Studies on applications of *Clostridium* species for biorefinery

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Contents

Abbreviations

C. acetobutylicum	*Clostridium acetobutylicum*
C. cellulovorans	*Clostridium cellulovorans*
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
EC	Electrochemical detector
E. coli	*Escherichia coli*
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
GPI	Glycosylphosphatidylinositol
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
i.d.	Inside diameter
IgG	Immunoglobulin G
LB	Luria-Bertani
MES	2-(N-morpholino)ethanesulfonic acid
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
N.C.	Negative control
OD	Optical density
PBS	Phosphate buffered salts
PCR	Polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SD	Synthetic dextrose
TCA	Tricarboxylic acid
YPD	Yeast-peptone-dextrose

Introduction

In the last several decades, our lifestyle has dramatically changed and become much more comfortable owing to developments in science and technology. However, the advantages afforded by such progress have caused many environmental problems such as air pollution, climate change, and global warming. Global warming is mainly caused by the accumulation of greenhouse gases such as CO_2, CH_4, and N_2O. The major cause of increasing CO_2 concentration in the atmosphere is excessive consumption of fossil fuels, e.g., petroleum, coal, and natural gas, together with the production of products such as plastics, which are derived from petroleum and natural gas. Moreover, excessive consumption of fossil fuel has caused not only global warming but also global economic problems. Paradigm-changing new ideas and technologies are required to overcome these problems.

Biorefinery

Biorefinery is a technology that produces fuels and chemical products such as plastics, medicines, and foods from sustainable and renewable feedstock, e.g., biomass (Ohara, 2003; Kamm & Kamm, 2004). Biomass is a collective term for the biological material from living organisms, and it is regarded as a possible alternative energy source and source of raw materials because biorefinery process are 'carbon-neutral'; that is, the net CO_2 emission from biorefinery processes is zero. Since plants absorb CO_2 and photosynthesize it to produce energy and to grow, the net CO_2 emission from the use of biomass in biorefinery processes should be zero in theory. In addition, biomass can be used to produce fuel and many chemical products which are currently produced from petroleum. Thus, biomass has the potential to completely replace petroleum.

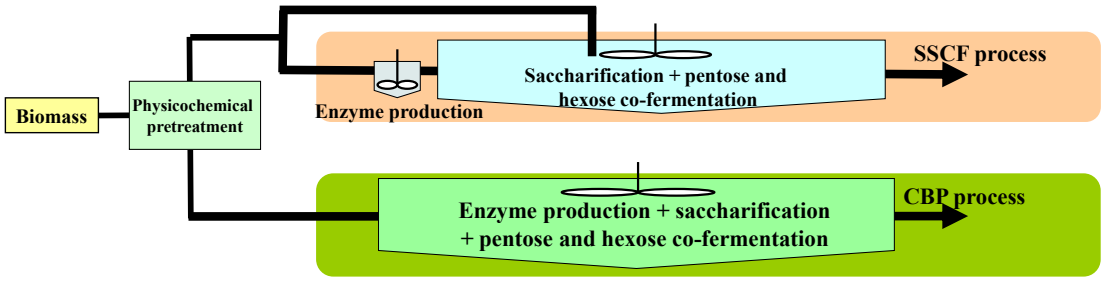
Biofuels

The utilization of biomass energy can potentially reduce the emission of greenhouse gases. This is because the CO_2 released during the combustion of biomass energy is recycled through

photosynthesis, without affecting the overall CO_2 balance in the atmosphere. Therefore, the introduction of biofuels is considered a promising approach to reduce dependence on fossil resources. Bioethanol and biobutanol are examples of biofuels.

Bioethanol fermentation is one of the largest-scale microbial processes using sugars or polysaccharides that can be depolymerized to a fermentable sugar (Demain, 2009). Most of the raw materials currently used for bioethanol production are grain biomasses such as corn and sugar cane; however, the increasing demand for bioethanol has led to a global increase in the prices of food crops. Thus, future restriction of the supply of these materials is inevitable. Therefore, lignocellulosic biomasses are considered attractive raw materials for bioethanol production (the so-called second-generation bioethanol). To construct an energy-saving and sustainable society, the development of technologies to utilize lignocellulosic biomass is required.

There are four major bioprocesses in bioethanol production from cellulosic biomass: production of cellulases and hemicellulases, hydrolytic degradation of cellulose and hemicelluloses, C6 sugar fermentation, and C5 sugar fermentation. Consolidated bioprocessing (CBP) is the system by which these four bioprocesses take place in a single fermenter (Fig 1). The simultaneous saccharification and cofermentation (SSCF) of starch is currently the primary method for ethanol fermentation from starch, but simplification and cost reduction of the process are highly desired. SSCF requires the process of enzymes for bioethanol production, whereas CBP can produce the enzymes required on its own, thus enabling more efficient production of bioethanol from cellulose containing hexose and pentose sugars in a smaller fermenter. Therefore, CBP has an advantage over SSCF in terms of reduced cost of production and equipment. To perform CBP, however, it is essential that a single and advanced species of microorganism is used in the production of saccharification enzymes, saccharification of biomasses of cellulose, and fermentation of saccharified sugar (Lynd *et al.*, 2005). Thus, yeasts with saccharification ability would be ideal for use in bioethanol production via CBP. As yeasts can perform ethanol fermentation of sugars but not saccharification, they must be engineered in order to be made suitable for CBP.



SSCF : Simultaneous saccharification and co-fermentation
CBP : Consolidated bioprocessing

Fig. 1 A model of SSFC and CBP systems.

Recently, biobutanol has been attracting more attention than other biofuels because butanol has more significant advantages as a biofuel than ethanol. First, butanol can be used either in its pure form or mixed with gasoline in any proportion, whereas ethanol can only comprise up to 85% of an ethanol-gasoline mixture. Second, combustion of butanol does not require modification of existing car engines because butanol is less soluble in water than ethanol. Third, the energy content of butanol is about 40% higher than that of ethanol (Table 1) (Durre, 2007; Atsumi *et al.*, 2008). Biobutanol is produced by fermentation using *Clostridium* species, which naturally possess metabolic pathways for the conversion of sugar into solvents such as acetone, butanol, and ethanol. Although the process of biofuel conversion by clostridia is known (acetone-butanol-ethanol [ABE] fermentation), the mechanisms that regulate the metabolic fluxes in these organisms is still obscure. For the industrial-level production of butanol, various factors affecting clostridia fermentation have been widely studied, including pretreatment of substrates, techniques of butanol recovery, and induction of solventogenesis. In addition, the introduction into hosts of clostridial metabolic pathways or keto-acid pathways for synthesis of butanol has also been investigated (Atsumi & Liao, 2008; Inui *et al.*, 2008).

Table 1 Comparison of butanol with ethanol

Fuel	Caloric value (MJ/L)	Air-fuel ratio	Vapor pressure (mm Hg, 20°C)	Research octane number
Butanol	29.2	11.2	4	96
Ethanol	21.2	3.0	45	129

The major problems in the production of biofuel include low and nonspecific productivity, high cost, and solvent-sensitivity of living cells. However, advances in genetic manipulation and metabolic engineering can offer solutions to these problems. Thus, there is a strong incentive to produce butanol from user-friendly organisms such as yeast *Saccharomyces cerevisiae*, which are useful for the following reasons:

・Yeast is safer to use than *Escherichia coli* and other bacteria.

・Yeast can be cultivated in aerobic conditions, whereas *Clostridium* requires anaerobic conditions.

・Yeast, which is a eukaryotic organism, has more CoA and NADH than *E. coli* and other bacteria.

・Yeast can be engineered to improvement tolerance to organic solvents.

・It is possible to use yeast to produce butanol from cellulosic biomass via CBP.

Due to these reasons, yeast was used to produce butanol in this study.

Clostridium species

Clostridia comprise a diverse group of anaerobic, spore-forming, and gram-positive bacteria that include notable pathogens as well as industrially significant microorganisms. *Clostridium acetobutylicum* was found to produce acetone/butanol/ethanol at a ratio of 3:6:1 via ABE fermentation (Fig. 2) (Antoni *et al.*, 2007; Inui *et al.*, 2008). Bacterial production of butanol and acetone via the ABE fermentation process was valuable in the production of the lacquer solvent butylacetate and in the development of the synthetic rubber industry. However, bacterial production has declined with the advancement of the petrochemical industry, which can produce acetone and butanol at low costs. As biofuel production is increasingly being practiced worldwide due to its usefulness, research and development into microbial butanol production is once again being actively pursued.

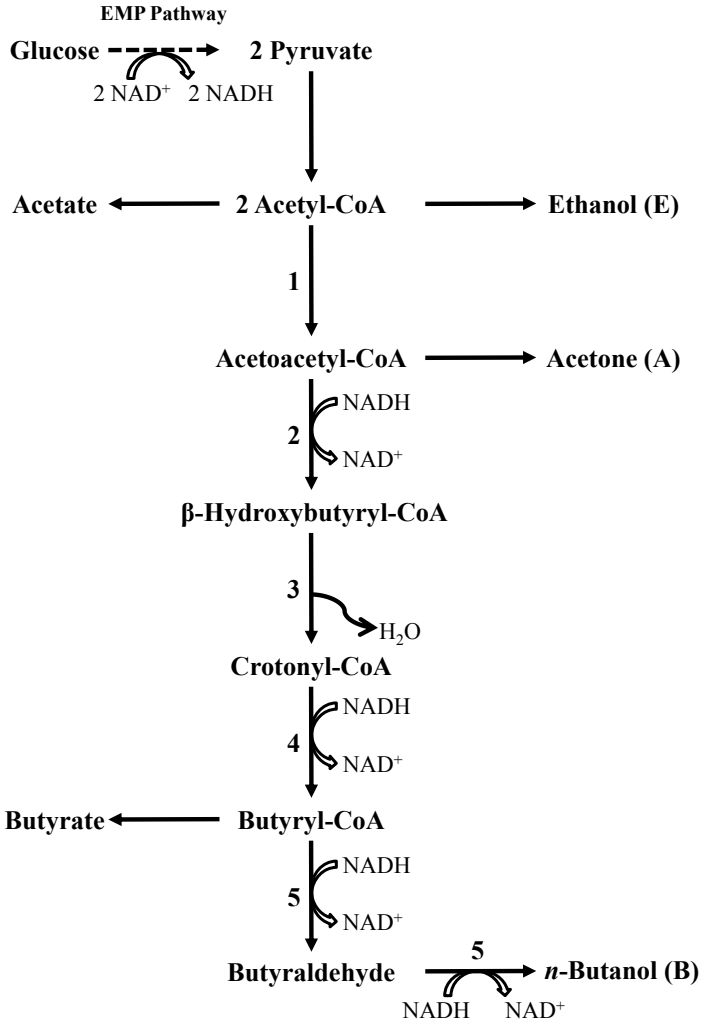


Fig. 2 ABE fermentation by *C. acetobutylicum*. Numbers refer to the enzymes; 1: acetyl-CoA acetyltransferase (thiolase; THL), 2: β-hydroxybutyryl-CoA dehydrogenase (HBD), 3: 3-hydroxybutyryl-CoA dehydratase (crotonase; CRT), 4: butyryl-CoA dehydrogenase (BCD), electron transfer flavoprotein A (ETFA) and B (ETFB), 5: aldehyde dehydrogenase (ADHE)/ aldehyde-alcohol dehydrogenase (ADHE1), EMP pathway: Embden-Meyerhof-Parnas pathway.

C.cellulovorans is an anaerobic, mesophilic bacterium that effectively degrades and utilizes plant cell walls (Sleat *et al.*, 1984), and recently, its genome was completely sequenced (Tamaru *et al.*, 2010a). *C. cellulovorans* has been reported to utilize many kinds of biomass and derived pure compounds such as cellulose, xylan, pectin, cellobiose, glucose, fructose, galactose, and mannose as carbon sources (Koukiekolo *et al.*, 2005; Tamaru *et al.*, 2010b). This wide

spectrum of degradation is thought to be due to the extracellular complex called 'cellulosome', which contains scaffoldin and various kinds of cellulases. Whole genome sequencing, and subsequent comparative genomics research has shown that *C. cellulovorans* has a small number of cellulosomal proteins, compared to other cellulosome-producing clostridia (Tamaru *et al.*, 2010a; Tamaru *et al.*, 2010b). This indicates that *C. cellulovorans* has the most basic cellulosome, so it would be an ideal *Clostridium* species to study in terms of cellulosome reconstruction. In addition, although several cellulosome-producing *Clostridium* species have been reported to date, most of the researches on these species have focused on the cellulosome itself. To understand biomass degradation and adapt it for practical application, it will be important to elucidate the basic biology of clostridia, especially the metabolic processes that are highly associated with the conversion of carbohydrates to final products. Moreover, because *C. cellulovorans* can completely degrade several kinds of biomass, its saccharification enzymes have more potent degradation activity than those from other cellulolytic microorganisms; thus, the saccharification enzymes from *C. cellulovorans* would be more useful for biomass degradation.

