Appl Microbiol Biotechnol (2012) 94:1667–1678
DOI 10.1007/s00253-012-4094-0

BIOENERGY AND BIOFUELS

Ethanol production from wood hydrolysate using genetically engineered *Zymomonas mobilis*

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Received: 26 December 2011 / Revised: 1 April 2012 / Accepted: 10 April 2012 / Published online: 11 May 2012

Abstract An ethanologenic microorganism capable of fermenting all of the sugars released from lignocellulosic biomass through a saccharification process is essential for secondary bioethanol production. We therefore genetically engineered the ethanologenic bacterium *Zymomonas mobilis* such that it efficiently produced bioethanol from the hydrolysate of wood biomass containing glucose, mannose, and xylose as major sugar components. This was accomplished by introducing genes encoding mannose and xylose catabolic enzymes from *Escherichia coli*. Integration of *E. coli manA* into *Z. mobilis* chromosomal DNA conferred the ability to co-ferment mannose and glucose, producing 91 % of the theoretical yield of ethanol within 36 h. Then, by introducing a recombinant plasmid harboring the genes encoding *E. coli xylA*, *xylB*, *tal*, and *tktA*, we broadened the range of fermentable sugar substrates for *Z. mobilis* to include mannose and xylose as well as glucose. The resultant strain was able to ferment a mixture of 20 g/l glucose, 20 g/l mannose, and 20 g/l xylose as major sugar components of wood hydrolysate within 72 h, producing 89.8 % of the theoretical yield. The recombinant *Z. mobilis* also efficiently fermented actual acid hydrolysate prepared from cellulosic feedstock containing glucose, mannose, and xylose. Moreover, a reactor packed with the strain continuously produced ethanol from acid hydrolysate of wood biomass from coniferous trees for 10 days without accumulation of residual sugars. Ethanol productivity was at 10.27 g/l h at a dilution rate of 0.25 h^{-1}.

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Keywords Bioethanol · Lignocellulose · Hydrolysates · Mannose · Xylose · Ethanol · Metabolic engineering · *Zymomonas mobilis*

Introduction

Bioethanol production from lignocellulosic biomass is considered to be a milestone for sustainable development. Although bioethanol production from crop-based substrates such as sugar cane juice and cornstarch is a well-developed technology, lignocellulosic biomass like crop waste, forestry residues, and municipal solid waste represents a potentially superior source of feedstock for bioethanol fuel (Perlack et al. 2005; Vertes et al. 2006) since it is an inexpensive, abundant, and sustainable raw material that is available worldwide and occurs as a by-product without competing uses. Hydrolysates from lignocellulosic biomass include a variety of pentoses and hexoses, and their specific compositions will depend on the target biomass (Lui et al. 2010; Chandel and Singh 2011). There is no single organism yet known that can efficiently convert all of these sugars to ethanol (Fu and Paul 2008), though ethanol-producing bacteria have attracted much attention in recent years because their growth rate is substantially higher than that of *Saccharomyces* presently used for production of fuel alcohol. Moreover, with recent advances in biotechnology, they have the potential to play a key role in making ethanol production much more economical (Weber et al. 2010).

Zymomonas mobilis is a well-known ethanol-producing bacterium used in tropical areas to make alcoholic beverages from plant sap (Swings and De Ley 1977). The advantageous features of *Z. mobilis* are its high growth rate and high specific ethanol production; unfortunately, its fermentable carbohydrate substrates are limited to glucose, fructose, and sucrose (Rogers et al. 2007). Among the first challenges that

must be overcome to facilitate bioethanol production from lignocellulosic biomass is the development of ethanologenic microorganisms capable of simultaneously fermenting the mixtures of derived pentoses and hexoses.

We have been focusing on the production of ethanol from wood biomass hydrolysates using an ethanologenic bacterium that can co-ferment the major sugar components: glucose, mannose, and xylose. In that regard, Weisser et al. (1996) reported that a genetically engineered *Z. mobilis* assimilated mannose as the sole carbon source following introduction of genes encoding fructokinase (FK) from *Z. mobilis* and phosphomannose isomerase (PMI) from *Escherichia coli*. However, they did not address the issue of ethanol production from mannose by the recombinant *Z. mobilis*. For the pentose fermentation, Zhang et al. (1995) and Deanda et al. (1996) demonstrated ethanol production from xylose and arabinose, respectively. Introduction of *E. coli* genes encoding the four xylose catabolic enzymes, xylose isomerase (XI), xylulokinase (XK), transaldolase (TA) and transketolase (TK), into *Z. mobilis* enable to co-ferment xylose with the theoretical yield of ethanol. In addition, introduction of *E. coli* genes encoding the three arabinose catabolic enzymes, arabinose isomerase (AI), ribulokinase (RK), and ribulose-5-phosphate-4-epimerase (PE), with *talB* and *tktA* conferred the ability of arabinose co-fermentation. Since then a method for co-fermentation of a mixture of glucose, xylose and arabinose by recombinant *Z. mobilis* has been improving to enhance ethanol productivity (Lawford and Rousseau 2002, Mohagheghi et al. 2004), but there have been few reports concerning the breeding of a strain capable of producing ethanol from a mixture of glucose, mannose and xylose.

In the present study, we focused on wood, rather than herbaceous, biomass and investigated the possibility of conferring the ability to simultaneously ferment glucose, mannose and xylose on *Z. mobilis* through coexpression of genes encoding mannose and xylose catabolic enzymes. Furthermore, using the genetically engineered strain, the efficiency of ethanol production was tested using actual acid hydrolysate from coniferous Japanese Cedar residues. On the basis of our results, we then assessed the ability of a reactor packed with immobilized engineered *Z. mobilis* to continuously ferment acid hydrolysate from the coniferous trees.

