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Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*

Masayuki Inui · Masako Suda · Sakurako Kimura · Kaori Yasuda · Hiroaki Suzuki · Hiroshi Toda · Shogo Yamamoto · Shohei Okino · Nobuaki Suzuki · Hideaki Yukawa

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Abstract A recombinant butanol pathway composed of *Clostridium acetobutylicum* ATCC 824 genes, *thiL, hbd, crt, bcd-etfB-etfA*, and *adhe1* (or *adhe*) coding for acetyl-CoA acetyltransferase (THL), β-hydroxybutyryl-CoA dehydrogenase (HBD), 3-hydroxybutyryl-CoA dehydratase (CRT), butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (BYDH), and butanol dehydrogenase (BDH), under the *tac* promoter control was constructed and was introduced into *Escherichia coli*. The functional expression of these six enzymes was proved by demonstrating the corresponding enzyme activities using spectrophotometric, high performance liquid chromatography and gas chromatography analyses. The BCD activity, which was not detected in *E. coli* previously, was shown in the present study by performing the procedure from cell extract preparation to activity measurement under anaerobic condition. Moreover, the *etfA* and *etfB* co-expression was found to be essential for the BCD activity. In the case of BYDH activity, the *adhe* gene product was shown to have higher specificity towards butyryl-CoA compared to the *adhe1* product. Butanol production from glucose was achieved by the highly concentrated cells of the butanologenic *E. coli* strains, BUT1 with *adhe1* and BUT2 with *adhe*, under anaerobic condition, and the BUT1 and BUT2 strains were shown to produce 4 and 16-mM butanol with 6- and 1-mM butyrate as a byproduct, respectively. This study reports the novel butanol production by an aerobically pregrown microorganism possessing the genes of a strict anaerobe, *Clostridium acetobutylicum*.

M. Inui · M. Suda · S. Kimura · K. Yasuda · H. Suzuki · H. Toda · S. Yamamoto · S. Okino · N. Suzuki · H. Yukawa (✉)
Research Institute of Innovative Technology for the Earth,
9-2 Kizugawadai, Kizugawa,
Kyoto 619-0292, Japan
e-mail: mmg-lab@rite.or.jp

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Introduction

"Biofuels" produced from renewable resources are attracting ever-increasing attention as the public concerns on the global warming and energy security have been growing worldwide. Among the biofuels, butanol is currently under the spotlight due to its advantages as a new-generation biofuels over ethanol as it contains more energy (1.5 times greater volumetric energy content than ethanol), is less corrosive and less water-soluble, and is easier to blend with gasoline or diesel fuels.

The microbial butanol production was first reported by Louis Pasteur in 1861 and was industrialized using *Clostridium acetobutylicum* isolated by Chaim Weizmann in the early 20th century. The bacterium produces butanol/acetone/ethanol in the ratio of 6:3:1 from sugars and starch (Mitchell 1998), and the process is termed acetone, butanol, and ethanol (ABE) fermentation. This process still remains to be the foundation of the current biological butanol production process.

Bacterial butanol production studied to date is carried out exclusively by the members of the genus *Clostridium*, which is known to contain ABE fermentation and butanol–isopropanol–ethanol fermentation microorganisms and include *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, *C. saccharoacetobutylicum*, *C. aurantibutyricum*, *C. pasteurianum*, *C. sporogenes*, *C. cadaveris*, and *C. tetanomorphum* (George et al. 1983; Gottwald et al. 1984; Keis et al. 1995). Of those butanol producers, the whole genome sequences of *C. acetobutylicum* ATCC 824^{TS} (Nölling et al. 2001) and *C. beijerinckii* NCIMB 8052 (NCBI accession

number CP000721) have been analyzed reflecting the active research in the ABE fermentation process.

Despite the long research history into the ABE fermentation process, obstacles still remain today. Firstly, as *Clostridium* are strict anaerobes, the growth condition must remain anaerobic, and the growth rate is significantly slow compared to many of the aerobic microorganisms resulting in low butanol production rate. To solve these problems, aerobic microorganisms with fast growth rate could be utilized; however, there has been no report on such microorganism with ability to produce butanol. Secondly, butanol production has been shown to require change in the culture condition. Acetate and butyrate produced during the growth phase reduce the culture pH and the acidic condition at the stationary phase is widely regarded to trigger the shift in metabolism resulting in solvent production (Jones and Woods 1986). Another factor shown to trigger the butanol production is the regulator protein Spo0A, which is responsible for sporulation as well as induction of genes involved in butanol production (Brown et al. 1994; Ravagnani et al. 2000). These findings imply that the fermentation process should be tightly regulated to maximize the solvent production. Finally, there have been several reports on the loss of *C. acetobutylicum*'s ability to produce solvent. This degeneration was found to be due to the loss of megaplasmid, pSOL1, which carries genes required for solventogenesis (Cornillot et al. 1997), and this phenomenon is one of the element causing instability in solventogenesis during the ABE fermentation process. Owing to the negative aspects described above, developments of a novel butanol producing microorganisms and production process are anticipated.

The butanol production pathway or the metabolic pathway from acetyl-CoA to butanol in *Clostridium* spp. is illustrated in Fig. 1 and involves the following enzymes: acetyl-CoA acetyltransferase (thiolase; THL), β-hydroxybutyryl-CoA dehydrogenase (HBD), 3-hydroxybutyryl-CoA dehydratase (crotonase; CRT), butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (BYDH) and butanol dehydrogenase (BDH; Jones and Woods 1986). The BYDH and BDH reactions in the *Clostridium* spp. are catalyzed by the bifunctional enzyme and the enzyme is coded by *adhe1* and *adhe* (Nölling et al. 2001). Among the genes of *C. acetobutylicum* ATCC 824 involved in the butanol production, HBD, CRT, and BCD coding genes have been cloned in *Escherichia coli* and their activities have been measured after growing the microorganisms under the aerobic or anaerobic conditions. Although HBD and CRT activities were measured, BCD activity was not detected (Boynton et al. 1996). Advantages of using *E. coli* as a host microorganism in the butanol production process might include the vast amount of information available on its genetic and physiological characteristics and the existing variety of genetic tools to carry out the host modification. Using the