Metabolism of Clostridium spp.

To take advantage of the opportunities for biorefinery provided by cellulosome-producing bacteria, we must first collect a lot of information about the physiological aspects of bacteria themselves as well as those metabolites directly associated with the process of converting cellulosic carbohydrates to other products. Then the metabolism of cellulosome-producing bacteria during fermentation should be closely investigated. However, there are few researches into carbon central metabolism of *Clostridium* spp. aside from that on *C. kluyveri* and *C. acetobutylicum*. In *C. kluyveri*, approximately 30% of the cell carbon is derived from CO_2 and about 70% from acetate, using $^{14}CO_2$ as the ^{14}C substrate to trace metabolic processes. (Tomlinson & Barker, 1954). The use of ^{13}C-labeled carbon such as [U-^{13}C]-glucose and $NaH^{13}CO_3$ to study central carbon metabolism in *C. acetobutylicum* revealed that this species has CO_2 fixation activity and bidirectional TCA cycle (Fig. 3) (Amador-Noguez *et al.*, 2010),

although at that time the study was performed, several genes in the TCA cycle (in particular, the gene encoding 2-oxoglutarate dehydrogenase) had not yet been identified. However, an alternative pathway for the conversion of 2-oxoglutarate to succinate via succinic semialdehyde was reported in *Mycobacterium tuberculosis* and cyanobacteria *Synechococcus* sp. PCC7002 (Tian *et al.*, 2005; Zhang & Bryant, 2011).

In particular, ^{13}C-labeling studies of metabolic products are useful for studying *in vivo* metabolism since they can distinguish fluxes through different pathways when the fluxes result in different positional isotopic enrichments in metabolic intermediates (Ratcliffe & Shachar-Hill, 2006; McKinlay *et al.*, 2007). In *C. cellulovorans*, there have been few studies of metabolic compounds and metabolic pathways; thus, the metabolic pathways of *C. cellulovorans* are still unknown.

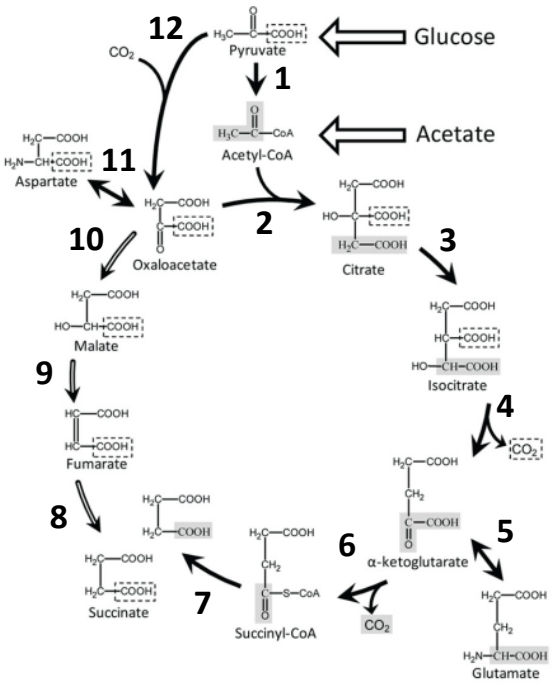


Fig. 3 CO_2 fixation activity and bidirectional TCA cycle in *C. acetobutylicum*. Numbers refer to the enzymes; 1: pyruvate dehydrogenase, 2: citrate synthase, 3: aconitase, 4: isocitrate dehydrogenase, 5: glutamate dehydrogenase, 6: α-ketoglutarate dehydrogenase, 7: succinyl-CoA synthase, 8: succinic dehydrogenase, 9: fumarase, 10: malate dehydrogenase, 11: aspartate aminotransferase, 12: pyruvate carboxylase. Gray boxes show the fate of the carbons in the incoming acetyl group from acetyl-CoA, and dotted boxes show the fate of the carbons in the carboxyl group from pyruvate.

Cell-surface engineering of yeast

Cell surface engineering technology is a very useful strategy for the molecular breeding of yeasts as CBP microorganisms. Using this technology, functional proteins can be displayed on the cell surface of the microorganism. Therefore, cell surface engineering technology can provide intact cells with new functions, and construct various arming cells with novel functions (Murai *et al.*, 1997; Ueda & Tanaka, 2000a; Ueda & Tanaka, 2000b; Kondo & Ueda, 2004). The cell surface is a functional interface between the inside and the outside of the cell. *S. cerevisiae* has the rigid cell wall that is about 200 nm thick, mainly composed of mannoproteins and β-linked glucans, and lies outside the plasma membrane. In yeasts, several proteins on the cell surface have secretion signal peptides at the N-terminal and glycosylphosphatidylinositol (GPI) anchors at the C-terminal. GPI anchors play important roles in surface production and are essential for cell viability. Upon completion of protein synthesis, the secreted proteins are translocated into the lumen of the endoplasmic reticulum (ER), and transported from the ER to the Golgi apparatus and then to the plasma membrane in membrane-enclosed vesicles. GPI-anchored proteins are further transported to the outside of the plasma membrane through the secretory pathway; they are released from the plasma membrane by a phosphatidylinositol-specific phospholipase C (PI-PLC), and transferred to the outermost surface of the cell wall (Fig. 4a) (Ueda & Tanaka, 2000b). α-Agglutinin, which is encoded by *AGα1* and interacts with the binding subunit of the agglutinin complex of **a**-type cells, is one of the GPI-anchored cell surface proteins. α-Agglutinin is composed of a secretion signal region, an active region, an anchoring region rich in serine and threonine, and a GPI anchor-attachment signal (Fig. 4b). Using this molecular information about the mechanism underlying the localization of proteins to the cell wall, it has become possible to display target heterologous proteins on the yeast cell surface by genetic engineering techniques (Fig. 4c) (Kondo & Ueda, 2004). Furthermore, cell surface engineering is an innovative molecular tool by which the function of a displayed protein can be analyzed on intact cells. Since the mutated proteins can be analyzed by treating the cells as microparticles covered with proteins, protein purification and concentration is not required (Ueda, 2004). Therefore, cell surface engineering technology

enables the construction of various biocatalysts with potential for industrial utilization.

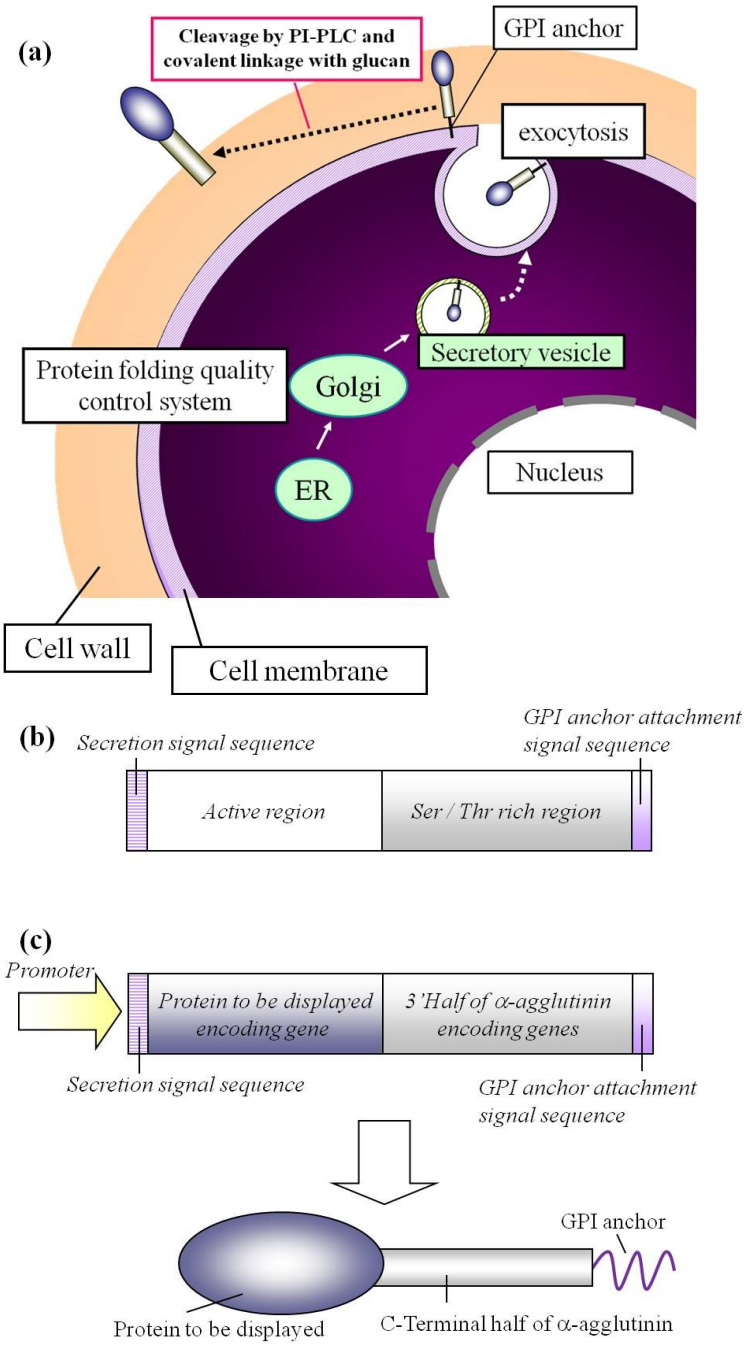


Fig. 4 (a) Mechanism of cell surface display of proteins by cell surface engineering, (b) molecular structure of α-agglutinin, and (c) molecular design of cell-surface-displayed enzyme.

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Chapter I Fixation of CO_2 in *Clostridium cellulovorans* analyzed by ^{13}C-isotopomer-based target metabolomics

C. cellulovorans has been suggested to have a CO_2 fixation pathway, because of its ability to grow under a higher concentration of '100%' CO_2 compared to other *Clostridium* species (*C. cellulovorans*, 20% (atm/atm); *C. acetobutylicum*, 5%; *C. thermocellum*, 10%; *C. difficile*, 10%; and *C. kluyveri*, 5%) (Thauer *et al.*, 1968; Sleat *et al.*, 1984; Amador-Noguez *et al.*, 2010; Saujet *et al.*, 2011; Waller *et al.*, 2013). Previously, a few studies have characterized the metabolic pathway of *C. kluyveri* and *C. acetobutylicum* (Jungermann *et al.*, 1970; Amador-Noguez *et al.*, 2010). In the genome analysis of *C. cellulovorans* (Tamaru *et al.*, 2010), the genes of 2 important CO_2 fixation enzymes, namely pyruvate:ferredoxin oxidoreductase (PFOR) and phosphoenolpyruvic acid (PEP) carboxylase (PEPC) were annotated. Notably, PFOR of glycolysis and PEPC of the TCA cycle are both in the node of main metabolic pathways in *C. cellulovorans*. Therefore, the study of CO_2 fixation by metabolome analysis would help to clarify the complete metabolic pathway of *C. cellulovorans*. In particular, ^{13}C-labeling studies of metabolic products are useful for understanding the *in vivo* metabolism since 13-carbon isotope can distinguish fluxes through different pathways when these fluxes result in different positional isotopic enrichments in metabolic intermediates (Ratcliffe & Shachar-Hill, 2006; McKinlay *et al.*, 2007).

As illustrated in Fig. 1, we carried out labeling experiments of metabolic intermediates by allowing *C. cellulovorans* to grow in medium with an atmosphere of '100%' CO_2 containing either $NaH^{13}CO_3$ or [U-^{13}C]-glucose as a labeling reagent, followed by the GC/MS analysis. We demonstrated metabolic fluxes of *C. cellulovorans* and discussed the physiological meaning of CO_2 fixation in the metabolic pathway of *C. cellulovorans*.

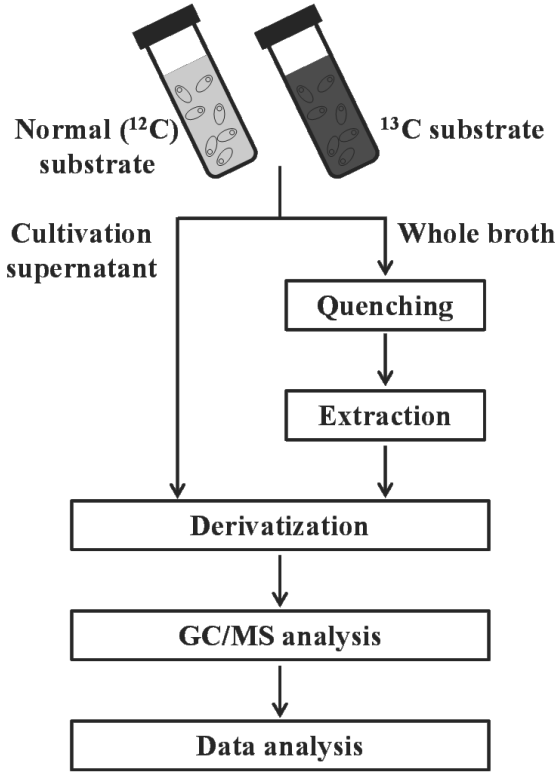

Fig.1 Workflow for the investigation of CO_2 incorporation and the quantification of metabolites of interest.