Materials and methods

Bacterial strains, plasmids, and cultural conditions

E. coli JM 109 [*recA*, *supE*, *endA*, *hsdR*, *gyrA*, *relA*, *thi*, Δ(*lac-proAB*), *F'tra*, Δ*proAB*, *lacIq*, *lacZdM15*] was used as the host strain for recombinant DNA manipulation. *Z. mobilis* ATCC29192 was used as the breeding host strain for the fermentation of a mixture of glucose, mannose, and xylose. *E. coli* K12 was used as the source of xylose isomerase (*xylA*), xylulokinase (*xylB*), transaldolase (*tal*), and transketolase (*tktA*) genes, encoding the xylose catabolic enzymes (Blattner et al. 1997) as well as phosphomannose isomerase (*manA*). *Z. mobilis* ATCC29191 was used as the source for the gene promoter from glycelaldehyde-3-phosphate dehdyrogenase (P*gap*) (Conway et al. 1987). *E. coli* was grown in Luria–Bertani medium (10 g/l Bacto trypton, 5 g/l Bacto yeast extract, 5 g/l sodium chloride, pH 7.2) containing 100 μg/ml Ap (ampicillin). *Z. mobilis* was cultured statically in RM medium (20 g/l of glucose, 10 g/l Bacto yeast extract, 2 g/l potassium phosphate, pH 6.0) at 30 °C. The *Z. mobilis*/*E. coli* shuttle vector pZA22 (Tc^r, tetracycline resistance; Cm^r, chloramphenicol resitance; 6.7 kbp) was used for gene transfer (Misawa et al. 1986), and pZA323 was also constructed through insertion of the Tc^r gene from the *E. coli* vector plasmid pBR322 (Ap^r, Tc^r; 4.6 kbp) into pZA32 (Tonomura et al. 1986; Yanase et al. 1994). pZA22 and pZA323 were compatible in *Z. mobilis* because they replicate from different replication origins. Transformation of *Z. mobilis* was accomplished using the spheroplast transformation method described previously (Yanase et al. 1986).

Construction of pSZ1-*manA* and pSZ1-*manA/frk*

An expression vector encoding a fused gene consisting of *E. coli manA* and *Z. mobilis frk* under the control of the strong, constitutive *Z. mobilis* P*gap* promoter was constructed using recombinant PCR (Fig. 1). Initially, a fused gene encoding *E. coli manA* under the control of P*gap* was constructed. A DNA fragment containing the P*gap* promoter was amplified from *Z. mobilis* chromosomal DNA using primers 5′- CG*G AAT TC*G TTC GAT CAA CAA CCC GAA TCC TAT CG (PMI1: 5′-primer, *Eco*RI site is italicized) and 5′- TTG CAC TGA GTT AAT GAG TTT TTG CAT GTT TAT TCT CCT AAC TTA TTA AG (PHI3: 3′-primer). *E. coli manA* with the *Z. mobilis* P*gap* flanking region was amplified from *E. coli* K12 chr. DNA using primers 5′- CTT AAT AAG TTA GGA GAA TAA ACA TGC AAA AAC TCA TTA ACT CAG TGC AA (PHI2: 5′-primer) and 5′-CGC *GGA TCC* TTA CAG CTT GTT GTA AAC ACG CGC TA (PHI4: 3′-primer; *Bam*HI site is italicized). The resultant two DNA fragments were mixed, heated at 94 °C for 20 min, and then incubated for an additional 45 min at 37 °C to form a heteroduplex having an *Eco*RI site at its 5′-end and a *BamH* I site at its 3′-end. The fused *Pgap-manA* was then amplified from the heteroduplex using primers PHI1 and PHI4. The amplified 1,491-bp P*gap-manA* fragment was digested with *Eco*RI and *Bam*HI and inserted into the *Eco*RI and *Bam*HI sites within the multicloning site of pSTV29 (TAKARA Bio. Co. Ltd), yielding pSTV29-P*gap*/*manA*. To introduce fused P*gap-manA* into *Z. mobilis*, the *rep* gene arising from