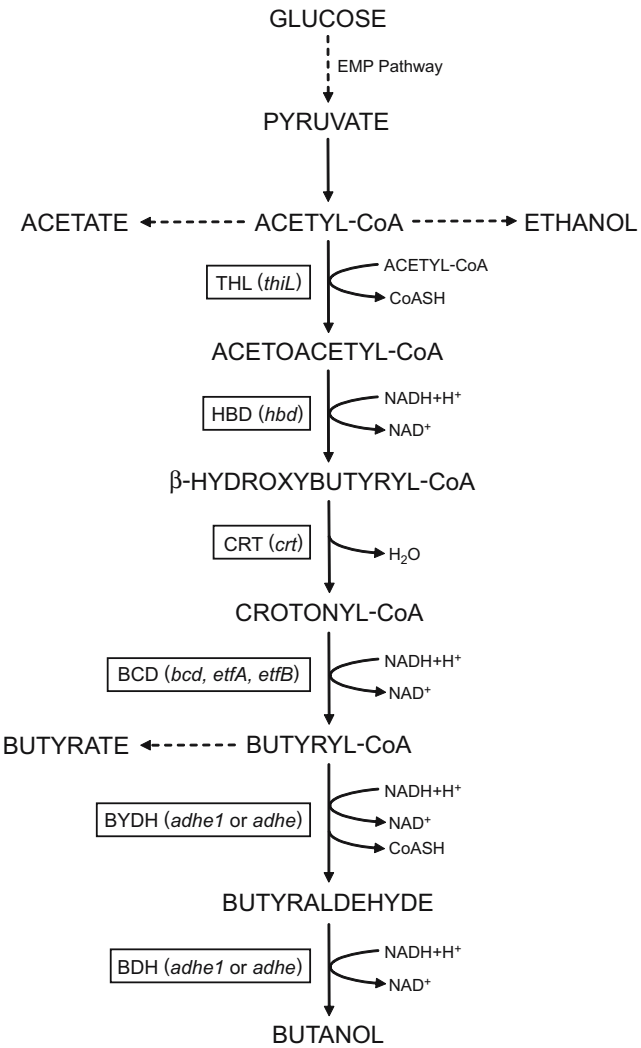


Fig. 1 Butanol synthetic pathway. The *open boxes* indicate the relevant eyzymes (*uppercase*) and genes (*italicized letters in parentheses*) *THL*, acetyl-CoA acetyltransferase (thiolase); *HBD*, β-hydroxybutyryl-CoA dehydrogenase; *CRT*, 3-hydroxybutyryl-CoA dehydratase (crotonase); *BCD*, butyryl-CoA dehydrogenase; *BYDH*, butyraldehyde dehydrogenase; *BDH*, butanol dehydrogenase

information and tools available, the host could be modified to be more suitable for the butanol production process by increasing its tolerance to butanol and minimizing the byproduct formation.

In the current paper, the expression of five genes coding for the enzymes involved in the six steps from acetyl-CoA to butanol in *E. coli*, and their enzymatic activity measurements are reported. Moreover, butanol production by the *E. coli* strains possessing the genes involved in butanol production is demonstrated.

Materials and methods

Bacterial strains and plasmids The bacterial strains and plasmids used in this study are listed in Table 1.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F'[*traD36 proAB*$^+$*lacI*^{q} *lacZ*ΔM15]	Takara
BUT1	Cm^r; *E.coli* JM109 bearing pBUT1	This work
BUT2	Cm^r; *E.coli* JM109 bearing pBUT2	This work
BUT1ΔETF	Cm^r; *E.coli* JM109 bearing pBUT1ΔETF	This work
ETF	Cm^r; *E.coli* JM109 bearing pETF	This work
ADH1	Ap^r; *E.coli* JM109 bearing pADH1	This work
ADH2	Ap^r; *E.coli* JM109 bearing pADH2	This work
C. acetobutylicum ATCC824	Type strain	ATCC
Plasmids		
pKK223–3	Ap^r; expression vector, source of *tac* promoter	Pharmacia
pCRB1	Cm^r; α-*lac* multicloning site. *E. coli-Corynebacterium* sp. shuttle vector derived from pHSG398 and pBL1	Nakata et al. (2003)
pTHI	Ap^r; pKK223-3 with a 1.2-kb *Sma*I PCR fragment containing the *C. acetobutylicum thiL* gene.	This work
pBCS	Ap^r; pKK223-3 with a 4.8-kb *Sma*I PCR fragment containing the *C. acetobutylicum crt-bcd-etfB-etfA-hbd* (BCS) operon	This work
pADH1	Ap^r; pKK223-3 with a 2.8-kb *Sma*I PCR fragment containing the *C. acetobutylicum adhe1* gene	This work
pADH2	Ap^r; pKK223-3 with a 2.7-kb *Sma*I PCR fragment containing the *C. acetobutylicum adhe* gene	This work
pCRB1-PtacBCS	Cm^r; pCRB1 with a 5.1-kb *Bam*HI–*Bgl*II DNA fragment containing the P*tac*-BCS operon	This work
pCRB1-PtacBCS-PtacADH1	Cm^r; pCRB1-PtacBCS with a 3.4-kb *Sal*I–*Xho*I DNA fragment containing the P*tac-adhe1* gene	This work
pCRB1-PtacBCS-PtacTHI	Cm^r; pCRB1-PtacBCS with a 1.7-kb *Sal*I–*Xho*I DNA fragment containing the P*tac-thiL* gene	This work
pBUT1	Cm^r; pCRB1-PtacBCS-PtacADH1 with a 1.7-kb *Sal*I–*Xho*I DNA fragment containing the P*tac-thiL* gene	This work
pBUT2	Cm^r; pCRB1-PtacBCS-Ptac THI with a 3.3-kb *Sal*I–*Xho*I DNA fragment containing the P*tac-adhe* gene	This work
pBUT1ΔETF	Cm^r; pBUT1 deleting a 1.9-kb DNA fragment containing the *etfB* and *eftA* genes	This work
pETF	Cm^r; pCRB1 with a 2.1-kb PCR fragment containing the P*tac-etfB-etfA* genes	This work

ATCC, American Type Culture Collection; *Ap^r*, ampicillin resistance gene; *Cm^r*, chloramphenicol resistance gene.

Chemicals All chemicals used in this study were of the highest available purity and were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma-Aldrich (MO, USA).

Conditions for growth and butanol production For genetic manipulations, *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (Sambrook et al. 1989).

For butanol production, 10-ml LB liquid medium in a test tube was inoculated with *E. coli* strains and incubated at 30°C under aerobic condition with shaking at 200 rpm for 20 h. The 10-ml culture was used to inoculate 500-ml LB medium in a 1-l conical flask and incubated as described above. The cells were collected by centrifugation at 8,000×*g*, 4°C for 20 min, and the collected cells were resuspended in M9 liquid medium [0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.0001% thiamine hydrochloride, 0.025% $MgSO_4$, and 0.001% $CaCl_2$; (*w*/*v*)] to the final cell density of OD_{660} of 20. The cell suspension (50 ml) was transferred to a 100-ml bottle, and glucose was added to the final concentration of 4% (*w*/*v*). The culture was incubated with gentle stirring in an anaerobic chamber filled with gas comprised of 95% N_2 and 5% H_2 (Coy Laboratory Products, CA, USA), and maintained at 30°C. The pH was kept above 6.5 by a pH controller (DT-1023; ABLE & Biott, Tokyo, Japan) by pumping 2.5-N NaOH solution.

Where appropriate, media were supplemented with antibiotics. The final antibiotic concentrations were 50 μg/ml of ampicillin and 50 μg/ml of chloramphenicol.