Materials and methods

Cultivation conditions and growth rate analysis

C. cellulovorans 743B (ATCC 35296) was grown anaerobically at 37°C in an atmosphere of '100%' CO_2 unless otherwise noted. Liquid cultivation media contained the following reagents: 0.45 g/l $KH_2PO_4 \cdot H_2O$, 0.45 g/l K_2HPO_4, 0.9 g/l NaCl, 0.3675 g/l NH_4Cl, 0.1575 g/l $MgCl_2 \cdot 6H_2O$, 0.12 g/l $CaCl_2 \cdot 2H_2O$, 5.2 mg/l Na_2-EDTA, 1.5 mg/l $FeCl_2 \cdot 4H_2O$, 0.942 mg/l $CoCl_2 \cdot 6H_2O$, 0.85 mg/l $MnCl_2 \cdot 4H_2O$, 0.07 mg/l $ZnCl_2 \cdot 6H_2O$, 0.062 mg/l H_3BO_4, 0.036 mg/l $Na_2MoO_4 \cdot 2H_2O$, 0.024 mg/l $NiCl_2 \cdot 6H_2O$, 0.017 mg/l $CuCl_2 \cdot 6H_2O$, 5g/l $NaHCO_3$, 4 g/l Bacto™ Yeast Extract (Becton and Dickinson Company), 3 g/l glucose, and 1 g/l L-cysteine. For labeling experiments, $NaHCO_3$ and glucose were replaced by $NaH^{13}CO_3$ and [U-^{13}C]-glucose, respectively (both 99% purity; Cambridge Isotope Laboratories, Andover, MA).

Quenching and extraction of intracellular metabolites

Quenching and metabolite extraction were carried out as previously described (Winder *et al.*, 2008), with some modifications. In brief, culture broths were injected rapidly into 4 volumes of 60% aqueous methanol solution (–40°C) for quenching. Supernatants after centrifugation at 3000 × *g* at –9°C for 10 min for quenching were removed rapidly, and washed with 1 ml of 60% aqueous methanol (–40°C), followed by centrifugation at 3000 × *g* at –9°C for 10 min. Subsequently, supernatants were thoroughly removed, and cell pellets were frozen in liquid nitrogen and kept at –80°C until the following extraction procedures. Cell pellets were suspended in 500 μl of 100% methanol (–40°C), frozen in liquid nitrogen, and allowed to thaw on dry ice. After, the freeze-thaw cycle was performed 3 times in total, the suspensions were centrifuged at 16000 × *g*, at –9°C, for 5 min. Supernatants were retained and stored on dry ice, and another aliquot (500 μl) of 100% methanol (–40°C) was added to each pellet. The procedure was repeated twice, and the second aliquot of methanol was combined with the first one.

Metabolite derivatization

Extract aliquots and cultivation medium supernatants (20 μl each), as well as dilution series of standard mixtures of target metabolites (Table 1), were spiked with internal standards (ribitol, 10 or 1 μg for the extracellular or intracellular analysis, respectively) and lyophilized. Dried samples were subsequently derivatized in 2 stages, as previously described (Tsugawa *et al.*, 2011). For oximation, 100 μl (50 μl for intracellular metabolites) of methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO) in pyridine (20 mg/l) (Wako, Tokyo, Japan) was added and incubated at 30°C for 90 min. For trimethylsilylation, 50 μl (25 μl for intracellular metabolites) of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (GL Science, Tokyo, Japan) was added and incubated at 37°C for 30 min. Insoluble residues were removed by centrifugation at 12000 × *g* at 4°C for 5 min, and cultivation supernatants were transferred to clean vials.

Table 1 Target metabolites detected by GC/MS

Name	Retention time (min)	Formula	*m/z* Range
Pyruvate + OAA	**6.34**	$C_6H_{12}NO_3Si$	**174–177**
Lactate	**6.72**	$C_8H_{19}O_3Si_2$	**219–222**
Succinate	**14.45**	$C_9H_{19}O_4Si_2$	**247–251**
Fumarate	**15.31**	$C_9H_{17}O_4Si_2$	**245–249**
Malate	**17.42**	$C_{12}H_{27}O_5Si_3$	**335–339**
PEP	**18.39**	$C_{11}H_{26}O_6PSi_3$	**369–372**
Citrate	**19.79**	$C_{17}H_{37}O_7Si_4$	**465–471**
Ribitol (i.s.[a])	**19.32**	$C_{19}H_{49}O_5Si_5$	**219**

(i.s.[a]), internal standard.

GC/MS analysis and data processing

Derivatized metabolites were analyzed using GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with a 30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25-μm CP-SIL 8 CB low bleed (Agilent Technologies, Santa Clara, CA). Aliquots (1 μl) were injected in the split mode (25/1, supernatant analysis; 5/1, intracellular analysis) at 230°C, using helium as carrier gas at a flow rate of 1.12 ml/min. The column temperature was held at 80°C for 2 min isothermally, raised to 130°C (4°C/min) and then to 330°C (25°C/min), and maintained for 6 min isothermally. The interface and MS source temperatures were 250°C and 200°C, respectively, and the ion voltage was 1 kV. Data were collected by GCMS solution (Shimadzu), and identified metabolites are shown in Table 1. Mass isotopomer distributions were corrected for natural isotope abundance as previously described (Nanchen *et al.*, 2007). The GC/MS analysis was performed on 3 biological replicates of each sample.

Results

CO_2 incorporation into C. cellulovorans metabolites

According to previous reports that CO_2 was required for culturing some *Clostridium* species, we speculate that *C. cellulovorans* also has the activity of CO_2 fixation. Our speculation is further supported by the fact that *C. cellulovorans*, whose genes related to CO_2 fixation were

also annotated in the genome of *C. cellulovorans* (Tamaru *et al.*, 2010), can be cultivated in media containing higher CO_2 concentrations, even at '100%', compared to other *Clostridium* species. Therefore, in this study, we cultivated *C. cellulovorans* in media containing $NaH^{13}CO_3$ instead of $NaHCO_3$ and then examined a massive number of metabolites derived from *C. cellulovorans* and cultivation supernatants using GC/MS. Fig. 2 shows the ratios of each metabolite in media containing either $NaHCO_3$ or $NaH^{13}CO_3$. Higher values of relative fractions when *C. cellulovorans* was cultivated in media containing $NaH^{13}CO_3$ indicate that ^{13}C atoms derived from $NaH^{13}CO_3$ were incorporated into specific metabolites. Our results demonstrate that when *C. cellulovorans* was cultivated in media containing $NaH^{13}CO_3$, the relative fractions of pyruvate + oxaloacetate (OAA), lactate, fumarate, and malate inside (Fig. 2a) and outside (Fig. 2b) cells were significantly higher than those from *C. cellulovorans* cultivated in media containing $NaHCO_3$. Based on these findings, *C. cellulovorans* evidently is able to incorporate ^{13}C atoms into abovementioned metabolites, and therefore has the ability to fix CO_2.

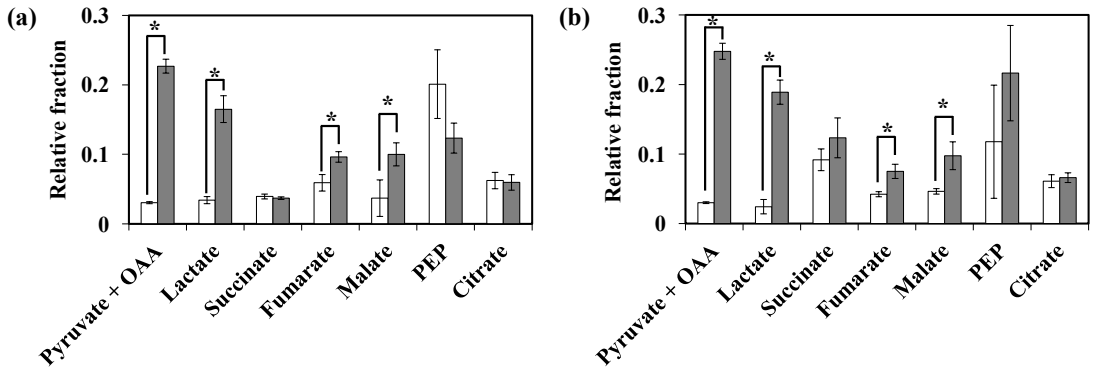

Fig. 2 Incorporation of CO_2 into several metabolites inside and outside of the cells. The vertical axis represents the relative fraction of ^{13}C-labeled metabolites, which was calculated as follow as: $m_1/\sum_{i=0}^{n} m_i$, where m_1 and m_i are the corrected intensities and n is the number of carbon atoms in metabolites (n = 3 in the case of lactate, for example). (a) Intracellular metabolites. (b) Extracellular metabolites. White and gray bars represent the ratios of each metabolite in media containing $NaHCO_3$, and $NaH^{13}CO_3$, respectively. Error bars and asterisks indicate standard deviations and significant difference (*$P < 0.01$), respectively.

Glucose metabolism into metabolic pathway intermediates

Next, to understand the whole strategy of glucose metabolism in *C. cellulovorans*, we examined a massive number of metabolites inside bacterial cells that were cultivated in media containing [U-^{13}C]-glucose. In this way, there is a report how metabolites flow in metabolic pathway of *C. acetobutylicum* have been analyzed (Amador-Noguez *et al.*, 2010). To more understand metabolites flow in *C. cellulovorans*, we observed how ^{13}C atoms were incorporated into some metabolites. The results shown in Fig. 3 indicate that ^{13}C atoms derived from [U-^{13}C]-glucose were incorporated into pyruvate + OAA, lactate, fumarate, and malate inside the cells. These results also demonstrate the following 4 points. First, both PFOR and PEPC fixed CO_2. It is because that pyruvate had only two ^{13}C atoms of three carbons (Fig. 3). The results indicated that pyruvate was converted from acetyl-CoA associated with CO_2 fixation once pyruvate became acetyl-CoA, which is constructed two carbons in acetyl group. In the same way, as malate and fumarate had three ^{13}C atoms, they could be prepared from PEP by PEPC associated with CO_2 fixation. Second, PFOR initiated the reversible conversion of pyruvate to acetyl-CoA. Third, the amount of PEP flowing into the TCA cycle could be much less than that flowing into pyruvate, acetyl-CoA, and lactate. Fourth, under this condition, ^{13}C atoms were not incorporated into succinate and citrate.

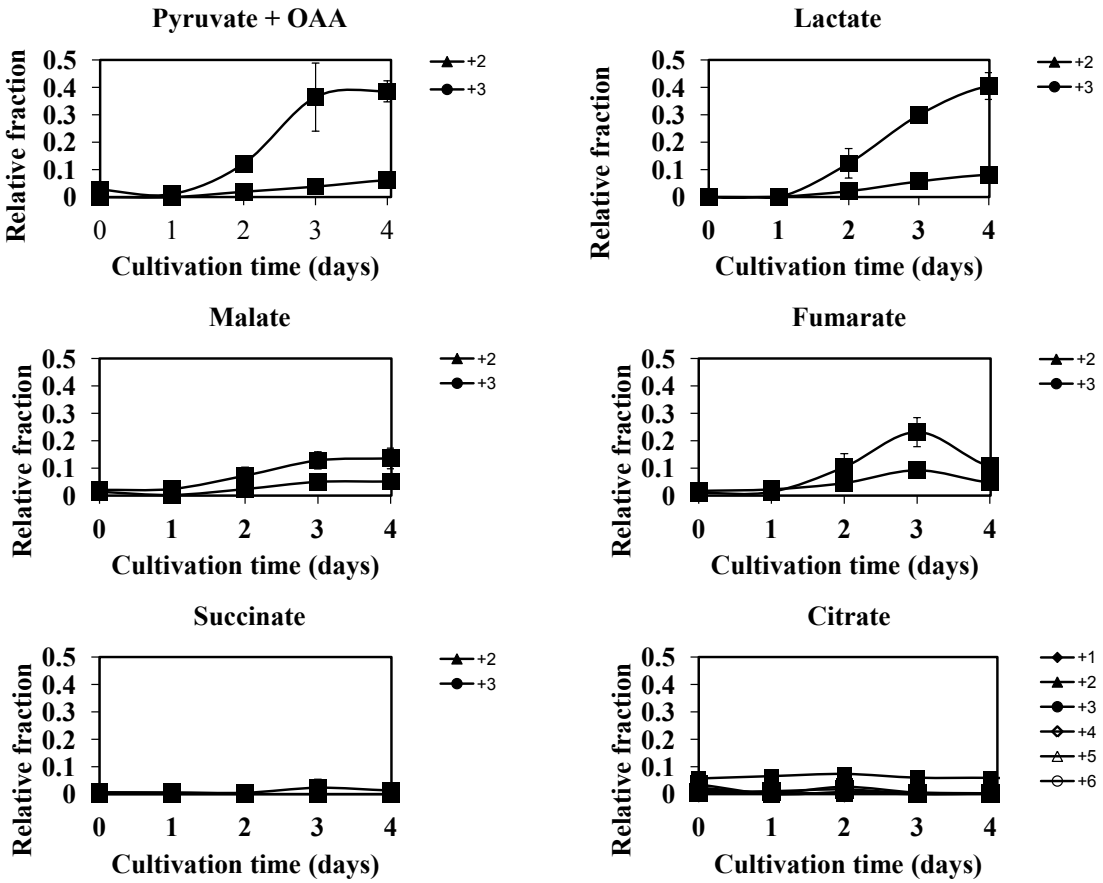

Fig. 3 Dynamic incorporation of [U-^{13}C]-glucose into target metabolites. The abscissa and vertical axes represent the cultivation time of *C. cellulovorans* and the relative fraction of indicated labeled compounds. '+1', '+2', '+3', '+4', '+5', and '+6' mean the number of 13-carbon isotope in each metabolite incorporated from [U-^{13}C]-glucose. Error bars represent standard deviations.