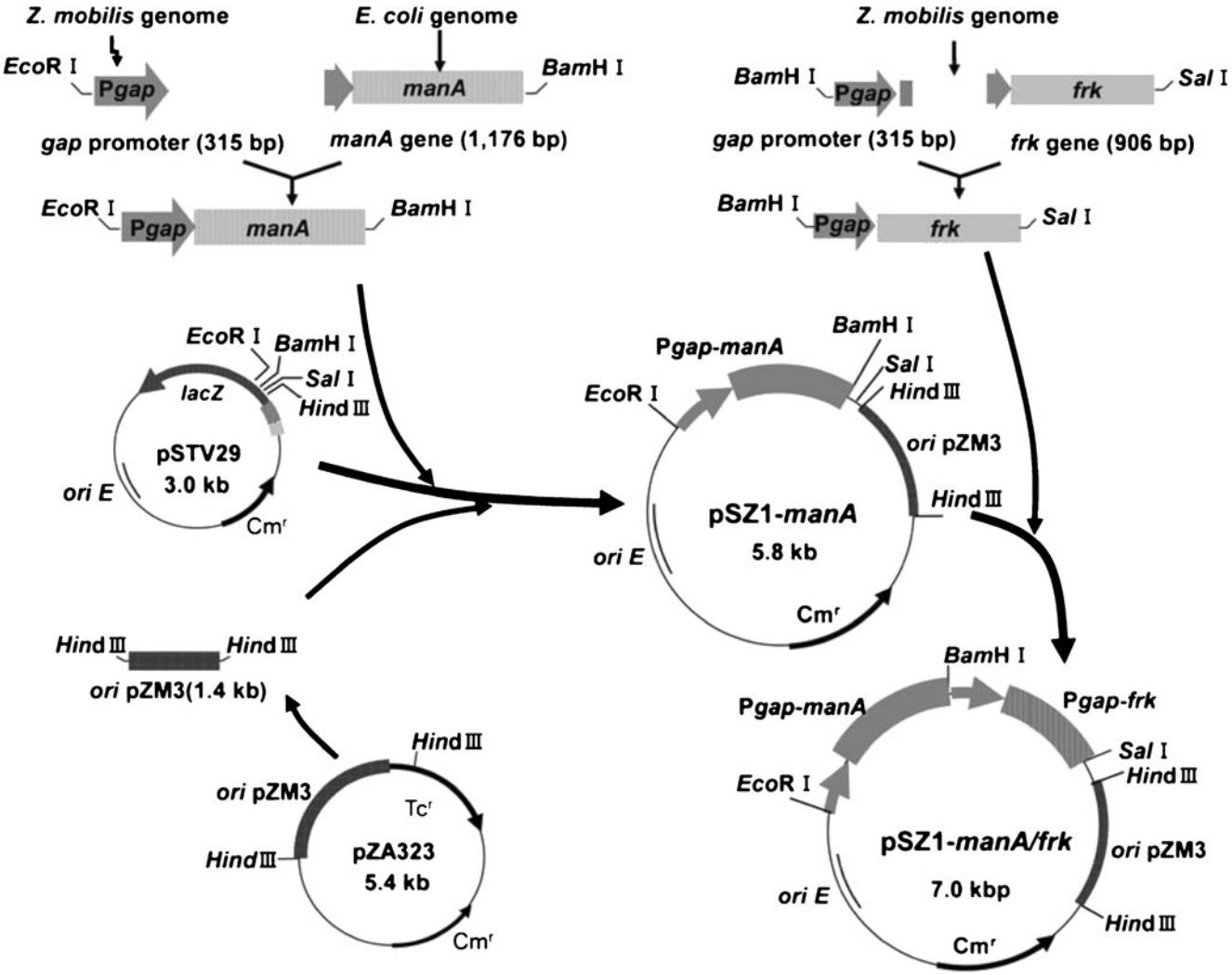

Fig. 1 Construction of pSZ1-*manA* and pSZ1-*manA/frk*. *Arrows* indicate the transcriptional direction. The *heavy solid arrows* indicate the promoter of the *Z. mobilis* glycelaldehyde-3-phosphate dehydrogenase gene (P*gap*). The *dotted bars* indicate the *E. coli* phosphomannose isomerase gene (*manA*), while the *striped bars* indicate the *Z. mobilis* fructokinase gene (*frk*). The *dotted light arrow* indicates *E. coli lacZ*, and the *solid medium arrows* indicate the CAT gene. The *reverse hatched bars* indicate the *Z. mobilis* replication gene on the endogenous cryptic plasmid pZA3 (*ori pZM3*). The *solid line* indicates the vector plasmid pSTV29

an endogenous *Z. mobilis* plasmid, pZM3 (3.9 Kbp), was inserted into pSTV29-P*gap/manA*. The 1.4-kb *Hin*dIII fragment encoding *ori pZM3* was excised from pZA323, which was constructed previously as a shuttle vector for use between *E. coli* and *Z. mobilis* (Yanase et al. 1994), and inserted into the *Hin*dIII site of pSTV29-P*gap*/*manA*, yielding pSZ1-*manA*.

A fused gene encoding *Z. mobilis frk* under the control of P*gap* was constructed in an analogous fashion. A DNA fragment containing P*gap* was amplified from *Z. mobilis* chr. DNA using primers 5′-CGC *GGA TCC* GTT CGA TCA ACA ACC CGA ATC CTA TC (FK1:5′-primer, *Bam*HI site is italicized) and 5′- TCC ATA AAT TTT TTT ATC GTT TTT CAT GTT TAT TCT CCT AAC TTA TTA AG (FK3: 3′-primer), while *frk* with the P*gap* flanking region was amplified from *E. coli* K12 chr. DNA using primers 5′-CTT AAT AAG TTA GGA GAA TAA ACA TGA AAA ACG ATA AAA AAA TTT ATG GA (FK2: 5′-primer) and GGC *GTC GAC* TTC CAA AAT CCC TTT TCG GTT AAG AA (FK4: 3′-primer, *Sal*I site is italicized). The two DNA fragments were mixed, heated at 94 °C for 20 min, and then incubated for an additional 45 min at 37 °C to form a heteroduplex having a *Bam*HI site at its 5′-end and a *Sal* I site at its 3′-end. Thereafter, *frk* was amplified from the heteroduplex using primers FK1 and FK4. The amplified 1,221-bp fused P*gap-frk* fragment was then digested with *Bam*HI and *Sal*I and inserted into the *Bam*HI and *Sal*I sites of pSZ1-*manA*, yielding pSZ1-*manA/frk*. The plasmid pSZ1

was also constructed to insert the *Hin*dIII fragment encoding *ori* pZM3 into the *Hin*dIII site of pSTV29 as a control vector.