Analytical methods Culture samples were centrifuged (15,000×*g*, 4°C, 10 min), and the supernatants were analyzed for butyric acid, butanol, and glucose. Butyric acid and butanol were analyzed with high performance liquid chromatography (HPLC 8020; Tosoh Corporation, Tokyo, Japan) with following conditions, column (TSK-Gel SCX(H+), 7.8 mm×30.0 mm, ϕ5 μm; TOSOH Corporation), column temperature at 40°C, eluent (0.1-M phosphoric acid) with elution rate of 1.0 ml/min, injection volume 30 μl, with a reflective index detector. Glucose was analyzed with enzyme electrode glucose sensor (BF-4, Oji Scientific Instruments, Hyogo, Japan). Cell growth was monitored by measuring the absorbance at 660 nm using a spectrophotomerter (Novaspec Plus, GE Healthcare Bio-Science, NJ, USA).

Enzyme assays For the enzyme assays, *E. coli* strains were grown in 100-ml LB liquid medium at 30°C, 200 rpm under aerobic condition for 13 h.

The cell extracts for THL, HBD, and CRT enzyme assays were prepared as follows. The cells were collected by centrifugation at 5,000×*g*, 4°C for 10 min and resuspended in a sonication buffer (100-mM Tris–(hydroxymethyl)aminomethane hydrochloride or Tris–HCl buffer and 2-mM 1,4-dithiothreitol; pH 7.5). The cells were collected, resuspended in 1 ml of sonication buffer before adding 0.5-g glass beads (150–212 μm; Sigma-Aldrich), and the mixture was homogenized for 30 min with ultrasonic homogenizer (UCD-200 Cosmo Bio, Tokyo, Japan). Cellular debris was removed by centrifugation at 15,000×*g*, 4°C for 15 min, and the supernatant was used as a cell extract. Protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA).

The THL activity was determined using acetoacetyl-CoA and CoA as substrates, and the decrease in acetoacetyl-CoA concentration was measured at 303 nm (Wiesenborn et al. 1988) using DU-800 UV/Visible spectrophotometer (Beckman Coulter, CA, USA). To the solution containing 100-mM Tris–HCl (pH 8.0), 10-mM $MgCl_2$, 1-mM 1,4-dithiothreitol, 50-μM acetoacetyl-CoA, and 0.2-mM CoA, the cell extract solution was added to start the enzymatic reaction at 30°C. Decrease in the absorbance was monitored in the sample solution and control solution, from which CoA was omitted, and the slope values were determined. The enzyme activity was determined by calculating the difference in the slope values of the sample and control, and using the molar extinction coefficient of 14,000 $M^{-1}cm^{-1}$. Furthermore, the change in substrate and product concentrations before the reaction and after the reaction had completed was determined with HPLC (8020; Tosoh Corporation). The cell extract was added to the mixture containing 100-mM Tris–HCl (pH 8.0), 10-mM $MgCl_2$, 1-mM 1,4-dithiothreitol, 1-mM acetoacetyl-CoA, and 1-mM CoA, and the reaction was carried out as before. The reaction was terminated by adding an aliquot of 60% (*v*/*v*) perchloric acid to the 1/100 volume of the reaction mixture. The resulting solution was neutralized by adding 1/100 reaction volume of 5-N NaOH solution before adding an equal volume of 200-mM phosphate buffer (pH 5.0). The protein in the solution was removed by ultracentrifugation (Ultracel YM-30 3,000MWCO; Millipore, MA, USA) and the cleared solution was analyzed with HPLC equipped with ODS column (TSK-GEL ODS-100Z; Tosoh Corporation), at the eluent (0.2-M sodium phosphate buffer pH 5.0 and 10% acetonitrile) flow rate of 1 ml/min, at 30°C. The absorbance was measured at 254 nm.

HBD activity was measured at 345 nm by monitoring the decrease in NADH concentration resulting from β-hydroxybutyryl-CoA formation from acetoacetyl-CoA (Hartmanis and Gatenbeck 1984). The cell extract was added to the mixture containing 100-mM MOPS buffer (pH 7.0), 1-mM 1,4-dithiothreitol, 0.1-mM acetoacetyl-CoA, and 0.15-mM NADH, and the enzyme activity was calculated as above with the control, from which acetoacetyl-CoA was omitted, using the molar extinction coefficient of 6,220 $M^{-1}cm^{-1}$. The activity was also measured using HPLC as described above in the reaction mixture containing cell extract, 50-mM MOPS buffer (pH 7.0), 1-mM 1,4-dithiothreitol, 0.5 mM acetoacetyl-CoA, and 0.5 mM NADH.

CRT activity was measured by monitoring the decrease in crotonyl-CoA concentration at 263 nm, which results from β-hydroxybutyryl-CoA formation from crotonyl-CoA (Hartmanis and Gatenbeck 1984). The reaction mixture contained cell extract prepared as described before, 100 mM Tris–HCl buffer (pH 7.6) and 50 μM crotonyl-CoA. The enzyme activity was determined as described above using the molar extinction coefficient of 6,700 $M^{-1}cm^{-1}$. The activity was assayed with HPLC as described before by carrying out the reaction in the reaction mixture containing cell extract, 100-mM Tris–HCl buffer (pH 7.6), and 0.5-mM crotonyl-CoA. The analysis was achieved with the condition above except for using gradient elution method (eluent containing 0.2-M sodium phosphate buffer pH 5.0 and acetonitrile concentration increasing from 12 to 36% over 27 min).

The cell extract for BCD assay was prepared as follows and the following procedure was carried out in an anaerobic chamber filled with gas comprised of 95% N_2 and 5% H_2. The collected cells were resuspended in a sonication buffer (50-mM MOPS buffer, pH 7.0), collected again before resuspension in 1-ml sonication buffer, and 0.5-g glass beads was added to the solution. The mixture was homogenized by repeating the following steps for ten

times; vortexing for 1 min and incubation on ice for 1 min. The debris was removed by centrifugation at 15,000×*g*, 4°C for 15 min, and the BCD activity was assayed by monitoring the ferricenium ion, which acts as an electron donor in butyryl-CoA formation from crotonyl-CoA, at 300 nm (Lehman et al. 1990) using Ultraspec 2100 pro (GE Healthcare Bio-Science, NJ, USA) at 30°C. To the mixture containing cell extract and 50-mM MOPS buffer (pH 7.0), crotonyl-CoA was added to 0.4 mM and, following 10 min equilibration ferricenium ion was added to the final concentration of 0.2 mM. The decrease in the absorbance of the sample solution and control solution without crotonyl-CoA was monitored and the activity was determined as above using the molar extinction coefficient of 4,300 $M^{-1}cm^{-1}$. The activity measurement was also carried out with HPLC as described above and the reaction mixture contained cell extract, 50-mM MOPS buffer (pH 7.0), 0.2 mM crotonyl-CoA and 0.2 mM ferricenium ion. The analysis was performed with the condition described above except for using gradient elution method [eluent containing 0.2-M sodium phosphate buffer (pH 5.0) and acetonitrile concentration increasing to 36% over 42 min].