Lactate secretion accompanied with CO_2 fixation

As shown in Fig. 2 and 3, a flux of lactate was observed in *C. cellulovorans*, in agreement with the previous report (Sleat *et al.*, 1984). Therefore, we checked the amount of secreted lactate by *C. cellulovorans* cultivated in media containing $NaH^{13}CO_3$ (Fig. 4a). We further calculated the percentage of ^{13}C incorporation into secreted lactate. The results show that, accompanied with CO_2 fixation, *C. cellulovorans* produced lactate at a constant rate after 2 days (Fig. 4b).

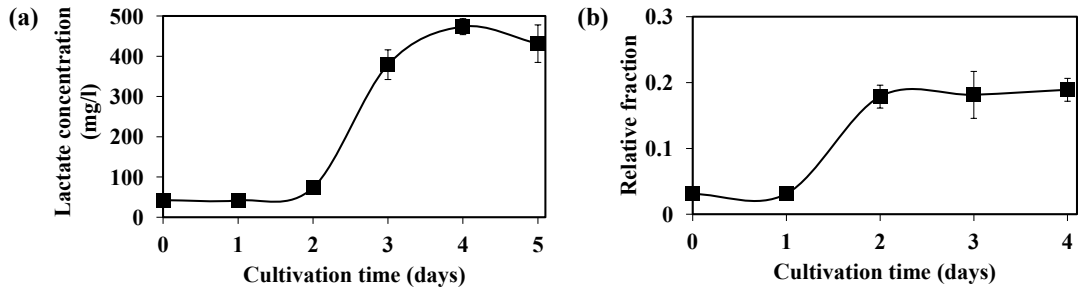

Fig. 4 Lactate secretion accompanied with CO_2 fixation. (a) The time course of lactate concentration in cultivation supernatants. (b) The time course of relative fraction of lactate in cultivation supernatants. Relative fractions were calculated as described in Figure 2. Error bars represent standard deviations.

Discussion

Using target metabolomics, we demonstrate here that *C. cellulovorans* produces lactate, malate, and fumarate. As illustrated in the metabolic map, including the TCA cycle of *C. cellulovorans* (Fig. 5), we propose that *C. cellulovorans* produces lactate accompanied with CO_2 fixation and generates fumarate by partly operating the TCA cycle in a reductive manner (Fig. 5a). The reason why *C. cellulovorans* operates these metabolic pathways (lactate and fumarate production and CO_2 fixation, except for the PEPC reaction) could be the preservation of redox balance in the cell. That is, the reactions of lactate and malate production (operated by lactate dehydrogenase and malate dehydrogenase, respectively) might be accompanied with the regeneration of 1 molecule of $NAD(P)^+$. In addition, the reaction of CO_2 fixation by PFOR produces oxidized ferredoxin, and a molecule of oxidized ferredoxin subsequently produces 2 molecules of NAD^+. These reactions may help oxidizing agents to be used in glycolysis. We also examined the existence of CO_2 fixation enzymes (PFOR and PEPC) by the proteome analysis. Compared to the flux from glycolysis to the TCA cycle, the flux to lactate could be dominant, since our results show that higher amounts of ^{13}C atoms were incorporated into lactate, but not malate and fumarate (Fig. 3).

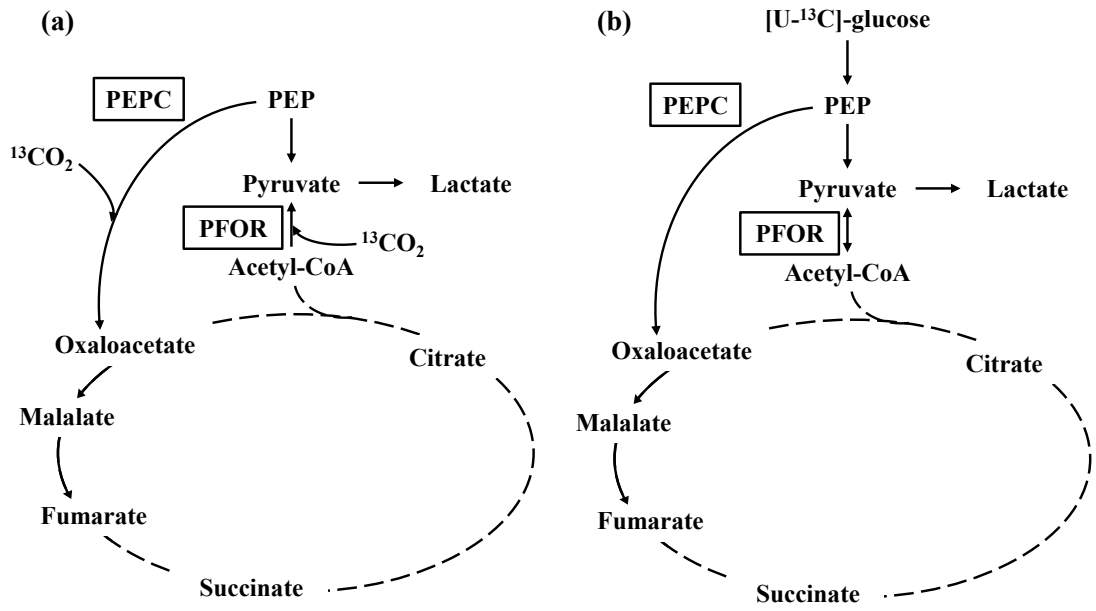

Fig. 5 Proposed pathway for CO_2 incorporation in *C. cellulovorans*. Possible metabolic maps of *C. cellulovorans* in media containing $NaH^{13}CO_3$ (a) and [U-^{13}C]-glucose (b). Solid lines, possible pathways with directions indicated by arrows; dashed lines, impossible pathways in our studies. Lactate is produced from pyruvate, whereas fumarate is generated from oxaloacetate and malate. CO_2 is fixed by PFOR and PEPC. In contrast, no citrate is produced from oxaloacetate.

Our findings also indicate that little citrate and succinate was produced from glucose (Fig. 3). Isocitrate dehydrogenase, which operates downstream of citrate in the TCA cycle and operates in an oxidative manner with $NAD(P)^+$, could not be used. It is known that citrate is produced from glutamate in some organisms. The metabolic information of *C. acetobutylicum* (Amador-Noguez *et al.*, 2010) also suggested that *C. cellulovorans* could use amino acids (glutamate/glutamine) to make other metabolites. If *C. cellulovorans* produces citrate from glutamate, redox balance would be better maintained because glutamate dehydrogenase or glutamate synthase uses $NADP^+$ and isocitrate dehydrogenase uses NADPH. In particular, when *C. cellulovorans* lives under a reductive condition, such a pathway is more reasonable than the pathway of citrate production from acetyl-CoA in an oxidative manner. To examine this hypothesis in the future investigation, it will be a promising approach to study how ^{13}C atoms

are incorporated into metabolites when using media containing ^{13}C-labeled glutamate. Some other amino acids may be needed to maintain the metabolic pathway in *C. cellulovorans*, because the bacterium cannot be cultivated in media without yeast extract, which has glutamate (Sleat *et al.*, 1984).

As mentioned above, we speculate that *C. cellulovorans* could use the mechanism to maintain redox balance, because the oxidizability (the ability to oxygenate other metabolites) is valuable for the condition which was absent from O_2. *C. cellulovorans* lives under anaerobic conditions because photosynthesis is not operated under the natural growth condition of *C. cellulovorans* (wood chip). It has been reported that CO_2 fixation is useful to maintain redox balance in microorganisms that have the Calvin cycle or TCA cycle (McKinlay & Harwood, 2010); therefore CO_2 fixation could be a common mechanism to regulate redox balance.

Notably, lactate production in cultivation supernatants after 4 days was 474 mg/l (= 31.6 mol per 100 mol glucose), which is about twice of that reported in the previous study (Sleat *et al.*, 1984), where cellobiose was used as a carbon source.

Here, we demonstrated that, accompanied with CO_2 fixation, *C. cellulovorans* produced several kinds of organic acids and that the TCA cycle was partly operated in a reductive manner at the metabolite level. These presented results would provide important information for the application of *C. cellulovorans* as industrial cellulosome-producing bacteria.

Summary

We carried out ^{13}C-isotopomer-based target metabolome analysis, or carbohydrate conversion process analysis, for more profound understanding of metabolic pathways of the bacterium. Our findings that pyruvate + OAA, fumarate, and malate inside and outside cells exhibited ^{13}C incorporation suggest that *C. cellulovorans* exactly fixed CO_2 and partly operated the TCA cycle in a reductive manner. Accompanied with CO_2 fixation, the microorganism was also found to produce and secrete lactate. Overall, our study demonstrates that a part of *C. cellulovorans* metabolic pathways related to glycolysis and the TCA cycle are involved in CO_2 fixation.

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Chapter II Display of *Clostridium cellulovorans* xylose isomerase on the cell surface of *Saccharomyces cerevisia*e and its direct application to xylose fermentation

Production of biofuels from lignocellulosic biomass is becoming an urgent issue to reduce our oil dependence. D-Xylose is a major component of lignocellulosic biomass, and efficient utilization of lignocellulosic biomass requires bioconversion of both xylose and glucose. *S. cerevisiae* is an attractive microorganism for bioethanol production due to its high ethanol productivity and high ethanol tolerance. However, xylose is difficult to be used for fermentation substrate because *S. cerevisiae* cannot easily uptake and metabolize xylose. Thus, the conversion of xylose to xylulose outside the cells would be a promising approach because xylulose could be taken up and metabolized by yeast (Chiang *et al.*, 1981).

There are two possible strategies to endow *S. cerevisiae* with the ability to assimilate xylose. One is the heterologous expression of xylose reductase (XR) and xylitol dehydrogenase (XDH) genes, and the other is the heterologous expression of xylose isomerase (XI) gene (Fig. 1a) (Kotter *et al.*, 1990; Kuyper *et al.*, 2003; Zhou *et al.*, 2012). Since XR and XDH require NADPH and NAD, respectively, this pathway leads to coenzyme imbalance and xylitol accumulation, which reduces the efficiency of xylose fermentation (Kotter & Ciriacy, 1993; Pitkanen *et al.*, 2003). Conversely, although it is advantageous that XI requires no coenzyme, it has been reported that only a few active XIs have been successfully produced 'inside' *S. cerevisiae* (Walfridsson *et al.*, 1996; Kuyper *et al.*, 2003; Brat *et al.*, 2009; Madhavan *et al.*, 2009). Difficulties in protein folding, posttranslational modifications, and intermolecular and intramolecular disulfide bridge formations have been suggested as the reasons for this low success rate (Amore *et al.*, 1989; Walfridsson *et al.*, 1996). Therefore it is necessary to find novel XI genes that are actively produced by *S. cerevisiae* and to determine how to express these genes. Moreover, no XI has been successfully produced 'on the cell surface' of *S.*

cerevisiae. Production of XI on the cell surface would allow for conversion of xylose to xylulose outside the cell, which would be an advantage over internal expression of the XI gene since *S. cerevisiae* can easily incorporate xylulose.