Integration of P*gap-manA* into *Z. mobilis* chr. DNA by using circularized DNA

The integration plasmid pUZE2d-*manA* was constructed using a DNA fragment that lacked the replicon gene of *Z. mobilis*, which allowed direct selection for integration of P*gap-manA* into the levansucrase gene (*sucZE2*) region of the chromosome. As we reported previously, a fragment of about 4.9 kb encoding *sucZE2* and *sucZE3* (invertase gene) cloned from *Z. mobilis* chr. DNA was inserted into pUC118 to construct the recombinant plasmid pUZ45, after which the *sucZE2* was subcloned into pUC118, yielding pUZE2 (Yanase et al. 1998). The P*gap-manA* fragment was excised from pSZ1-*manA* by digestion with *Bam*HI and *Eco*RI. The resultant 1,491-bp DNA fragment was blunted and inserted into the blunted *Nde*I site of pUZE2, within which the *Nde*I site was located at base 617 of *sucZE2* (1,272 bases), yielding pUZE2-*manA*. The plasmid was then used to transform *Z. mobilis* with selection for double homologous recombination that enables assimilation of mannose as the carbon source.

Construction of pZA22-*xylA/xylB-tal/tkt*

To confer the ability to ferment xylose, four *E. coli* genes, *xylA*, *xylB*, *tal*, and *tktA*, were introduced into *Z. mobilis* (Fig. 2). First, shuttle vector pZA22 was modified to exchange the *Sal*I site on the Tc-resistance gene with the *Not*I site by inserting p*Not*I-linker (TAKARA Bio. Co. Ltd) into the blunted *Sal*I site, yielding pZA22n. The operon encoding *E. coli xylA* and *xylB* under the control of *Z. mobilis* P*gap* was excised from pUC118-P*gap-xylA/xylB*, which was constructed previously (Yanase et al. 2007). The *Eco*RI site upstream of P*gap-xylA/xylB* was exchanged with the *Not*I site by insertion of p*Not*I-linker, after which the 3,212-bp P*gap-xylA/xylB* fragment was excised by digestion with *Bgl*I. In addition, the operon encoding *E. coli tal* and *tktA* under the control of P*gap* was excised from pUC118-P*gap-tal/tktA*, which also had been constructed previously (Yanase et al. 2007). The *Sac*I site downstream of P*gap-tal/tktA* was exchanged with *Not*I site by inserting p*Not*I-linker, after which the 3,256-bp P*gap-tal/tktA* fragment was excised by digestion with *BglI*. The *NotI/BglI*-digested P*gap-xylA/xylB* and P*gap-tal/tktA* fragments were then ligated to pZA22n digested with *Not*I, yielding pZA22-xtR.

Preparation of crude extracts and enzyme assays

Cells were harvested by centrifugation for 10 min at 5,000× *g*, and then washed with buffer (20 mM potassium phosphate buffer, pH 7.0). After sonication (two times for 30 s at 35–40 W) in an ice bath using INSONICATOR (Kubota), the resultant lysate was cleared by centrifugation for 15 min at 35,000×*g*, and the supernatant was used as a cell-free extract. The protein content of the extracts was determined using a protein assay kit (BioLad Laboratories, Inc.), with bovine serum albumin serving as the standard.

PMI activity was assayed in a reaction mixture containing 250 μl of 34 mM Mops buffer (pH 7.0), 50 μl of 42 mM $MgCl_2$, 50 μl of 6 mM ATP, 50 μl of 6 mM $NADP^+$, 1 μl of 1U/μl PGI (phosphoglucose isomerase), 1 μl of 1U/μl G6PDH (glucose-6-phosphate dehydrogenase), 448 μl of water, and

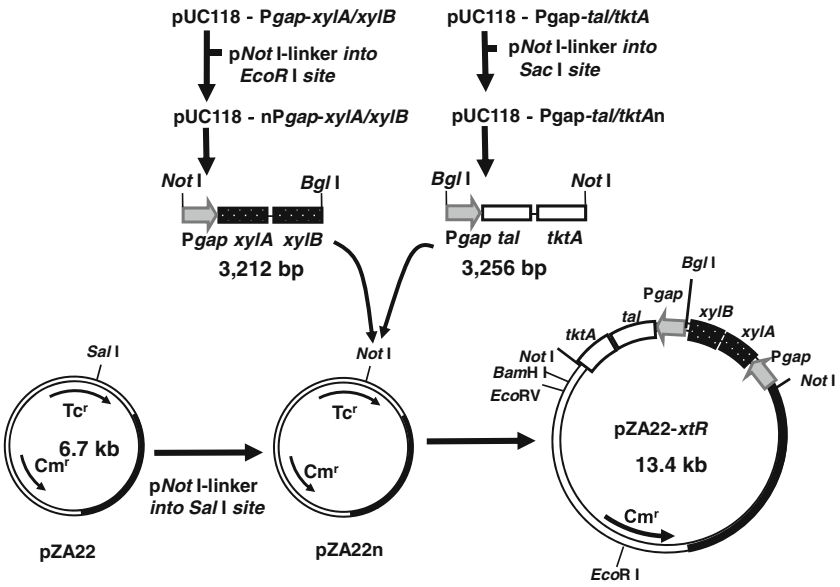

Fig. 2 Construction of pZA22-xtR. The *heavy solid arrows* indicate the promoter of the *Z. mobilis* glycelaldehyde-3-phosphate dehydrogenase gene (P*gap*). The *sparsely dotted bars* indicate *E. coli* XI (*xylA*) and XK (*xylB*), respectively. The *white bars* indicate *E. coli* TA (*tal*) and TK (*tkt*), respectively. The *open line* indicates the vector plasmid pACYC184, and the *black line* indicates the *Z. mobilis* endogenous cryptic plasmid, pZA2