The cell extract for BYDH and BDH assays was prepared as follows in an anaerobic chamber at 30°C, and the following procedure was carried out at the same temperature. The aerobically grown culture was transferred into a 50-ml bottle, incubated under anaerobic condition for 3 h with gentle stirring, and the cell extract was prepared as described in BCD assay method. BYDH activity assay was performed with the yeast-derived alcohol dehydrogenase (F. Hoffmann-La Roche, Switzerland; Dürre et al. 1987). In this coupled assay, BYDH converts butyryl-CoA to butyraldehyde, which is further converted to butanol by the alcohol dehydrogenase resulting in consumption of 2 NADH molecules by a BYDH molecule. The mixture containing cell extract, 50-mM MES buffer (pH 6.0), 100-mM KCl, 0.15-mM NADH and 3 U of yeast-derived alcohol dehydrogenase was incubated for 10 min and 0.2-mM butyryl-CoA was added to the mixture. Decrease in NADH was measured with the sample solution and control solution without butyryl-CoA at 345 nm and the activity was determined as above. The BYDH activity was measured chromatographically in a reaction mixture containing cell extract, 50-mM MES buffer (pH 6.0), 100-mM KCl, 1-mM NADH, and 0.5-mM butyryl-CoA, and analysis was performed as described for CRT assay. Increase in butanol concentration was also monitored by gas chromatography (GC-2014; Shimadzu, Kyoto, Japan) fitted with packed-column (Thermon-1000, Shimadzu) under following condition; carrier-gas flow rate of 60 ml/min, temperature increase rate of 3°C/min with column temperature ranging from 160 to 196°C.

To measure the BDH activity, decrease in NADH concentration resulted from butanol formation from butyraldehyde, was monitored at 345 nm in a sample solution and control solution without butyraldehyde (Dürre et al. 1987). The reaction mixture contained cell extract, 50-mM MES buffer (pH 6.0) and 0.15-mM NADH and the mixture was incubated for 10 min before butyraldehyde was added to the final concentration of 35 mM. The changes in substrate and product concentrations in the reaction mixture containing cell extract, 50-mM MES buffer (pH 6.0), 0.5-mM NADH, and 0.5-mM butyraldehyde were monitored with gas chromatography as described above.

DNA techniques Plasmid DNA was isolated by the alkaline lysis procedure (Sambrook et al. 1989) or using a QIAprep Spin Miniprep Kit (QIAGEN, CA, USA) according to the manufacturer's instructions. Restriction endonucleases were purchased from Takara (Shiga, Japan) and used following the manufacturer's instructions. To construct the expression plasmids, pBUT1 and pBUT2 (Fig. 2), THL coding *thiL* gene (locus_tag; CA_P0078), the *crt-bcd-etfB-etfA-hbd* (*b*utyryl-*C*oA *s*ynthesis; BCS) operon coding for CRT, BCD, electron transfer flavoprotein α (ETF-A) and β (ETF-B) subunits and HBD (CA_C2708-C2712; Boynton et al. 1996), *adhe1* gene (CA_P0162) coding for BYDH and BDH, and *adhe* gene (CA_P0035) which is an isozyme gene of *adhe1* were amplified using the primers listed in Table 2 with *C. acetobutylicum* ATCC 824 (American Type Culture Collection) genome as a template. The gene fragments were extracted from agarose gel using QIAquick Gel Extraction Kit (QIAGEN). PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) in a total volume of 50 μl with 50 ng of chromosomal DNA, 0.4-mM dNTP in LA PCR™ Buffer II with $MgCl_2$ and 2 U of TaKaRa LA Taq™ (Takara) for 30 cycles at temperatures of 94°C for denaturation (1 min), 52°C for annealing (1 min), and 72°C for extension (3 min). The PCR fragments were purified with a QIAquick PCR purification kit (QIAGEN). Transformation of *E. coli* cells was performed by the $CaCl_2$ procedure (Sambrook et al. 1989).

Results

Construction of butanologenic *E. coli* strains

To construct an expression plasmid harbouring the butanol synthesis pathway genes of *C. acetobutylicum* ATCC 824, the genes were amplified with PCR. Each of the resultant PCR products containing *thiL* (1.2 kb), *crt-bcd-etfB-etfA-hbd* operon (4.8 kb), *adhe1* (2.8 kb), and *adhe* (2.7 kb) genes were placed under the control of a constitutive tac promoter (P*tac*) by digesting the amplicons with *Sma*I and ligating into a *Sma*I-digested pKK223–3 (Pharmacia),

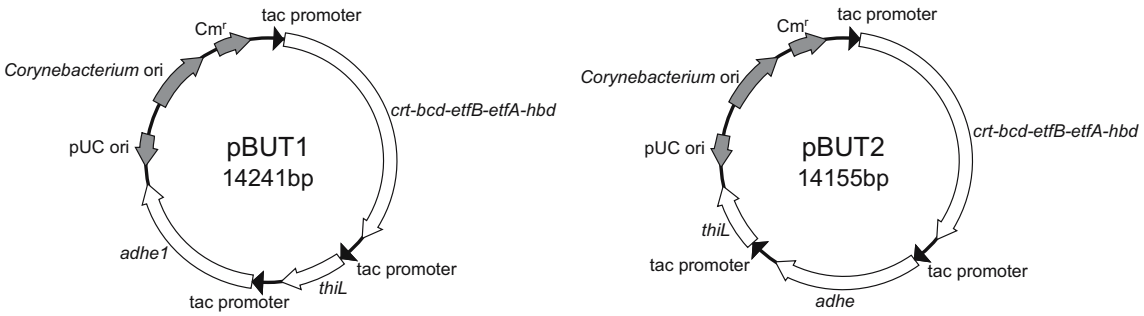


Fig. 2 The plasmids pBUT1 and pBUT2. The location and direction of transcription for relevant genes are indicated by *arrows*; *Cm*^{r}, chloramphenicol resistance gene

yielding plasmids pTHI, pBCS, pADH1, and pADH2, respectively (Table 1).

Subsequently, plasmid pBUT1, containing P*tac-crt-bcd-etfB-etfA-hbd*, P*tac-thiL*, and P*tac-adhe1*, was constructed by ligating the *tac* promoter controlled genes from pBCS, pADH1 and pTHI into pCRB1 (Nakata et al. 2003). pBUT2 with P*tac-crt-bcd-etfB-etfA-hbd*, P*tac-adhe*, and P*tac-thiL* was constructed in the same manner as pBUT1 except the fragment of pADH2 harboring *adhe* was used instead of *adhe1*. The constructed plasmids, pBUT1, and pBUT2 (Fig. 2) were used to transform *E. coli* JM109 to obtain butanologenic *E. coli* strains, BUT1 and BUT2, respectively (Table 1).

Enzyme assays for butanol synthetic enzymes expressed in *E. coli*

Cell extracts obtained from *E. coli* JM109, BUT1, and BUT2 were analyzed for THL, HBD, CRT, BCD, BYDH, and BDH enzyme activities.