C. cellulovorans is an anaerobic, mesophilic bacterium that effectively degrades and utilizes plant cell walls (Sleat *et al.*, 1984). Recently, the genome of *C. cellulovorans* was sequenced (Tamaru *et al.*, 2010), and genomic analysis revealed the presence of XI-related genes. The XI produced by this bacterium is expected to work well at the mid temperature since it can grow in a medium containing xylan, a polymer of xylose, as the sole carbon source at 37^{o}C (Foong & Doi, 1992). Many metabolic enzyme-encoding genes as well as hemicellulolytic enzyme-associated genes related to pentose metabolism exist in *C. cellulovorans*. Although fewer of these genes are found in other *Clostridium* species such as *C. thermocellum* (Tamaru *et al.*, 2010), *C. cellulovorans* can vigorously assimilate pentose sugars. D. Brat *et al.* reported that XI from *C. phytofermentans* was produced as an active form 'inside' *S. cerevisiae* (Brat *et al.*, 2009).

A yeast molecular display system has been employed to produce XI 'on the cell surface' of *S. cerevisiae*. This system enables the enzyme-displaying yeast to be treated as a whole-cell biocatalyst, and the enzymes displayed on the yeast cell surface can be analyzed and utilized without purification and concentration (Ueda & Tanaka, 2000a; Kadonosono *et al.*, 2008; Meguro *et al.*, 2011). In fact, many types of heterologous proteins have been functionally displayed on the yeast cell surface, leading to the generation of yeast cells with new functions (Kuroda & Ueda, 2003; Fujita *et al.*, 2004; Kuroda & Ueda, 2011). Cell-surface display of XI might allow for extracellular conversion of xylose to xylulose, which can be assimilated by even wild-type *S. cerevisiae* (Fig. 1b). Moreover, the reaction conditions for the displayed XI can be controlled, which is impossible for intracellularly expressed XI gene.

Therefore, to provide yeast cells with the ability to assimilate xylose, XI from *C. cellulovorans* was first displayed 'on the yeast cell surface'. Furthermore, the xylose assimilation and fermentation capabilities of the XI-displaying yeast were demonstrated.

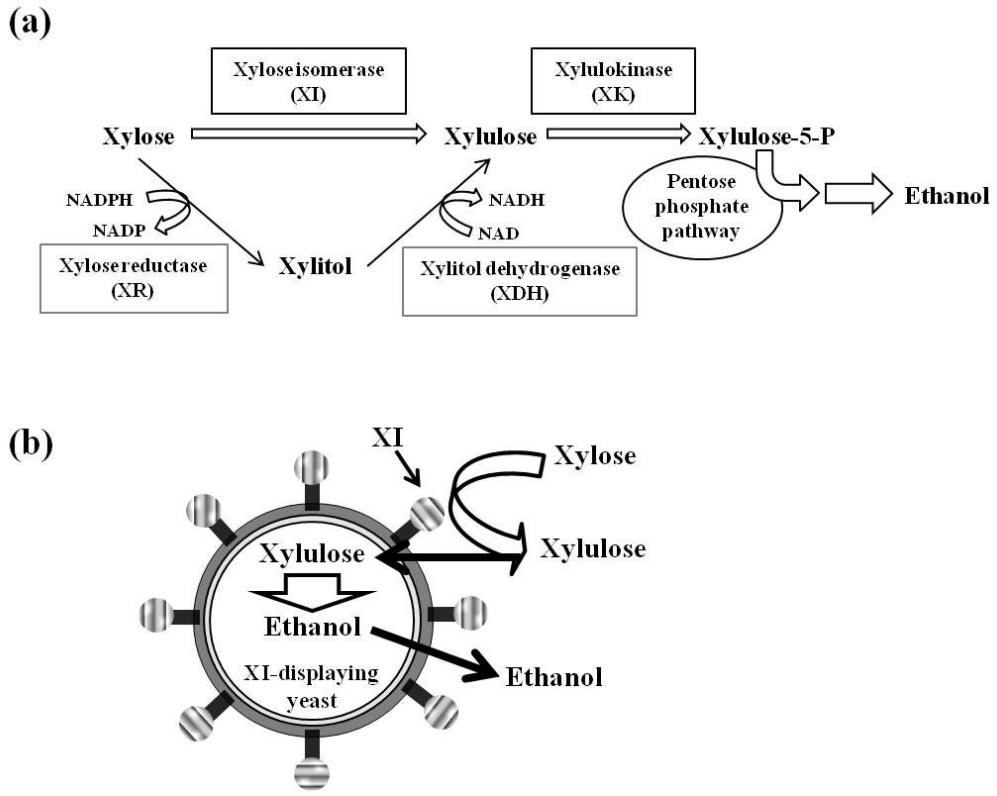

Fig. 1 Schematic illustration of xylose fermentation. (a) Conversion of xylose to ethanol *via* XR and XDH, or XI in *S. cerevisiae* cells and (b) direct xylose fermentation by display of XI on the cell surface of *S. cerevisiae*.

Materials and methods

Genome analysis

An analysis of the *C. cellulovorans* genome 743B (NCBI accession no. NC_014393; ATCC 35296) was performed using in silico Molecular Cloning™ Genomic Edition software version 3.0.26 (In silico Biology Co., LTD., Japan).

Strains and media

Escherichia coli strain DH5α (*F*⁻, *end*A1, *hsd*R17 [r_K^- m_K^+], *sup*E44, *thi*-l, λ–, *rec*A1, *gyr*A96, *deo*R, *rel*A1*Δ*[*lac*ZYA-*arg*F]*U169, ϕ80dlacZΔM15*)(Toyobo, Japan) was used as a host for DNA manipulation. *S. cerevisiae* strain BY4741/*Δsed1* (Kuroda *et al.*, 2009) (*MAT***a**, *his3*,

leu2, *met15*, *ura3, YDR077w::kanMX4*) was used as the host for cell-surface displaying protein construction. *E. coli* transformants were grown in Luria-Bertani medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride] containing 100 μg/ml ampicillin. Yeast host cells were grown in yeast-peptone-dextrose (YPD) medium (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) for transformation. For the activity assay, cells were cultivated in synthetic dextrose (SD) medium (0.67% (w/v) yeast nitrogen base without amino acids (Difco, USA); 2% (w/v) glucose, 2% (w/v) casamino acids, 0.002% (w/v) L-histidine, 0.003% (w/v) L-leucine, and 0.003% (w/v) L-methionine) buffered at pH 7.0 with 50 mM MES (2-(*N*-morpholino)ethanesulfonic acid)-NaOH buffer. For growth and xylose fermentation, cells were precultured in SD medium (0.67% (w/v) yeast nitrogen base without amino acids and 2% (w/v) glucose) supplemented with the appropriate amino acids and buffered with 50 mM MES buffer (pH 7.0), and then, inoculated into SXC medium (0.67% (w/v) yeast nitrogen base without amino acids, 5% (w/v) xylose, 2% (w/v) casamino acids, and 0.003% (w/v) L-methionine) buffered with 50 mM MES buffer (pH 7.0).

Construction of plasmids

The XI-encoding gene was amplified from *C. cellulovorans* genomic DNA by PCR using the following primers (restriction sites are underlined): 5'-CAGTAGATCTAGAGAATATTTTG CAAATGTACCGAAAATAAAATACG-3' and 5'- CAGTCTCGAGGTCGTTGAAGATGTATT GGTTAACAAC-3'. The xylulokinase (XK)-encoding gene was isolated from *S. cerevisiae* genomic DNA using the following primers: 5'-CAGTGAATTCATGTTGTGTTCAGTAATTCA GAGACAGAC-3' and 5'-CAGTGTCGACTTAGATGAGAGTCTTTTCCAGTTCGCTTAAG-3'. The amplified products were digested with *Bgl*II and *Xho*I or *Eco*RI and *Sal*I, and then inserted into pULD1 (Kuroda *et al.*, 2009) or p423GPD vector (Mumberg *et al.*, 1995). The constructed plasmids were named pULD1-XI and p423-XK, respectively. As a control for immunofluorescence microscopy, the pULD1-s vector (Kuroda *et al.*, 2009), which only displays the Strep-tag, was used.

Yeast transformation

Constructed plasmids were introduced into yeast using the lithium acetate method (Ito *et al.*, 1983), with the EZ-Yeast transformation kit (BIO 101, CA, USA). Transformants were selected on SD plate medium containing the appropriate amino acids.

Immunofluorescence microscopy

To confirm the display of XI on the yeast cell surface, cells were immunofluorescently labeled using a FLAG epitope tag as described below. Cells were fixed with 3.7% (v/v) formaldehyde/phosphate-buffered saline (PBS; pH 7.4) for 1.5 h and incubated in PBS (pH 7.4) containing 1% (w/v) bovine serum albumin for 30 min prior to immunostaining. A mouse monoclonal anti-FLAG M2 antibody (1:300 dilution; Sigma Chemical Co., USA) was used as the primary antibody. A mixture of cells and antibody was incubated for 1.5 h at room temperature on a rotator. The cells were then washed with PBS (pH 7.4). Next, an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:300 dilution; Invitrogen, USA) was incubated with the cells for 1.5 h at room temperature on a rotator. After washing with PBS (pH 7.4), the cells were suspended in 30 μl of PBS (pH 7.4) and observed by microscopy. Fluorescence was detected using an inverted microscope IX71 (Olympus, Japan) through a U-MNIBA2 mirror unit with a BP470-490 excitation filter, a DM505 dichroic mirror, and a BA510-550 emission filter (Olympus). Live images were obtained using a digital charge-coupled device camera (C4742-95-12ER; Hamamatsu Photonics, Japan), controlled by Aqua-Cosmos 2.0 software (Hamamatsu Photonics).

Measurement of enzyme activity

The XI-displaying yeast was cultivated in buffered SDC medium at 30^{o}C for 36 h and collected by centrifugation for 5 min at 4000 × *g* and 4^{o}C. The cells were then washed with distilled water, and were added to a reaction solution containing 50 mM D-xylose, 10 mM $CoCl_2$, and 50 mM HEPES buffer (pH 4.5-9.5) to obtain an optical density of 10 at 600 nm (OD_{600}); and incubated at 40-90^{o}C. After a given length of time (1.5 or 50 min), the reaction solutions

were centrifuged for 1 min at 14,000 × *g* and 4°C, and the supernatant was collected. The supernatant was filtered by Ultrafree-MC 0.45mm centrifugal filter device (Millipore, USA) and used for further analysis. Xylose and xylulose concentrations were determined by high-performance liquid chromatography (Shimadzu, Kyoto, Japan), equipped with a sugar-D column (Nacalai Tesque, Inc., Kyoto, Japan) and Coulochem III electrochemical detector (EC) (ESA, Chelmsford, MA). The optimum temperature was measured in the range of 40-90°C at pH 7.5. To determine the optimum pH, the cells were incubated at pH 4.5-9.5 at 60°C. Measurement of enzyme activity was performed in triplicate.

Cell growth in xylose-containing medium and xylose fermentation

Cells were pre-cultivated in 10 ml of SD medium buffered with 50 mM MES (pH 7.2) for 24 h, then transferred into 100 ml fresh medium at an OD_{600} of 0.1, and cultivated at 30°C for 24 h. Cells were washed with distilled water and inoculated at an OD_{600} of 1 into 100 ml of SXC medium containing 5% (w/v) xylose as the sole carbon source. Cells were grown at 30°C, and cell growth was monitored by measuring the OD_{600} in triplicate.

For xylose fermentation, harvested cells were inoculated at an OD_{600} of 10 into 100 ml of SXC medium containing 5% (w/v) xylose. Xylose fermentation was examined at 30°C. Fermentation samples were filtered using an Ultrafree-MC 0.45-mm centrifugal filter device (Millipore). Ethanol concentration was determined by high-performance liquid chromatography (Shimadzu) together with a RID-10A refractive index detector (Shimadzu) using a YMC-Pack Polyamine II column (YMC Co., LTD, Japan). This experiment was performed in triplicate.

Results

Construction of a yeast strain displaying XI from C. cellulovorans

Six putative XI-related genes were found in the genome of *C. cellulovorans* (ATCC 35296); one gene is annotated as XI (Clocel_0590), and the five other genes are annotated as proteins containing XI domains (Clocel_2257, Clocel_4188, Clocel_1063, Clocel_2267, and Clocel_1493). The product of Clocel_0590 was thought to have the highest contribution to

xylose metabolism in *C. cellulovorans*.

To display XI from *C. cellulovorans* on the yeast cell surface, the multicopy expression plasmid pULD1-XI was constructed and introduced into the *S. cerevisiae* BY4741/ *Δsed1* strain for enhanced display efficiency (Fig. 2a) (Kuroda *et al.*, 2009). To confirm the display of the XI protein on the cell surface, the FLAG-tag fused to XI was labeled by immunofluorescence. Yeast harboring pULD1-s, which contains a strep-tag instead of a FLAG-tag, was used as a negative control. Cells harboring pULD1-XI had green fluorescence on their cell surface (Fig. 3). These results indicated that XI from *C. cellulovorans* was displayed correctly on the yeast cell surface.