100 μl of cell-free extract. The reaction was started by adding 50 μl of 50 mM mannose-6-phosphate, and the NADPH formed was determined spectroscopically based on the absorbance at 340 nm recorded at 25 °C (Weisser et al. 1996). One unit of PMI was defined as the amount of enzyme needed to produce 1 μmol of NADPH in 1 min. FK activity was assayed using the same reaction mixture used to assay PMI activity. The reaction was started by adding 50 μl of 50 mM fructose, and again the NADPH formed was determined spectroscopically based on the absorbance at 340 nm recorded at 25 °C. One unit of FK was defined as the amount of enzyme needed to produce 1 μmol of NADPH in 1 min. In analogous fashion, mannose kinase (MK) activity was also assayed in a reaction mixture containing 250 μl of 34 mM Mops buffer (pH 7.0), 50 μl of 42 mM $MgCl_2$, 50 μl of 6 mM ATP, 50 μl of 6 mM $NADP^+$, 1 μl of 1U/μl PMI, 1 μl of 1U/μl PGI, 1 μl of 1U/μl G6PDH, 447 μl of water, and 100 μl of cell-free extract. The reaction was started by adding 50 μl of 50 mM mannose, and the NADPH formed was determined spectroscopically based on the absorbance at 340 nm recorded at 25 °C. One unit of MK was defined as the amount of enzyme needed to produce 1 μmol of NADPH in 1 min. The activities of XI, XK, TA, and TK were assayed as described previously (Yanase et al. 2007).

Batch fermentation

After preculturing recombinant *Z. mobilis* for 48 h in RM medium supplemented with 20 g/l glucose, xylose, or mannose, the cells were harvested and washed with RM medium lacking a carbon source. The washed cells were then inoculated into 100 ml of RM medium supplemented with the tested sugars in a 200-ml bottle with a screw cap and cultivated statically at 30 °C. The culture medium initially had an optical density at 610 nm (OD_{610}) of 0.1 (about 10^6 cells/ml).

Fermentation experiments with acid hydrolysate of wood biomass

Acid hydrolysate of wood biomass from coniferous trees, prepared after pretreatment with concentrated sulfuric acid, was provided by JGC Corporation (Yokohama, Japan) (Yamada et al. 2002). The typical sugar composition of the hydrolysate was (g/l) glucose—68.5, mannose—22.4, and xylose—14.3. The pH was adjusted to 5.5 by adding $CaCO_3$, after which the mixture was allowed to rest overnight. The resultant supernatant was sterilized at 110 °C for 10 min and was then mixed with an equal volume of autoclaved twofold concentrated RM medium, yielding a mixture designated as 1:1 acid hydrolysate–RM medium. To make 9:1 acid hydrolysate–RM medium, the sterilized acid hydrolysate was mixed with an autoclaved tenfold concentration of RM medium at a ratio of 9:1 (*v*/*v*).

Continuous fermentation of acid hydrolysate of wood biomass

A laboratory-scale bioreactor packed with the immobilized growing *Z. mobilis* [*sucZE2::manA*, pZA22-xtR] cells was used for continuous fermentation fed with acid hydrolysate of wood biomass. Based on the results of preliminary tests of various combinations of immobilization procedures and carriers, we selected the adsorption method for immobilization of cells on ceramic beads. To immobilize cells through adsorption, a 20-ml inoculum of pre-cultured *Z. mobilis* [*sucZE2::manA*, pZA22-xtR] was added to 200 ml of 40 g/l xylose–RM medium supplemented with 2 g of ceramic beads (spherical diameter, 3 to 5 mm) and then cultured statically at 30 °C for 48 h. The ceramic beads with the adsorbed cells were collected and packed in a glass column (inner diameter, 25 mm; height, 130 mm) to a height of 75 mm. The 9:1 acid hydrolysate of wood biomass was fed into the bottom of the reactor using a peristaltic pump, and the fermented broth overflowed from the top of the reactor. During continuous fermentation, the temperature was maintained by circulating 30-°C water through the jacket of the reactor.

Analysis

The growth of strains was measured based on OD_{610}: a reading of 1.0 was equivalent to 0.41 mg of cells (dry weight)/ml. Levels of mannose, xylose, and glucose in the culture broth were determined by high-performance liquid chromatography using a Shodex Ionpack KS801 (4.6×300 mm: Soko Co. Ltd.) and Aminex 87-P (4.6×300 mm: Bio-Rad Laboratories, Inc,) tandem column at a column temperature of 75 °C. The mobile phase was degassed water, the flow rate was 0.75 ml/min, and a refractive index detector (model RID-10A; Shimazu Corp) was used. For metabolites such as lactate and acetate, an Aminex 87 H column (Bio-Rad Laboratories, Inc.) was used with 0.1 M hydrogen sulfate as the mobile phase at a column temperature of 40 °C. Ethanol was assayed using gas chromatography with a glass column (0.26×200 cm) filled with Porapak Type QS (80–100 mesh, Waters Corp.) at 180 °C; nitrogen was the carrier gas (40 ml/min), and a FID detector was used. DNA sequencing was carried out using the dideoxy chain termination method in an automated DNA sequencer (Applied Biosystems, model 373A). The sequencing reaction was carried out using a BigDye