The THL, HBD and CRT activities in *E. coli* JM109, BUT1, and BUT2, were measured spectrophotometrically, and the results showed that the THL, HBD, and CRT activities of BUT1 and BUT2 were *ca*. 30, 20, and 500 times greater than those of control (JM109), respectively (Table 3). This finding was in agreement with the previous work carried out by Boynton et al. (1996) in which HBD and CRT activities in *E. coli* possessing the pUC19 vector containing *crt-bcd-etfB-etfA-hbd* operon from *C. acetobutylicum* ATCC 824 were shown to be greater than those of the control harboring pUC19 vector (Boynton et al. 1996). The same activities were also analyzed in *E. coli* BUT1 strain with chromatographic method in vitro. As a result of THL activity, 2 acetyl-CoA molecules are formed from acetoacetyl-CoA and CoA, and decrease in acetoacetyl-CoA and increase in acetyl-CoA were detected over the reaction time as illustrated in Fig. 3a. The THL specific activity calculated based on the decrease in acetoacetyl-CoA was 2.9-U/mg protein and that determined from the increase in acetyl-CoA was 4.3-U/mg protein [Fig. 3b(1)]. The HBD specific activity determined from the decrease in acetoacetyl-CoA was 2.4-U/mg protein, whereas that calculated from the increase in β-hydroxybutyryl-CoA was 2.5-U/mg protein [Fig. 3b(2)]. The specific activity of CRT was also determined as above and that calculated by the decrease in crotonyl-CoA was 16.0-U/mg protein while the activity determined by the increase in β-hydroxybutyryl-CoA was 19.0-U/mg protein [Fig. 3b(3)]. It thus appears that the specific activities of THL, HBD, and CRT determined by two independent methods, spectrophotometric enzyme assay and chromatographic determination of substrates and products, are in good agreement.

Table 2 Oligonucleotides used in this study

Oligonucleotide	Target gene	Sequence (5'-3')	The overhanged restriction sites[a]
Primer 1	*thiL*	ATCCCGGGGAGGAGTAAAACATGAGAGA	*Sma*I
Primer 2		ATCCCGGGCTCGAGTTAGTCTCTTTCAACTACGA	*Sma*I, *Xho*I
Primer 3	*crt-bcd-etfB-etfA-hbd*	ATCCCGGGATATTTTAGGAGGATTAGTC	*Sma*I
Primer 4		ATCCCGGGAGATCTCCATATTATAATCCCTCCTC	*Sma*I, *Bgl*II
Primer 5	*adhe1*	ATCCCGGGATATCTATCTCCAAATCTGC	*Sma*I
Primer 6		ATCCCGGGCTCGAGCCAATCATAATTGTCATCCC	*Sma*I, *Xho*I
Primer 7	*adhe*	CTCTCCCGGGTATAAGGCATCAAAGTGTGT	*Sma*I
Primer 8		CTCTCCCGGGCTCGAGGTCTATGTGCTTCATGAAGC	*Sma*I, *Xho*I

[a] The restriction site overhangs used in the cloning procedure are underlined.

Table 3 Enzyme activities of *E. coli* JM109, BUT1 and BUT2 strains

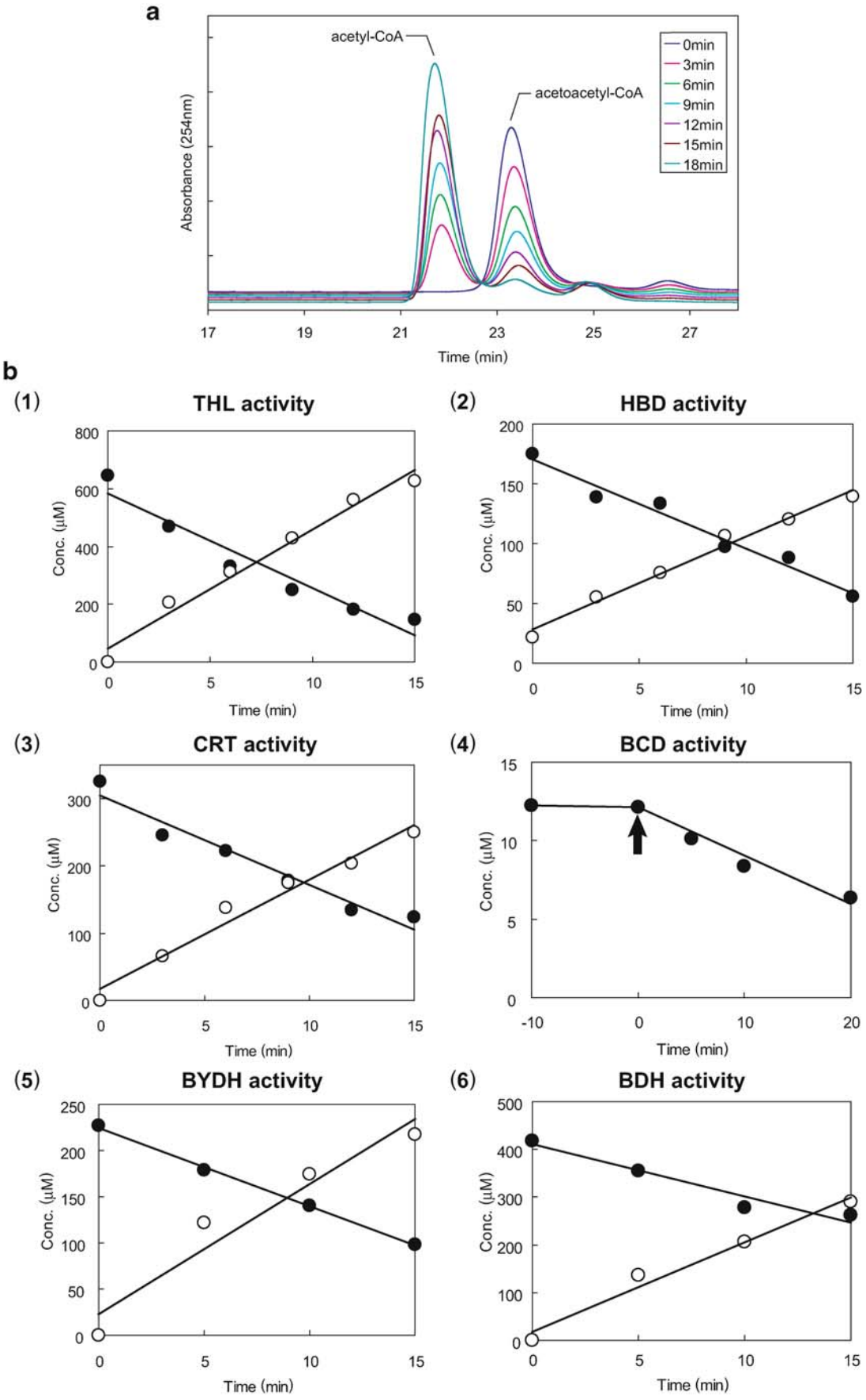
Strain	Enzyme activity (units/mg protein)[a]					
	THL	HBD	CRT	BCD	BYDH	BDH
JM109	0.043	0.047	0.031	n.d.	0.0020	0.13
BUT1	1.2	1.0	15	0.010	0.0028	0.11
BUT2	1.4	0.90	16	0.019	0.024	0.13

[a] Data represent the mean values calculated from triplicate experiments. One unit is the amount of enzyme that converts 1 μmol of the substrate to the product per min.
n.d., not detectable; the minimum measurable specific activity of BCD is less than 0.0010 U/mg-protein.