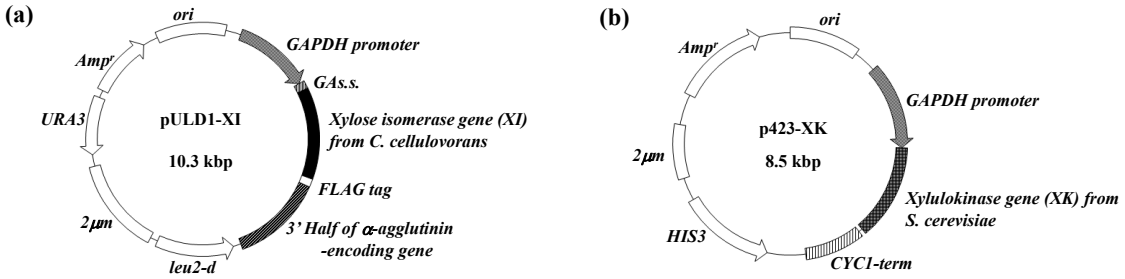

Fig. 2 Plasmid map of pULD1-XI (a), which was used for the cell-surface display of XI from *C. cellulovorans*, and p423-XK (b), which was used for intracellular production of XK from *S. cerevisiae*. *Amp*^{r}, ampicillin resistance gene for bacterial selection; *2 μm*, replication origin of yeast; *ori*, replication origin of *E. coli*; *URA3*, uracil marker gene; *leu2-d*, leucine marker gene; *HIS3*, histidine marker gene; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GA s.s.*, glucoamylase signal sequence; *CYC1-term*, transcription terminator.

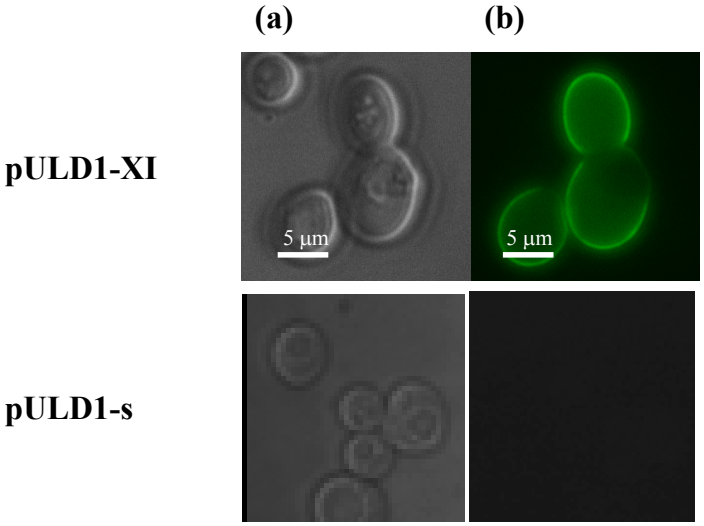

Fig. 3 Immunofluorescence labeling of yeast cells using an anti-FLAG antibody and an Alexa488-conjugated goat anti-mouse IgG antibody. (a) Phase contrast micrographs and (b) immunofluorescence micrographs of *S. cerevisiae* harboring pULD1-XI and pULD1-s.

Activity of XI derived from C. cellulovorans

The characteristics of XI from *C. cellulovorans* on the XI-displaying yeast were confirmed because the properties of enzymes displayed on the cell surface were previously shown to be almost equal to those of purified enzymes (Kadonosono *et al.*, 2008). The XI-displaying yeast produced xylulose from xylose in SXC medium buffered with 50 mM MES buffer (pH 7.2), whereas the control yeast did not. This result indicated that the XI displayed on the yeast cell surface was functional and maintained isomerization activity. Metal ion specificity was determined by incubating the yeast in reaction solutions containing 10 mM Co^{2+} or Mg^{2+}. XI showed higher activity in solutions containing Co^{2+} than in solutions containing Mg^{2+}. The effects of temperature and pH on XI activity were examined. Although the relative activity of XI was the highest at 70°C (Fig. 4a), the amount of xylulose after incubation for 50 min was the greatest at 60°C (Fig. 4b). This may be caused by instability or denaturing of XI in 70°C. As for optimum pH, XI had the highest activity at pH 6.5 in both incubation conditions, and production of 2.4 mg/ml xylulose from 7.5 mg/ml xylose was detected after the reaction for 50 min (Fig. 4c and 4d).

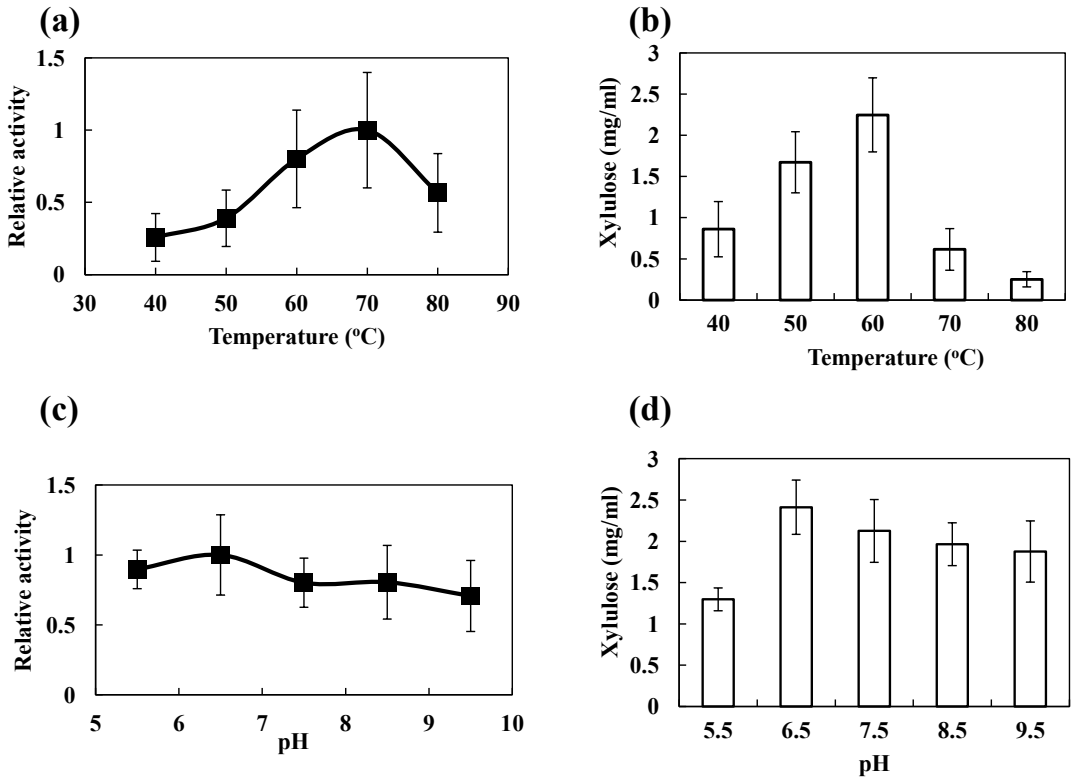

Fig. 4 Activity assay of XI from *C. cellulovorans*. Relative activity for reaction within 1.5 min (a, c) and the amounts of produced xylulose from xylose after 50 min of reaction (b, d) are shown. A value of 1 for relative activity means that the largest amount of produced xylulose (140 μg/ml (a) and 91 μg/ml (c)) from 7.5 mg/ml xylose among samples. Reactions were performed at a temperature range of 40-80°C (a, b), or at a pH range of 5.5-9.5 (c, d). Values represent the mean ± standard deviation of three independent experiments.

Cell growth on xylose medium

Once the isomerization activity of the surface-displayed XI was confirmed, the growth of XI-displaying yeast was examined on xylose medium. However, XI-displaying yeast almost did not grow. This may be because the activity of the yeast XK was too low, and little of the xylulose was changed to xylulose-5-P (Fig. 1a). To enhance xylose assimilation ability (Chang & Ho, 1988; Toivari *et al.*, 2001), XK was overproduced 'inside' the XI-displaying yeast by introducing p423-XK (Fig. 2b) into BY4741*Δsed1*/pULD1-XI. A yeast strain harboring the empty vectors pULD1 and p423GPD was used as a control. The XI-displaying yeast with XK

overproduction grew in SXC medium buffered with MES (pH 7.0) under aerobic conditions by assimilating xylose as the sole carbon source although the negative control strain did not (Fig.5). These results indicate that cell-surface-displayed XI contributed to xylose assimilation by catalyzing the extracellular production of metabolizable xylulose from xylose.

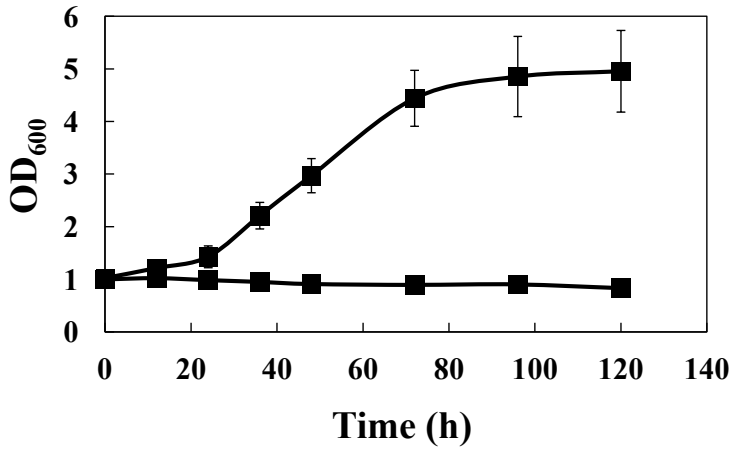

Fig. 5 Growth curve of XI-displaying yeast in SXC medium containing 5% (w/v) xylose as the sole carbon source. ♦, BY4741*Δsed1*/pULD1-XI and p423-XK; ×, BY4741*Δsed1*/pULD1 and p423GPD. The values represent the mean ± standard deviation of the results from three independent experiments.

Xylose fermentation

To analyze ethanol production from xylose by the XI-displaying yeast, yeast was anaerobically cultivated in SXC medium buffered with MES (pH 7.0). Yeast harboring both pULD1-XI and p423-XK successfully produced ethanol in SXC medium, while negative control yeast harboring pULD1 and p423GPD did not produce significant ethanol (Fig. 6). These results, including the growth experiments, indicate that cell-surface display of XI on yeast might be an effective strategy for direct fermentation of xylose.

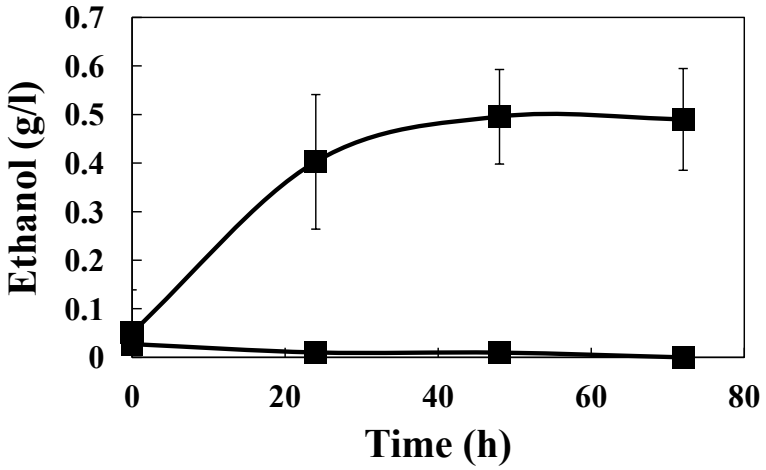

Fig. 6 Xylose fermentation by XI-displaying yeast in SXC medium containing 5% (w/v) xylose as the sole carbon source. ♦, BY4741*Δsed1*/pULD1-XI and p423-XK; ×, BY4741*Δsed1*/pULD1 and p423GPD. The results show the mean ± standard deviation of the results from three independent experiments.

Discussion

In this study, we successfully displayed XI derived from the *C. cellulovorans* gene found by genome analysis (Tamaru *et al.*, 2010). This XI was believed to be functional at the growth temperature of yeast. Using the cell-surface display system, it is possible to directly measure the activity of the enzyme produced 'on the cell surface' of *S. cerevisiae* without protein purification and concentration (Kuroda & Ueda, 2011). As a result, the novel *C. cellulovorans*-derived XI was successfully displayed 'on the cell surface' of *S. cerevisiae*. Furthermore, it is easily controllable to provide the optimum pH for a surface-displayed enzyme than for an intracellularly produced enzyme because the pH of the fermentation medium is easily controlled by chemical methods. In addition, the thermal stability, optimal temperature, and optimal pH of enzyme displayed on the cell surface were comparable to those of the secreted or intracellularly produced free enzymes (Ueda & Tanaka, 2000b).