Terminator v.3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

Results

Expression of mannose catabolic enzyme genes in *Z. mobilis*

The primary reason that *Z. mobilis* cannot ferment mannose appears to be the absence of *manA*, which encodes PMI. Furthermore, the phosphorylation of mannose by FK might be the rate-limiting step in mannose fermentation. Therefore, to enable *Z. mobilis* fermentation of mannose to ethanol, the organism was first transformed with the recombinant plasmid pSZ1-*manA* or pSZ1-*manA/frk*. Following transformation, the expression of PMI and FK activities driven by the *Z. mobilis* P*gap* promoter was assessed in cells grown in glucose under standard conditions (Table 1). *Z. mobilis* harboring pSZ1 showed some endogenous FK activity and catalyzed the phosphorylation of incorporated mannose, demonstrating FK's broad substrate specificity. On the other hand, there was no detectable PMI activity, which catalyzes the isomerization of mannose 6-phosphate to fructose 6-phosphate. *Z. mobilis* harboring pSZ1-*manA* or pSZ1-*manA/frk* efficiently expressed PMI and FK in cells grown in glucose, and the activity of FK in the strain harboring both *manA* and *frk* was twofold higher than that in the strain harboring *manA*. When cells harboring pSZ1-*manA/frk* were grown in mannose, the levels of both PMI and FK were slightly higher than in cells grown in glucose. This suggests that the carbon source affects the expression of the enzymes involved in carbohydrate utilization.

Table 1 Expression of *E. coli manA* and *Z. mobilis frk* in *Z. mobilis*

Plasmid	Carbon source	Activity[a]	
		PMI (mU/mg)	FK (mU/mg)
pSZ1	Glucose	<1	145
pSZ1-*manA*	Glucose	110	204
	Mannose	180	255
pSZ1-*manA/frk*	Glucose	273	559
	Mannose	324	746
sucZE2::*manA*	Glucose	193	280
	Mannose	254	364

[a] Cells were cultured in RM medium containing 20 g/l glucose or mannose and 100 μg/ml Cm for 48 h at 30 °C, after which the harvested cells were disrupted by sonication. The resultant supernatant was used as a cell-free extract. One unit of PMI=1 μmol of NADPH produced/min; 1 U of KF=1 μmol of NADPH produced/min

Fermentation of mannose by *Z. mobilis* harboring pSZ1-*manA* or pSZ1-*manA/frk*

After preculturing *Z. mobilis* harboring pSZ1-*manA* or pSZ1-*manA/frk* for 48 h in RM medium supplemented with 20 g/l glucose, the cells were harvested and washed with RM medium lacking a carbon source. The washed cells were then inoculated into RM medium supplemented with 20 g/l mannose and cultivated statically (Fig. 3). The culture medium initially had an OD_{610} of about 0.1 (about 10^6 cells/ml), and no growth of the strain harboring empty pSZ1 was detected when mannose was the sole carbon source. The strains harboring pSZ1-*manA* and pSZ1-*manA/frk* grew in mannose, reaching OD_{610} levels of 1.3 and 1.55, respectively, after 72 h of cultivation. During cultivation, 20 g/l mannose was consumed to produce 9.5 and 9.8 g/l ethanol or >95 % of the theoretical yield (Fig. 3a). While the consumption of mannose by the strain harboring *manA* was delayed by about 24 h compared with the strain harboring both *manA* and *frk*. In the presence of 20 g/l glucose, both recombinant strains utilized the 20 g/l mannose perfectly within 48 h of cultivation with co-fermentation of glucose, producing 90 and 97 % of the theoretical yield of ethanol, respectively (Fig. 3b). These results suggest that introduction of *manA* alone into *Z. mobilis* enables fermentation of mannose because actual hydrolysates of cellulosic biomass contain glucose as a major sugar component.

Integration of *E. coli manA* into *Z. mobilis* chromosomal DNA

To enable the strain to stably ferment mannose, *E. coli manA* was integrated into the *Z. mobilis* genome by transformation with pUZE2d-*manA*. *sucZE2* was selected as the target gene for homologous recombination because levansucrase reduces ethanol recovery during sucrose fermentation with accumulation of fructan. As indicated in the "Materials and methods", several of the transformants that appeared on mannose–RM agar plates were collected at random, and mannose fermentation was examined. Using transformants able to grow with mannose as the sole carbon source, the integration of *manA* into *sucZE2* within the *Z. mobilis* genomic DNA was analyzed by PCR using the genomic DNA as a template. A 1.5-kbp DNA fragment corresponding to P*gap-manA* and a 2.8-kbp DNA fragment corresponding to *sucZE2*::P*gap-manA* were detected by PCR (data not shown), indicating that *manA* had been integrated into *sucZE2* within the *Z. mobilis* genome. The resultant transformant strain was designated as *Z. mobilis* [*sucZE2::manA*].

The expression of *manA* in *Z. mobilis* [*sucZE2::manA*] was assessed in cells grown with glucose or mannose as the sole carbon source. The integrated strain exhibited PMI activity similar to that exhibited by the strain harboring