The spectrophotometric assay demonstrated BCD activity in both BUT1 and BUT2 strains, although such activity was not observed in JM109 (Table 3). The BCD activity in BUT1 was also monitored by chromatographic method by measuring the decrease in substrate and increase in product concentrations. As a result, the decrease in crotonyl-CoA concentration was observed immediately after the ferricenium ion addition demonstrating the BCD activity. However, the specific activity determined by the chromatographic method was 0.0019-U/mg protein [Fig. 3b(4)], which is one order of magnitude smaller than that determined by the spectrophotometric analysis (0.010-U/mg protein; Table 3) and this finding could be explained as follows. As the CRT activity (β-hydroxybutyryl-CoA↔crotonyl-CoA) was significantly higher than that of BCD (crotonyl-CoA↔butyryl-CoA) in BUT1 cell extract, the equilibrium was reached between β-hydroxybutyryl-CoA and crotonyl-CoA immediately after substrate, crotonyl-CoA, was added to the reaction mixture. To confirm that the equilibrium was maintained in the reaction mixture before reaction initiation, the mixture was incubated for 10 min before the addition of the ferricenium ion, which initiated the BCD activity [indicated with an arrow in Fig. 3b(4)]. As the decrease in crotonyl-CoA resulting from BCD activity shifts the equilibrium between β-hydroxybutyryl-CoA and crotonyl-CoA towards crotonyl-CoA, crotonyl-CoA was formed from β-hydroxybutyryl-CoA resulting in a smaller observed decrease in crotonyl-CoA than the actual decrease. From this assumption, the BCD activity determined by the spectrophotometric analysis was considered to more accurately reflect the true BCD activity in BUT1 cell extract. The increase in the product, butyryl-CoA, was not detected by the chromatographic analysis, and this was considered to be due to the immediate conversion of butyryl-CoA into butyraldehyde, butanol, and butyrate.

We further investigated whether the ETF-A and ETF-B are dispensable for the BCD activity. Plasmid pBUT1-ΔETF, which was constructed by removing the 1.9-kb fragment containing *etfA* and *etfB* genes coding for ETF-A and ETF-B from pBUT1, and plasmid pETF, which was constructed by introducing *etfA* and *etfB* genes under the control of *tac* promoter into pCRB1, were constructed (Table 1 and Fig. 3). The plasmids pBUT1ΔETF and pETF were introduced into *E. coli* JM109 to obtain *E. coli* BUT1ΔETF and ETF, respectively (Table 1), and the BCD activities were compared with BUT1 and JM109 strains. The activity measurement showed that the BCD activity was only detected in BUT1 strain in which the *bcd*, *etfA*, and *etfB* genes were expressed, and the activity was not detected in BUT1ΔETF, in which only the bcd gene was expressed, or in ETF, in which only the *etfA* and *etfB* genes were expressed (Fig. 4). Moreover, the BCD activity was not detected in the solution prepared by combining the cell extracts of individually grown BUT1ΔETF and ETF (Fig. 4). These results suggested that ETF-A and ETF-B were required for the BCD activity of the gene coded by *C. acetobutylicum* in *E. coli* and the *bcd*, *etfA*, and *etfB* had to be expressed in the same cell.

The BYDH and BDH activities in *E. coli* strains BUT1, BUT2, and JM109 were monitored, and the BYDH activity in BUT1 did not show a significant increase compared to JM109 strain whereas 12-fold increase in activity was recorded in BUT2 strain (Table 3). The BDH activity, on the other hand, showed no notable difference among the strains tested, although the *adhe1* and *adhe* transcription was confirmed by quantitative PCR analysis (data not shown). This finding could have been due to the high background BYDH and BDH activities of the native alcohol dehydrogenase in *E. coli* leading to no significant effect of the *adhe1* and *adhe* genes. Furthermore, the *adhe* gene product was thought to have higher specificity towards butyryl-CoA in terms of BYDH activity compared to the *adhe1* gene product. The BDH and BYDH activities in BUT2 strain were confirmed by chromatographic analysis and the BYDH specific activity determined measuring the decrease in butyryl-CoA concentration was 0.068-U/mg protein. Butyraldehyde was not detected with this method although the increase in butanol concentration was detected. This phenomenon could be explained by the immediate conversion of the product, butyraldehyde, to butanol by BDH activity in the sample. The BYDH activity calculated by this increase in butanol concentration was


a
acetyl-CoA
acetoacetyl-CoA
0min
3min
6min
9min
12min
15min
18min
Absorbance (254nm)
Time (min)
b
(1) THL activity
(2) HBD activity
(3) CRT activity
(4) BCD activity
(5) BYDH activity
(6) BDH activity
Conc. (μM)
Time (min)

◀ **Fig. 3** Enzyme assays by spectrophotometric method. **a** Chromatograph showing the change in acetyl-CoA and acetoacetyl-CoA concentrations in the THL activity assay reaction mixture. (The absorbance at 254 nm was monitored using the HPLC. The earlier peak corresponds to acetyl-CoA which is formed from acetoacetyl-CoA as a result of THL activity). **b** Changes in the substrate and product concentrations in the enzymatic reaction mixtures monitored by chromatographic analysis; (*1*) THL, (*2*) HBD, (*3*) CRT, (*4*) BCD, (*5*) BYDH and (*6*) BDH activities. The cell extracts added to the solution contained; 0.02, 0.006, 0.0015, 0.1, 0.5, and 0.2 mg/ml proteins, respectively. The *filled circle* indicates the substrate concentrations and the empty circle indicates the product concentrations. An *arrow* in the BCD activity result (*4*) designates the reaction initiation point by the ferricenium ion addition

0.051-U/mg protein [Fig. 3b(5)]. In the case of BDH activity, the specific activity determined by the decrease in butyraldehyde was 0.14-U/mg protein, whereas that calculated using the increase in butanol was 0.13-U/mg protein [Fig. 3b(6)]. The BYDH and BDH activities determined by the two individual assay methods; spectrophotometric and chromatographic methods were again shown to be in agreement.

Subsequently, the cell extracts were prepared from the *E. coli* strains expressing the *adhe1* and *adhe* genes, strains ADH1 and ADH2 (Table 1), respectively. The cell extracts were used to monitor (1) the aldehyde dehydrogenase activity—BYDH or acetoaldehyde dehydrogenase (ACDH) activity towards butyryl-CoA or acetyl-CoA, respectively—and (2) alcohol dehydrogenase activity—the BDH or ethanol dehydrogenase (EDH) activity towards butyraldehyde or acetaldehyde, respectively. As shown in Fig. 5, the ACDH activity towards acetyl-CoA increased 30- and 60-fold in ADH1 and ADH2, respectively, whereas the BYDH activity did not show a notable increase (twofold increase) with ADH1 although 20-fold increase was observed in the ADH2 cell extract (Fig. 5a) compared to JM109. When the ACDH activity in the ADH1 and ADH2 cell extracts was compared, the activity with the ADH2 was twofold greater than that of ADH1, whereas the BYDH activity with the ADH2 was 13-fold greater than that of ADH1. From this observation, the *adhe* gene product could be deduced to have higher specificity towards butyryl-CoA than towards acetyl-CoA. In the case of BDH activity measured with the ADH1 and ADH2 strains, no significant increase compared to JM109 using butyraldehyde as a substrate was recorded while twofold increase in the EDH activity was shown when acetaldehyde was used as a substrate (Fig. 5b).