Although displayed *C. cellulovorans* XI showed the highest activity at 60^{o}C and pH 6.5 (Fig. 4b and 4d), the XI-displaying yeast was cultivated at 30^{o}C, the optimal temperature for yeast growth. The XI-displaying yeast could grow and produce ethanol at 30^{o}C in medium

containing xylose as the sole carbon source (Fig. 5 and 6), suggesting that the displayed XI was active at 30°C and produced xylulose despite its higher optimal temperature. If other *S. cerevisiae* strains that can grow well at higher temperatures are used as the host for cell-surface display of XI, XI-displaying yeast could be used at higher temperatures, and the ethanol production would be improved by higher activity of XI. In addition, the introduction of some previously reported mutations into the displayed XI from *C. cellulovorans* might enhance its xylose assimilation and ethanol production abilities. The optimal pH of XI from *Thermoanaerobacterium* strain JW/SL-YS 489 was less than 7.0 (Liu *et al.*, 1996). Substitutions of some amino acids close to the active site were reported to reduce the negative charge in the surface of the active site and lead to a decrease in optimal pH. Since these mutations have not been observed in XI from *C. cellulovorans*, introduction of such mutations might be also effective.

XI displayed on the yeast cell surface exhibited isomerization activity, and thus enabled the extracellular conversion of xylose to xylulose. Xylose fermentation via xylulose catalyzed by XI-displaying yeast was demonstrated here for the first time (Fig. 6). When the ethanol production stopped after 48 h fermentation, 3.3% of xylose in the medium was converted into ethanol. In the experiment of the activity measurement of XI, the xylulose production from xylose reached a plateau (xylulose, 2.2 mg/ml) after 50min at 60°C (Fig. 4b). In the case that the same amount of xylulose was produced in fermentation medium, 74% of xylulose was converted into ethanol (0.5 g/l, Fig. 6). Therefore, the conversion of xylose to xylulose would be important in a fermentation process. However, conversion to xylulose by XI on the cell surface could be a useful strategy for ethanol production from xylose because it was reported that yeast more rapidly take up xylulose than xylose (Chiang *et al.*, 1981). To further improve ethanol production from xylose, fermentation conditions, such as temperature and pH, should be optimized to maintain the balance between xylose conversion and xylulose fermentation. The optimal conditions for xylulose fermentation by wild-type *S. cerevisiae* are 35°C and pH 4-6 (Chiang *et al.*, 1981), whereas those optimal conditions for XI activity are 60°C and pH 6.5. Furthermore, formation of XIs into dimeric or tetrameric complexes (Carrell *et al.*, 1984; Rey *et al.*, 1988;

Hess *et al.*, 1998) has been reported to be required for activity. However, fusion of XI to the 3' half of α-agglutinin likely reduced its ability to form multimers. In further studies, multimer formation of the displayed XI could be improved by producing a combination of secreted and cell-surface-displayed enzymes as reported previously (Lin *et al.*, 2003).

In conclusion, we demonstrated here the construction of a novel xylose-fermenting system using a strain of *S. cerevisiae* displaying XI 'on the cell surface'. Our study will provide a promising starting point for improving the direct fermentation of xylose by *S. cerevisiae*.

Summary

Xylose isomerase (XI) is a key enzyme in the conversion of D-xylose, which is a major component of lignocellulosic biomass, to D-xylulose. Genomic analysis of the bacterium *C. cellulovorans* revealed the presence of XI-related genes. In this study, XI derived from *C. cellulovorans* was produced and displayed using the yeast cell surface display system, and the xylose assimilation and fermentation properties of this XI-displaying yeast were examined. XI-displaying yeast grew well in medium containing xylose as the sole carbon source, and directly produced ethanol from xylose under anaerobic conditions.

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Chapter III Molecular breeding of 1-butanol-producing yeast *Saccharomyces cerevisiae* using the genes of *Clostridium acetobutylicum*

Biofuels are produced from renewable biomass by microbes, and butanol has more significant advantages as a biofuel than ethanol. It is well known that 1-butanol can be produced by clostridia fermentation. Clostridia comprise of a diverse group of anaerobic, spore-forming, and gram-positive bacteria that include notable pathogens or industrial significant microorganisms. *C. acetobutylicum* was found to produce acetone/butanol/ethanol at a ratio of 3:6:1 in a process called ABE fermentation (Antoni *et al.*, 2007; Inui *et al.*, 2008) (Fig. 1a). Bacterial production of butanol and acetone using the ABE fermentation process was valuable in the production of the lacquer solvent butylacetate and in the development of the synthetic rubber industry. However, bacterial production has declined with the advancement of the petrochemical industry, which can produce acetone and butanol at low costs. Biofuel production is currently increasingly being practiced worldwide, and research and development into microbial butanol production is again becoming actively pursued. Thus, we attempted to produce 1-butanol by introducing the 1-butanol production pathway from *C. acetobutylicum* into *S. cerevisiae* (Fig. 1b). *S. cerevisiae* has been considered another ideal host for butanol production. It has inherent tolerance to solvents due to its extensive use in industrial production of ethanol, and butanol toxicity is not a limiting factor for butanol production in *S. cerevisiae* because it can tolerate up to 2% butanol (Fischer *et al.*, 2008).

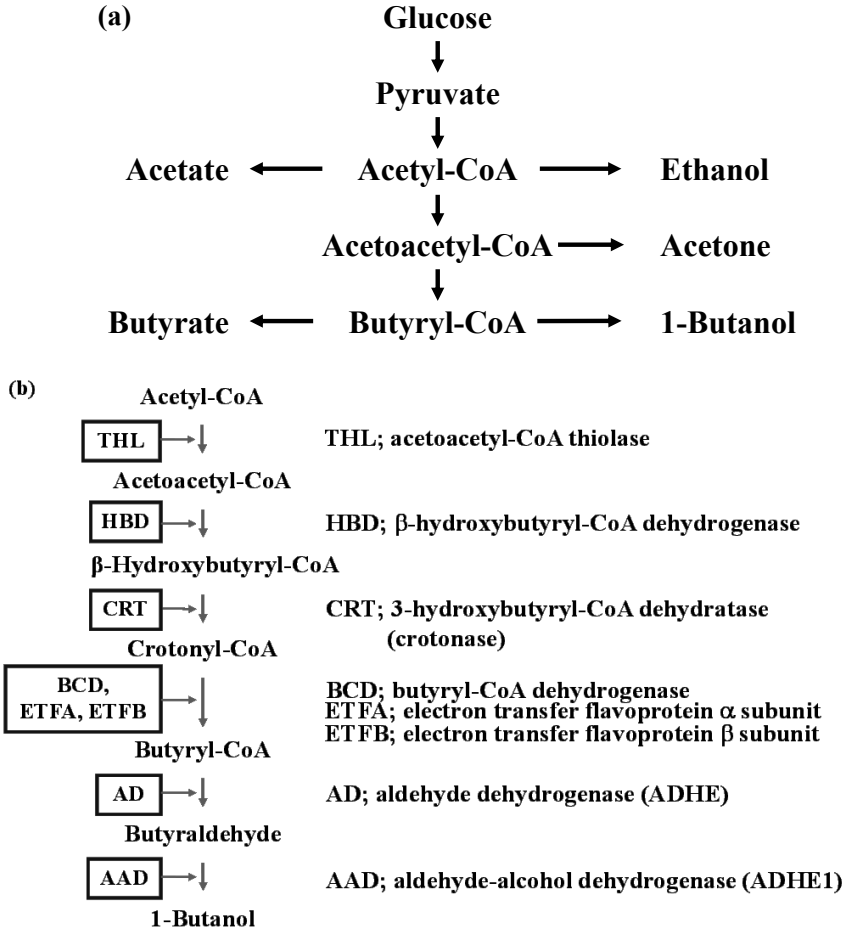

Fig. 1 Metabolic pathway. (a) ABE fermentation of *C. acetobutylicum*, and (b) the 1-butanol production pathway introduced into yeast. The words surrounded by boxes mean enzymes which need for 1-butnaol production pathway.

Materials and methods

Strains and media

Escherichia coli strain DH5α (*F*-, *end*A1, *hsd*R17 [r_K^- m_K^+], *sup*E44, *thi*-l, λ–, *rec*A1, *gyr*A96, *deo*R, *rel*A1Δ[*lac*ZYA-*arg*F]*U169, ϕ80dlacZΔM15*)(Toyobo, Japan) was used as a host for DNA manipulation. *S. cerevisiae* W303-1A (*MAT*a; *ade2-1*; *his3-11,15*; *leu2- 3,112*; *trp1-1*; *ura3-1*) was used as the host for the 1-buntanol pathway. *E. coli* was grown in Luria-Bertani medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride] containing 100 μg/ml ampicillin. Yeast host cells were grown in yeast-peptone-dextrose (YPD) medium (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) for transformation.

For the 1-butanol production, cells were cultivated in synthetic dextrose (SD) medium (0.67% (w/v) yeast nitrogen base without amino acids (Difco, USA); 2% (w/v) glucose, 0.5% (w/v) casamino acids, 0.002% (w/v) L-adenine , 0.002% (w/v) L-histidine, 0.003% (w/v) L-leucine, 0.002% (w/v) L-uracil and 0.002% (w/v) L-tryptophan).

Construction of 1-butanol-producing yeasts

The *thl* gene derived from *Candida tropicalis* was amplified from pWTIA (Kanayama *et al.*, 1997), and the other seven genes (*hbd*, *crt*, *bcd*, *etfA*, *etfB*, *ad*, and *aad*) were amplified from *C. acetobutylicum* (NBRC 13948) genomic DNA by PCR (the primers are listed in Table 1). The amplified DNA fragments were inserted into pULI1, a multicopy vector that with a constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (*PGAP*) (Miura *et al.*, 2012). The sequences of the inserted DNA fragments were confirmed via DNA sequencing, and the resulting plasmids were named pULI1-*thl*, pULI1-*hbd*, pULI1-*crt*, pULI1-*bcd*, pULI1-*etfA*, pULI1-*etfB*, pULI1-*ad*, and pULI1-*aad*, respectively. The eight genes were then amplified from these plasmids together with *PGAP* and GAPDH terminator (*TGAP*) and cloned into pRS403 (containing *bcd*, *etfA* and *etfB*), pRS405 (containing *ad* and *aad*), and pRS406 (containing *thl*, *hbd* and *crt*) (Fig. 2) (Sikorski & Hieter, 1989). The three constructed plasmids were introduced into the laboratory haploid strain W303-1A using the lithium acetate method (Ito *et al.*, 1983).

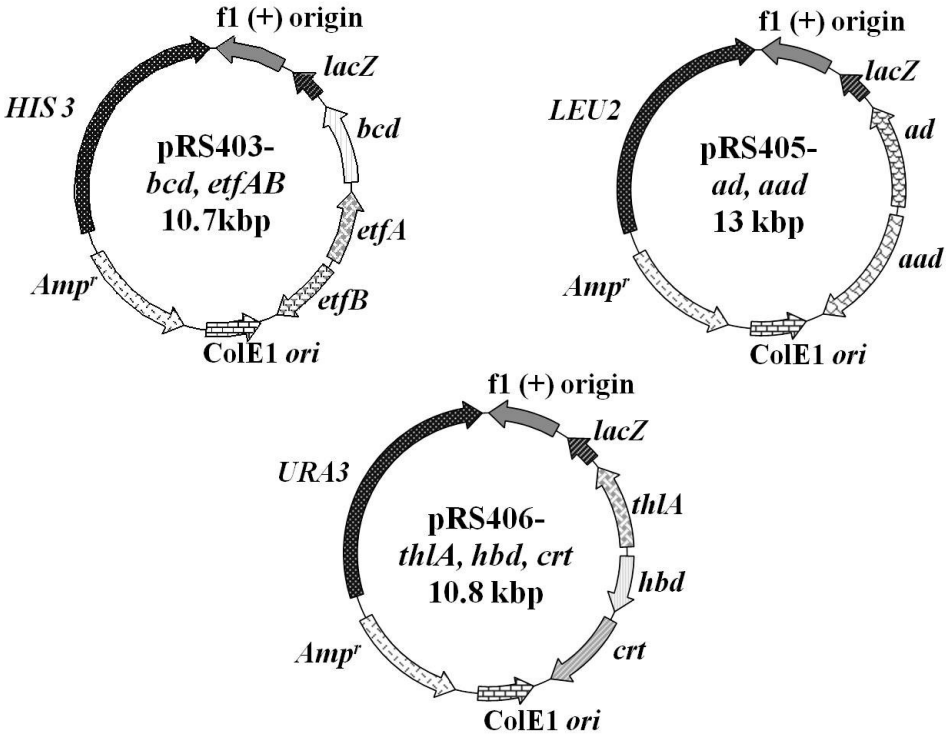

Fig. 2 Construction of reaction enzymes plasmids for 1-butanol production. *Amp*^{r}, ampicillin resistance gene for bacterial selection; f1(+) origin, replication of a f1 phage; ColE1 *ori*, replication origin of *E. coli*; *lacZ*, gene coding for β-galactosidase, *HIS3*, histidine marker gene; *LEU2*, leucine marker gene; *URA3*, uracil marker gene.