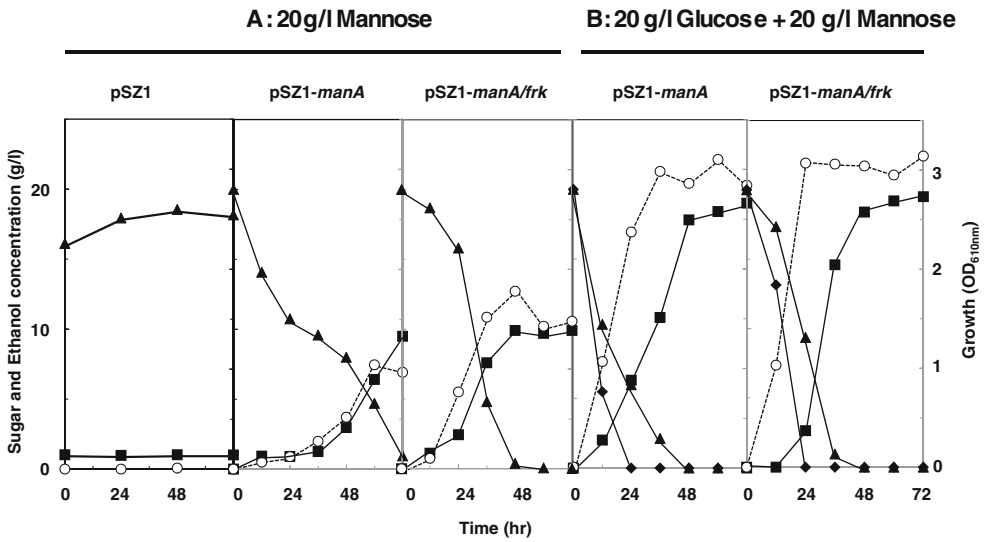

Fig. 3 Cell growth and fermentation of mannose or a mixture of glucose and mannose in *Z. mobilis* carrying the mannose catabolic enzyme genes. The recombinant strain was cultured statically in RM medium containing 20 g/l mannose (**a**) or a mixture of 20 g/l glucose and 20 g/l mannose (**b**) at 30 °C. *Z. mobilis* harboring the shuttle vector plasmid pSZ1, the strain harboring pSZ1-*manA*, and the strain harboring pSZ1-*manA/frk* were separately inoculated into the above medium. *Open circles* indicate the growth of the strain, *closed triangles* the residual mannose, *closed diamonds* the residual glucose, and *closed squares* the accumulation of ethanol

pSZ1-*manA*, in addition to FK activity (Table 1). After preculturing *Z. mobilis* [*sucZE2::manA*] for 48 h in RM medium supplemented with 20 g/l glucose, the cells were harvested and washed with RM medium lacking a carbon source (Fig. 4). The washed cells were then inoculated into RM medium supplemented with 20 g/l mannose or a mixture of 20 g/l mannose and 20 g/l glucose and cultivated statically. The integrated strain grew on mannose, reaching an OD_{610} of 0.91 after 48 h of cultivation, and produced 9.48 g/l ethanol or about 94.8 % of the theoretical yield. The fermentation profile for the mixture of 20 g/l glucose and 20 g/l mannose by the integrated strain showed simultaneous fermentation, producing 18.17 g/l ethanol or 91 % of the theoretical yield within 36 h of cultivation. Notably, the integrated strain fermented glucose and mannose simultaneously after being subcultured 20 times in glucose alone, which confirms that *manA* was stably maintained and expressed in *Z. mobilis* (data not shown).

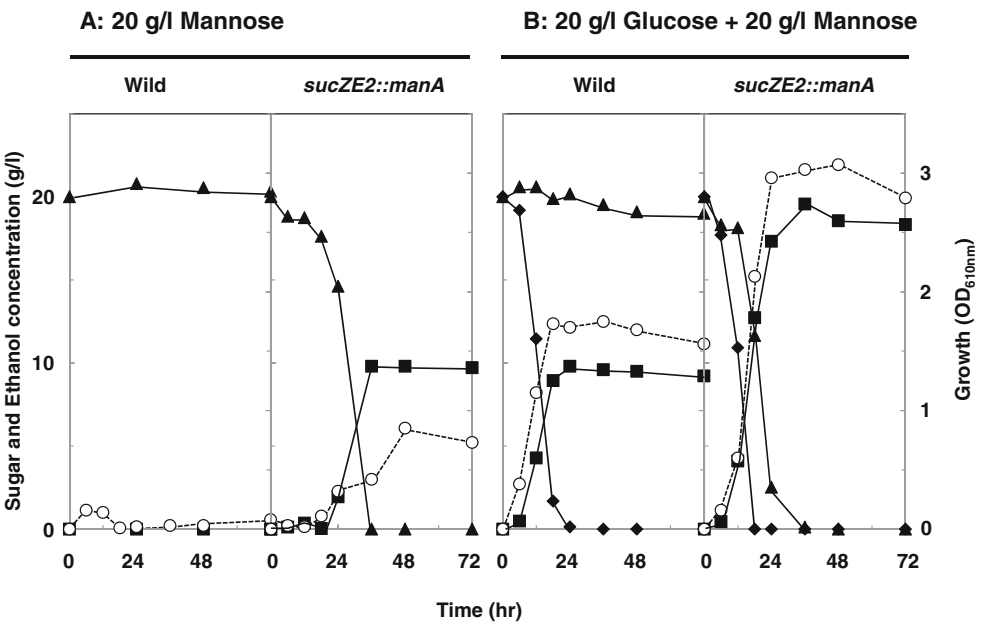

Fig. 4 Cell growth and fermentation of mannose or a mixture of glucose and mannose in *Z. mobilis* integrated with *manA*. The recombinant strain was cultured statically in RM medium containing 20 g/l mannose (**a**) or a mixture of 20 g/l glucose and 20 g/l mannose (**b**) at 30 °C. *Z. mobilis* integrated with *manA* was inoculated into the above medium, and the wild-type strain was also cultured as control. *Open circles* indicate the growth of the strain, *closed triangles* the residual mannose, *closed diamonds* the residual glucose, and *closed squares* the accumulation of ethanol

Co-expression of *E. coli* xylose catabolic genes in *Z. mobilis* [*sucZE2::manA*]

