 media. Nevertheless, the amount of 1-butanol anaerobically synthesised by the BOX-3 strain remained low. The lack of NADH equivalents required for redox-balanced 1-butanol biosynthesis is responsible for this phenomenon. In the presence of O_2, the NADH deficiency was caused by intensive oxidation; under anaerobic conditions, the NADH deficiency was caused by the insufficient generation of reducing equivalents. In the absence of O_2, the formation of 2 mol acetyl-CoA is accompanied by the generation of 2 mol glycolytic NADH, instead of the 4 mol that are required for 1-butanol biosynthesis. Moreover, in the case of the BOX-3 strain, the NADH availability for anaerobic production of 1-butanol decreases, due to the activation of lactic acid biosynthesis. The lactate formation competes with 1-butanol biosynthesis for both NADH and its direct metabolic precursor, pyruvate. The pathways that compete with 1-butanol biosynthesis for both carbon and reducing power (acetate, lactate and succinate biosynthesis) are responsible for the utilisation of about 80% of the glucose consumed by the strain (Table 2A).

The results presented above have demonstrated that 1-butanol biosynthesis via the inverted aerobic fatty acid β-oxidation pathway is possible even at conditions that are far from optimal. We did not delete the host pathways that compete with 1-butanol biosynthesis for reducing equivalents and carbon sources. However, it has been shown that the inactivation of the competitive reactions in recombinant *E. coli* strains harbouring clostridial 1-butanol biosynthesis genes on multi-copy plasmids can increase the production of 1-butanol from 1 mg/l (13 μM) to 0.5 g/l (6.7 mM) (Donaldson et al. 2006; Atsumi et al. 2008a).

The driving force required for efficient 1-butanol production includes the balanced activities of 1-butanol synthesising enzymes and the redox status of the producer (Bond-Watts et al. 2011; Shen et al. 2011). This concept for the construction of heterological recombinant 1-butanol producers can be applied to the optimisation of the inverted fatty acid β-oxidation pathway for 1-butanol production. The basic strain RB01 (MG1655 *fadR atoC(c)* Δ*arcA crp**), described

Table 2 Metabolite production and substrate consumption by the MG *adhE568* and BOX-3 strains under anaerobic conditions

Strain	IPTG	Pyruvate (mM)	Lactate (mM)	Acetate (mM)	Succinate (mM)	Ethanol (mM)	Butanol (μM)	Glucose[a] (mM)	DCW (g/l)
A (M9 + 20 g glucose/l)									
MG *adhE568*	−	0.6 ± 0.1	5.5 ± 0.3	9.8 ± 0.6	1.7 ± 0.1	0.8 ± 0.1	n.d.	22.9 ± 0.6	1.88 ± 0.12
MG *adhE568*	+	0.7 ± 0.1	5.2 ± 0.3	10.2 ± 0.5	1.5 ± 0.1	2.1 ± 0.2	n.d.	23.3 ± 0.8	1.92 ± 0.13
BOX-3	−	0.7 ± 0.1	5.1 ± 0.3	10.4 ± 0.6	1.6 ± 0.1	0.7 ± 0.1	n.d.	25.2 ± 0.8	2.24 ± 0.13
BOX-3	+	0.7 ± 0.1	4.7 ± 0.2	10.2 ± 0.5	1.5 ± 0.1	1.8 ± 0.1	8.3 ± 0.8	25.1 ± 0.7	2.32 ± 0.16
B (LB + 20 g glucose/l)									
MG *adhE568*	−	n.m.	n.m.	10.3 ± 0.5	n.m.	1.2 ± 0.1	n.d.	n.m.	2.32 ± 0.15
MG *adhE568*	+	n.m.	n.m.	10.4 ± 0.5	n.m.	1.9 ± 0.1	n.d.	n.m.	2.36 ± 0.16
BOX-3	−	n.m.	n.m.	10.5 ± 0.5	n.m.	2.1 ± 0.1	n.d.	n.m.	2.36 ± 0.16
BOX-3	+	n.m.	n.m.	10.8 ± 0.6	n.m.	2.9 ± 0.2	9.1 ± 0.8	n.m.	2.44 ± 0.16

Data are the means ± standard deviations from three replications

n.d. Not detected; *n.m.* not measured; "−" not added

[a] Consumed

by Dellomonaco et al. (2011), synthesised none of the products that can be derived from fatty acid β-oxidation intermediates; the 1-butanol production was achieved only after the inactivation of fermentative pathways by deleting *adhE*, *pta* and *frdAB* genes and overexpressing the *yqhD* gene, which codes for NADP-depended aldehyde dehydrogenase in the plasmid. In the present study, the inversion of fatty acid β-oxidation was achieved in the BOX-3 strain by induced expression of chromosomal copies of only aerobic *fad* regulon and *adhE* genes.

Conclusions

We have demonstrated the inversion of the entire aerobic fatty acid β-oxidation cycle in the biosynthetic direction. The constructed markerless and plasmidless strain BOX-3 synthesises 1-butanol as a result of acetyl-CoA to butyryl-CoA conversion via the inverted aerobic fatty acid β-oxidation pathway, followed by the reduction of butyryl-CoA to the corresponding alcohol by native alcohol/aldehyde dehydrogenase. This statement is based on the following results: (1) the only difference between the butanol-producing BOX-3 strain and the control strain MG *adhE568* is that the native regulatory regions of three genes coding for fatty acid β-oxidation enzymes were substituted in the BOX-3 strain by the artificial regulatory elements; (2) the BOX-3 strain synthesises 1-butanol only upon the induction of the expression of these three genes, which are under the control of the artificial regulatory elements; and (3) the induction of only alcohol/aldehyde dehydrogenase expression is not sufficient for 1-butanol biosynthesis in the control strain MG *adhE568*.

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