Butanol production by butanologenic *E. coli* strains

To investigate the abilities of *E. coli* BUT1 and BUT2 strains to produce butanol, these strains were grown in the LB liquid medium under aerobic condition. The cells were collected and resuspended in the M9 medium to OD_{660} value of 20 in the anaerobic chamber. The resuspended cells were incubated under anaerobic condition to monitor the butanol production, and no growth was observed during the incubation. After 60 h incubation, the maximum butanol concentration of 4.2 mM (320 mg/l) was recorded in BUT1 culture and 6.1-mM butyrate (540 mg/l), the fermentation byproduct, was detected. The butanol and butyrate yields calculated against the amount of glucose consumed were 1.4 and 2.3%, respectively. In the BUT2 culture, the maximum butanol concentration of 16.2 mM (1,200 mg/l) was measured after 60 h of incubation and 1.2 mM (100 mg/l) butyrate was detected. The butanol and butyrate yields were 6.1 and 0.4%, respectively (Fig. 6). It should also be noted that growing cultures of constructed butanologenic strains did not produce detectable butanol under either aerobic or anaerobic conditions (data not shown).

In the case of *E. coli* JM109 strain, which was monitored as a control, neither butanol nor butyrate was detected in the culture (data not shown).

Discussion

To investigate the butanol production in *E. coli*, the strains, BUT1, and BUT2, were created by introducing the *tac* promoter controlled genes of *C. acetobutylicum* ATCC 824

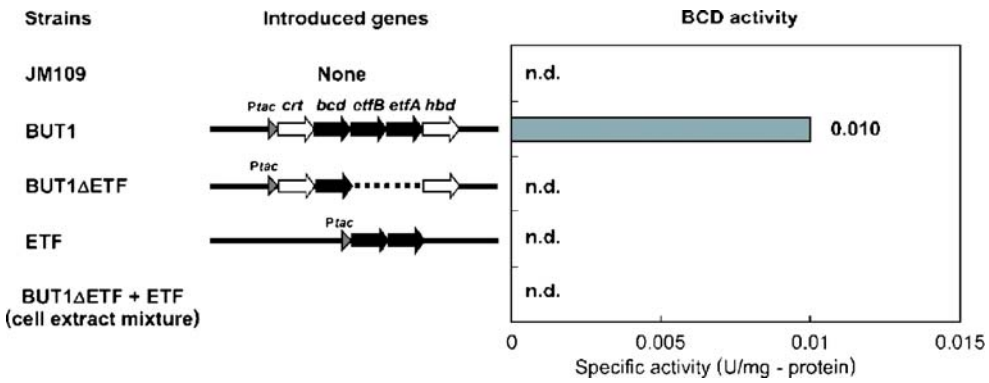


Fig. 4 BCD specific activity in the cell extracts of *E. coli* strains JM109, BUT1, BUT1ΔETF, ETF, and a mixture of BUT1-ΔETF and ETF cell extracts; *n.d.*, not detected; *Ptac*, *tac* promoter

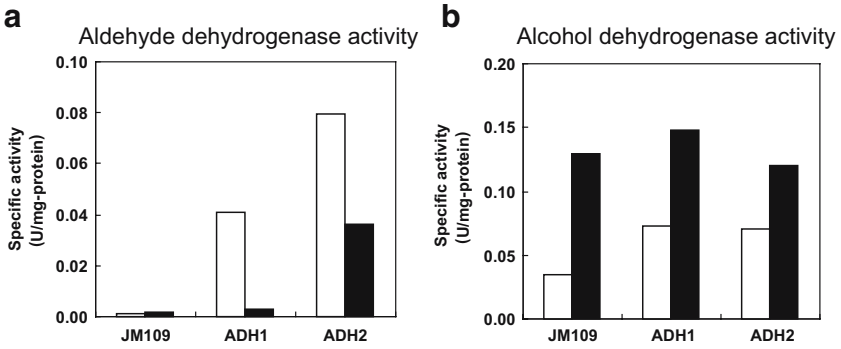


Fig. 5 **a** BYDH- and **b** BDH-specific activities in the cell extracts of *E. coli* JM109, ADH1 (*adhe1*) and ADH2 (*adhe*) strains. BYDH specific activity was measured using acetyl-CoA (*empty bar*) or butyryl-CoA (*filled bar*) as a substrate and BDH specific activity was monitored using acetylaldehyde (*empty bar*) or butyrylaldehyde (*filled bar*) as a substrate

coding for the enzymes that catalyze the six-step reactions from acetyl-CoA to butanol. The enzymatic activities in the BUT1 and BUT2 were measured by spectrophotometric and chromatographic analyses and the results demonstrated that the activities of the six enzymes showed a notable increase compared to the control, *E. coli* JM109 strain.

Boynton et al. (1996) reported that the BCD activity was not detected in the cell extracts of *E. coli* possessing *crt-bcd-etfB-etfA-hbd* operon from *C. acetobutylicum* ATCC 824 grown aerobically or anaerobically (Boynton et al. 1996). It is assumed that the BCD activity was successfully detected in this study as the cell extract preparation and enzyme assay were carried out under anaerobic condition although the cell extracts were prepared from the aerobically grown *E. coli* strains. When the same procedure was performed under aerobic condition, enzymatic activity was not detected (data not shown). This phenomenon is assumed to be due to oxygen sensitivity of the BCD and/or effect of metabolite oxidation in the cell extract on the enzymatic reaction.

Subsequently, the characteristics of the BCD were further investigated in the current study. The BCD and ETFs had been extracted from anaerobe *Megasphaera elsdenii* (Van Berkel et al. 1988; Whitfield and Mayhew 1974) and ETFs were reported to be essential for the electron transfer between BCD and NAD(H) (Engel and Massey 1971; Pace and Stankovich 1987; Whitfield and Mayhew 1974). On the *C. acetobutylicum* genome, the ETF-A and ETF-B coding genes are located immediately downstream of BCD coding gene as found with *Megasphaera elsdenii* (O'Neill et al. 1995); hence, it was predicted that the activity of the BCD coded by the *C. acetobutylicum*'s gene required ETF-A and ETF-B (Boynton et al. 1996). However, involvement of ETF-A and ETF-B in the activity of the BCD coded by the *C. acetobutylicum*'s gene had not previously been demonstrated by enzymatic characterization or gene disruption experiments. Therefore, the activity of the BCD coded by the *C. acetobutylicum*'s gene was shown to require the ETFs for the first time in this study. Furthermore, it was demonstrated that the *bcd*, *etfA*, and *etfB* genes had to be expressed simultaneously.