Table 1 Primers used in the experiments described in this chapter

Primers	Sequence
hbd-F	5'-CAGTGAATTCATGAAAAAGGTATGTGTTATAGGTGCAGGT-3'
hbd-R	5'-CAGTCTCGAGTTATTTTGAATAATCGTAGAAACCTTTTCC-3'
crt-F	5'-CAGTGAATTCATGGAACTAAACAATGTCATCCTTGAAAAG-3'
crt-R	5'-CAGTCTCGAGCTATCTATTTTTGAAGCCTTCAATTTTTCT-3'
bcd-F	5'-CAGTAGATCTATGGATTTTAATTTAACAAGAGAACAAGAATTAGT-3'
bcd-R	5'-CAGTCTCGAGTTATCTAAAAATTTTTCCTGAAATAACTAATTTCTGAA-3'
etfA-F	5'-CAGTGAATTCATGAATAAAGCAGATTACAAGGGCGTAT-3'
etfA-R	5'-CAGTCTCGAGTTAATTATTAGCAGCTTTAACTTGAGCTATTAATTCTG-3'
etfB-F	5'-CAGTGAATTCATGAATATAGTTGTTTGTTTAAAACAAGTTCCAGATACA-3'
etfB-R	5'-CAGTCTCGAGTTAATTTTGAGACAACATATCAGCTGCTTCC-3'
ad-F	5'-CAGTGAATTCATGAAAGTCACAACAGTAAAGGAATTAG-3'
ad-R	5'-CAGTCTCGAGTTAAGGTTGTTTTTTAAAACAATTTATATA-3'
aad-F	5'-CAGTGAGCTCATGAAAGTTACAAATCAAAAAGAACT-3'
aad-R	5'-CAGTCTCGAGTTAAAATGATTTTATATAGATATCCTTAAG-3'
GAPDH-Pro-F	5'-AAGCTTACCAGTTCTCACACGG-3'
GAPDH-Term-R	5'-GGTACCTCAATCAATGAATCG-3'

Underlined sequences indicate the restriction sites.

Cultivation conditions

The constructed yeasts were pre-cultivated in 10 ml medium at 30°C for 24 h under aerobic conditions. The yeasts were collected by centrifugation for 5 min at 3000 × *g* and 4°C and then transferred into 100 ml fresh medium at an OD_{600} of 0.1 and cultivated at 30°C for 24 h under aerobic conditions. Next, the yeasts were collected by centrifugation for 5 min at 3000 × *g* and 4°C and then transferred into 50 ml fresh medium at an OD_{600} of 10 at 30°C under anaerobic conditions with bubbling CO_2. The medium used throughout was SDC+AHLUW buffered with citric acid (pH 5.0).

1-Butanol measurement

For 1-butanol measurement, 380 μl samples were taken from the yeast cultures and the supernatants were collected by centrifugation for 5 min at 3000 × *g* and 4°C. 20 μl of 0.1% (w/w) 2-butanol were added to the supernatants as an internal standard. Then 400 μl ethyl acetate was added to the samples. The samples were vortexed for 1 min and left to stand for 10 min. The ethyl acetate was then recovered and analyzed with a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) using a DB-WAX capillary column (30 m, 0.32 mm i.d., 0.50 mm film thickness) (Agilent Technologies, Santa Clara, CA, USA) (Steen *et al.*, 2008). Aliquots (1 μl) were injected in the split mode (1/1) at 225°C, using helium as carrier gas at a flow rate of 7.7 mL/min. The column temperature was held at 40°C for 5 min isothermally, raised to 120°C (15°C/min) and then to 230°C (25°C/min); it was then maintained at this temperature for 4 min isothermally. The interface and mass MS source temperatures were 230°C and 240°C, respectively, and the ion voltage was −0.1 kV. Data were collected and calibrated using the GCMS solution software (Shimadzu).

Results

Construction of yeasts strain expressing 1-butanol production pathway enzymes

For 1-butanol production, glucose is converted into acetyl-CoA via the Embden-Meyerhof-Parnas pathway (EMP) pathway, and acetyl-CoA is converted into 1-butabol

via the 1-butanol production pathway from clostridia ABE fermentation (Fig. 1a, and 1b). Yeasts normally express the enzyme acetyl-CoA C-acetyltransferase (thiolase [*Erg10*]), which converts acetyl-CoA into acetoacetyl-CoA (Hiser *et al.*, 1994). However, in order to produce more acetoacetyl-CoA, *thl* (thiolase) derived from *C. tropicalis* was also overexpressed in yeasts, as it was reported that the protein could be successfully overexpressed and had enzyme activity (Kanayama *et al.*, 1997). Six strains were constructed with different combinations of introduced genes (Table 2). Strains #1 and #2 were created through the introduction of three plasmids, and strains #3 and #4 were created through the introduction of two plasmids (Fig. 2). Strains #5 and #6 were negative controls (strains #5 contained pRS403, pRS405, and pRS406, and strains #6 contained pRS403, and pRS406.).

Table 2 Constructed strains

strains / genes	*thl*	*hbd*	*crt*	*bcd*	*etfA&B*	*ad*	*aad*
#1	+	+	+	+	+	+	+
#2	-	+	+	+	+	+	+
#3	+	+	+	+	+	-	-
#4	-	+	+	+	+	-	-
#5 (N.C.)	-	-	-	-	-	-	-
#6 (N.C.)	-	-	-	-	-	-	-

1-Butanol fermentation of constructed yeasts

After pre cultivation of these strains, they were cultivated under anaerobic conditions and 1-butanol production was measured by GC/MS. As seen in Fig. 3, strains #1 and #2 strains produced 1-butanol at 36 h and 60 h. Strains #3 and #4 strains, which did not have pRS405-*ad*, *aad*, produced no 1-butanol.

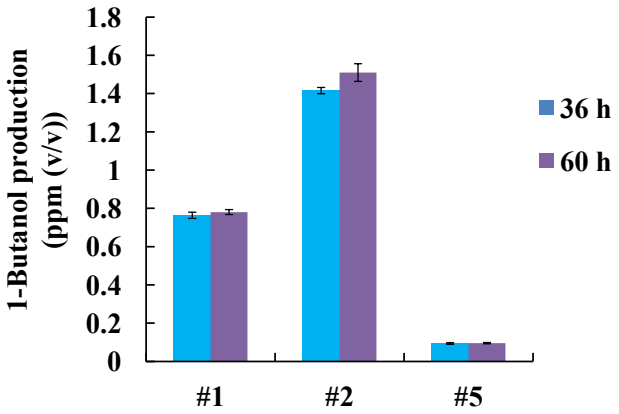

Fig. 3 1-Butanol production by constructed yeast strains. #1: W303-1A; pRS406-*thl*, *hbd*, *crt*, pRS403-*bcd*, *etfA&B*, and pRS405-*ad*, *aad*, #2: W303-1A; pRS406-*hbd*, *crt*, pRS403-*bcd*, *etfA&B*, and pRS405-*ad*, *aad*, #5: W303-1A; pRS406, pRS403, and pRS405 (N.C.).

Discussion

The *Clostridium* species, *C. acetobutylicum*, produces acetate, 1-butanol, and ethanol via ABE fermentation. *Clostridium* spp. are anaerobic and have a lower tolerance for solvent than the yeast *S. cerevisiae*. Thus, we modified *S. cerevisiae* to be a 1-butanol producing host. Unlike clostridia, *S. cerevisiae* is able to grow in aerobic conditions. In addition, as *S. cerevisiae* is a eukaryotic organism, *S. cerevisiae* is thought to produce more CoA and NADH, which are required for the *clostridium* metabolic pathway of butanol production (acetoacetyl-CoA to β-hydroxybutyryl-CoA, crotonyl-CoA to butyryl-CoA, butyryl-CoA to butyraldehyde, and butyraldehyde to 1-butanol). However, the constructed strains produced very little 1-butanol (Fig. 3). This may be because *S. cerevisiae* produces large amounts of ethanol under anaerobic conditions, and lacks a coenzyme factor involved in redox balance, NADH. The yeast *S. cerevisiae* ethanol production pathway comprises two reactions (Fig. 4). *PDC1*, *PDC5*, and *PDC6* encode the enzymes that convert pyruvate into acetaldehyde. In addition, *S. cerevisiae* produces large amounts of glycerol under anaerobic conditions (Fig. 5)(Costenoble *et al.*, 2000), and deletion of the glycerol pathway leads to higher ethanol production (Yu *et al.*, 2010; Jain *et al.*, 2011). Disruption of genes involved in ethanol production (*PDC1*, *PDC5*, and *PDC6*) and glycerol production (*GPD1*, and *GPD2*) should cause *S. cerevisiae* to produce 1-butanol.

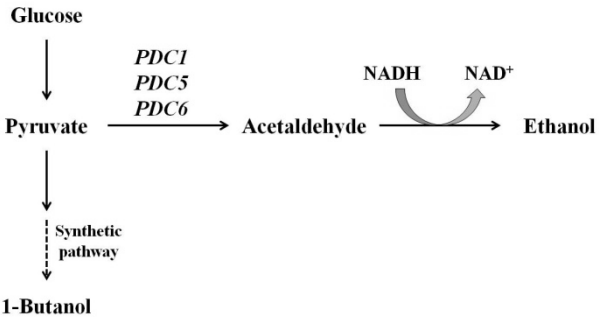

Fig.4 Ethanol production pathway.

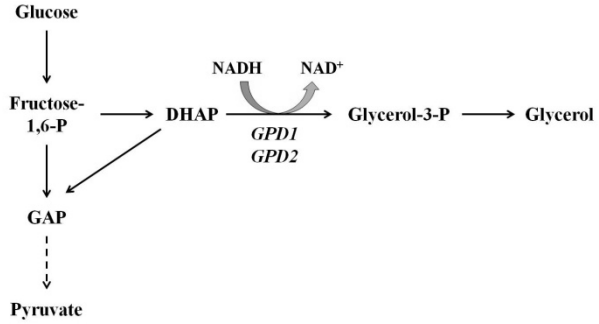

Fig. 5 Glycerol production pathway.

Summary

Biobutanol is a promising biofuel. We constructed 1-butanol-producing yeasts using 1-butanol enzymes derived from *C. acetobutylicum*. Under anaerobic cultivations, strains #2 strains produced 1.6 ppm (w/w) 1-butanol.

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Conclusion

The present study has been carried out to analyze and apply *Clostridium* species to biorefinery.

In chapter I, we attempted to use *Clostridium cellulovorans* for efficient biomass degradation. However, the metabolism of *C. cellulovorans* was not well understood. We carried out ^{13}C-isotopomer-based target metabolome analysis, to gain a carbohydrate conversion process analysis, for more profound understanding of biomass degradation by the bacterium. As a result, we were the first to demonstrate that, as well as having the ability to fix CO_2 fixation, *C. cellulovorans* is able to produce several kinds of organic acids and that the TCA cycle is partly operated in a reductive manner at the metabolite level. These results provide important information for the application of *C. cellulovorans* as a cellulosome-producing bacterium for use in industrial production.

In chapter II, to reduce our oil dependence, it has been needed to produce ethanol from $_D$-xylose, which is a major component of lignocellulosic biomass. We constructed a novel xylose-fermenting system using a strain of *Saccharomyces cerevisiae* displaying xylose isomerase (XI) 'on the cell surface'. Using this XI-displaying yeast, we produced ethanol using $_D$-xylose as a sole carbon source. Our work will provide a promising starting point for improving the direct fermentation of xylose by *S. cerevisiae*.

In chapter III, to construct an efficient system of biobutanol production, we attempted to create a butanol-producing yeast by introducing several genes ABE fermentation pathway from *Clostridium acetobutylicum* into *S. cerevisiae*. We successfully bred the 1-butanol-producing yeast and produced biobutanol. The strains would be a promising starting point for the efficient production of biobutanol, which could be a viable alternative to petroleum and bioethanol.

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Publications

Chapter I

Masahiro Shinohara, Hiroshi Sakuragi, Hironobu Morisaka, Hideo Miyake, Yutaka Tamaru, Eiichiro Fukusaki, Kouichi Kuroda and Mitsuyoshi Ueda (2013) Fixation of CO_2 in *Clostridium cellulovorans* analyzed by ^{13}C-isotopomer-based target metabolomics. *AMB Express*, **3**:61-66

Chapter II

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Chapter III

Hiroshi Sakuragi, Kouichi Kuroda, and Mitsuyoshi Ueda Molecular breeding of 1-butanol-producing yeast *Saccharomyces cerevisiae*. submitted

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