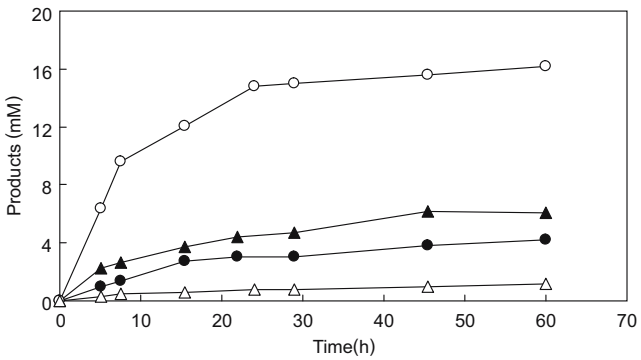


Fig. 6 Increase in butanol and butyrate concentrations in butanologenic *E. coli* BUT1 and BUT2 cultures. The results are the mean values of three independent experiments. (*filled circle*) butanol in BUT1 culture, (*open circle*) butanol in BUT2 culture, (*filled triangle*) butyrate in BUT1 culture and (*open triangle*) butyrate in BUT2 culture

According to the results obtained from the genome sequence analysis of *C. acetobutylicum* ATCC 824, six alcohol dehydrogenase genes have been annotated (Nölling et al. 2001). Of the six genes, two are ca. 2.6-kb bifunctional aldehyde/alcohol dehydrogenase genes, *adhe1* (CA_P0162) and *adhe* (CA_P0035). The other four genes are *ca.* 1.2 kb in length, and their sequences are similar to that of the C-terminal region of the bifunctional enzyme coding genes. Of the four genes, two were found to have the alcohol dehydrogenase function, *bdhA* (CA_C3299) and *bdhB* (CA_C3298), and the sequence of the other two, CA_P0059 and CA_C3392, are found to be similar to the former two genes.

In this study, *adhe1* or *adhe* gene was expressed in *E. coli*. When the *adhe1* gene was expressed on a plasmid in *C. acetobutylicum*, the ACDH and BDH activities were shown to increase, and BYDH activity was demonstrated to increase only at the late exponential growth phase (Nair et al. 1994). Moreover, the same study reported that the EDH activity showed no notable increase (Nair et al. 1994). The

present study showed that when *adhe1* gene was expressed in *E. coli*, the ACDH, BYDH, and EDH activities increased 30-, 2-, and 2-fold, respectively, while no increase in BDH activity was recorded compared to the control strain, JM109 (Fig. 5). A straightforward comparison cannot be made between the outcomes of the previous (Nair et al. 1994) and the present study as the hosts, *C. acetobutylicum* and *E. coli*, are different. However, high background activities of the native aldehyde dehydrogenase and alcohol dehydrogenase in *E. coli* could have resulted in masking of the enzymatic activity increase owing to the introduced gene. Therefore, it was necessary to analyze the characteristics of the purified enzyme to further investigate the specific activity of the *adhe1* gene product towards substrate.

The *C. acetobutylicum* ATCC 824 *adhe* gene product was reported to have high NADH-dependent BYDH and BDH activities when the gene was expressed in the *adhe1*-deficient *C. acetobutylicum* (Fontaine et al. 2002). Furthermore, by expressing the *adhe* gene fused with *Strep*-tagII in *E. coli* and by measuring the NADH-dependent BYDH and BDH activities with the purified protein, both BYDH and BDH activities were detected (Fontaine et al. 2002). This study, however, showed that by expressing the *adhe* gene in *E. coli*, the ACDH, BYDH, and EDH activities showed 60-, 20-, and 2-fold increases, respectively, when compared to the JM109 strain while no notable increase was detected with the BDH activity (Fig. 5). Moreover, by comparing the ACDH and BYDH activities of the *adhe1* or *adhe* gene expressed in *E. coli*, *adhe* product was assumed to have higher specificity towards butyryl-CoA than *adhe1* product (Fig. 5). From the results obtained in this study, *adhe* could be assumed to be more suited for the butanol production than *adhe1*, although a problem of the byproduct, ethanol, production arises as the *adhe* also possesses ACDH activity. Furthermore, among the four alcohol dehydrogenases in *C. acetobutylicum*, *bdhA* gene product was reported to have twofold greater specificity towards butyraldehyde compared to that towards acetaldehyde, whereas the *bdhB* gene product showed 46-fold greater specificity towards butyraldehyde (Petersen et al. 1991). As the increase in the BDH activity was not observed with the BUT1 and BUT2 compared to the JM109 strain in this study, the *bdhB* gene of *C. acetobutylicum* ATCC 824 has been introduced into the *E. coli* strains and its effect on the butanol production is currently under investigation.

The results obtained from analyzing the culture of the butanologenic *E. coli* strains, BUT1 and BUT2, showed that the amount of butanol produced was greater in BUT2 culture (16.2 mM) than in BUT1 culture (4.2 mM), whereas the amount of butyrate produced was greater in the BUT1 culture (6.1 mM) than in BUT2 culture (1.2 mM). This observation could be explained by the higher recorded BYDH activity in BUT2 than BUT1 and the higher BYDH activity should have lead to butanol production from butyryl-CoA rather than to butyrate production. As there is no phosphate butyryltransferase, which catalyzes the reaction to form butyrylphosphate from butyryl-CoA, or butyrate kinase, which catalyzes the reaction from butyrylphosphate to butyrate, coding gene on the *E. coli* genome, some assumptions had to be made on the butyrate production by the butanologenic *E. coli* strains. The expression of the *atoDA* gene of *E. coli* K-12, which encodes the acetyl-CoA:acetoacetyl-CoA transferase (ACT; EC:2.8.3.8), was reported to lead to butyrate utilization (Jenkins and Nunn 1987). As the ACT catalyzes the reaction in both directions, butyrate could be produced from butyryl-CoA by the ACT activity. Moreover, ACT has also been found in the *E. coli* O157:H7 strain. The fused predicted acetyl-CoA:acetoactyl-CoA transferase, coded by the *ydiF* gene was shown to produce butyrate from butyryl-CoA (Rangarajan et al. 2005). On the *E. coli* K-12 genome, the *ydiF* gene which shows 98% similarity to that of the O157:H7 strain is coded, and it can be assumed that the gene product possesses the same activity as the previously studied enzyme.

In BUT1 and BUT2 cultures, organic acids including lactate, formate, acetate, and succinate, etc., were found to be produced as byproducts (data not shown). Therefore, further investigation should be carried out by disrupting the genes responsible for producing the byproducts, including *ldhA*, *fdhF*, and *frdABCD*, to increase the butanol yield.

The current paper reports the development of the novel butanol production system by introducing the butanol synthetic genes of *C. acetobutylicum* ATCC 824 into the facultative anaerobe, *E. coli*. During the ABE fermentation, acetone, butanol, and ethanol are produced in the ratio of 6:3:1 from sugars and starch (Mitchell 1998); however, by focusing the butanol production pathway from glucose in particular, the redox is balanced, i.e., 4-mol NADH produced is consumed during the butanol production process (Jones and Woods 1986); hence, it is theoretically possible to solely produce butanol from glucose. This suggests that butanol production with higher yield could be achieved with a novel production system independent of the problems associated with the fermentation process using the strict anaerobes.

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