To confer the ability to ferment xylose along with mannose and glucose, xylose catabolic genes from *E. coli* were introduced into *Z. mobilis* [*sucZE2::manA*]. To confirm that *Z. mobilis* would acquire the ability to ferment xylose upon introduction of the recombinant plasmid pZA22-xtR, we initially transformed a wild-type strain with the vector. A transformant forming a large colony on 20 g/l xylose–RM agar containing 100 μg/l Cm was collected, and its expression of xylose catabolic genes was examined (Table 2). *Z. mobilis* harboring the empty pZA22 vector showed no detectable XI or XK activity, which catalyzes the initial steps of xylose utilization. Some TA and TK activity was observed in *Z. mobilis* cells as those enzymes are involved in the endogenous pentose phosphate cycle. In addition, the strain harboring pZA22-xtR efficiently expressed both XI and XK under the P*gap* promoter so that the levels of both TA and TK activity were threefold higher than the endogenous enzyme activities. When the strain harboring pZA22-xtR was grown with xylose as the sole carbon source, expression of XI, TA, and TK was increased slightly, though expression of XK was not. After preculturing of the strain harboring pZA22-xtR for 48 h in RM medium supplemented with 20 g/l xylose, the cells were harvested and washed with RM medium lacking a carbon source. The washed cells were then inoculated into RM medium supplemented with 40 g/l xylose or 40 g/l xylose and 40 g/l glucose and cultivated statically (Fig. 5). Neither the wild-type strain nor the strain harboring pZA22 grew when xylose was the sole carbon source (data not shown). By contrast, the strain harboring pZA22-xtR grew in xylose, reaching an OD_{610} of about 3.0 after 48 h of cultivation. Within 60 h, the 40 g/l xylose was entirely consumed to produce 17.8 g/l ethanol or 89 % of the theoretical yield (Fig. 5a). No accumulation of xylitol or organic acids (succinate, lactate, or acetate) as by-products was detected during the cultivation (data not shown). The fermentation profile for the mixture of glucose and xylose by *Z. mobilis* [pZA22-xtR] showed a diauxic shift from glucose to xylose (Fig. 5b). The recombinant strain consumed 40 g/l glucose entirely during 36 h of cultivation, after which the fermentation of xylose was initiated. Moreover, the rate of xylose consumption from the mixture of glucose and xylose was similar to that of xylose alone. During 72 h of cultivation, both glucose and xylose were utilized completely to produce 34.6 g/l ethanol or 88 % of the theoretical yield, without accumulation of by-products.

Table 2 Expression of *E. coli* xylose catabolic enzyme genes and *manA* in *Z. mobilis*

Plasmid	Carbon source	Activity[a] (mU/mg)					
		XI	XK	TA	TK	PMI	FK
pZA22	Glucose	<1	<1	42	68	nd	nd
pZA22-xtR	Glucose	211	521	123	187	nd	nd
	Xylose	389	316	181	312	nd	nd
sucZE2::manA/	Glucose	183	415	174	108	118	154
pZA22-xtR	Xylose	287	452	278	143	132	168
	Mannose	169	380	230	120	275	290

[a] Cells were cultured in RM medium containing 20 g/l glucose or xylose or mannose and 100 μg/ml of Cm for 48 h at 30 °C, and the harvested cells were disrupted by the ultrasonic oscillator. The resultant supernatant was used as a cell-free extract. One unit of XI=1 μmol of xylulose produced/min; 1 U of XK=1 μmol of NADH produced/min; 1 U of TA=1 μmol of NADH produced/min; 1U of TK=1 μmol NADH decreased/min; 1 U of PMI=1 μmol of NADPH produced/min; 1 U of FK=1 μmol of NADPH produced/min

To enable *Z. mobilis* to ferment all three major sugar components of lignocellulosic biomass, glucose, xylose, and mannose, we co-expressed the genes encoding mannose and xylose catabolism in *Z. mobilis* cells. *Z. mobilis* [*sucZE2::manA*] was transformed with pZA22-xtR, and its transformants were selected as colonies on xylose–RM agar plates. We collected a transformant capable of growing with either xylose or mannose as the sole carbon source and designated it as *Z. mobilis* [*sucZE2::manA*, pZA22-xtR]. We then assayed the activities of five genes, *xylA*, *xylB*, *tal*, *tktA*, and *manA*, using cell-free extracts prepared from cells grown in glucose, xylose, or mannose under standard conditions (Table 2). Although there were slight differences in the enzyme activities of the cells, depending upon a carbon source, the expression levels of the xylose and mannose catabolic genes, in addition to the endogenous *frk* gene, were sufficient to enable co-fermentation of glucose, xylose, and mannose by *Z. mobilis* (Table 2). When an aliquot of cells pre-cultivated in RM medium supplemented with xylose as the sole carbon source was inoculated into RM medium supplemented with the tested sugars, the recombinant strain grew well on xylose or mannose and, after a 12-h lag phase, exhibited a growth rate similar to that obtained with glucose (Fig. 6a–c). During 36 to 48 h of cultivation, the recombinant strain consumed 19 g/l xylose and 19 g/l mannose perfectly to produce 8.65 and 9.03 g/l ethanol or 91 and 95 % of the theoretical yield, respectively. When glucose was present, its consumption preceded that of xylose or mannose, and the rates of fermentation of xylose and mannose were similar to those of each sugar alone (Fig. 6d, e). When all three sugars were present at 20 g/l, all were consumed entirely to produce 26.5 g/l ethanol or 89.8 % of the theoretical yield during 60 to 72 h of cultivation (Fig. 6f). Under the analytical conditions used, the accumulation of noticeable by-products was not observed in the cultivation broth after 72 h of cultivation.

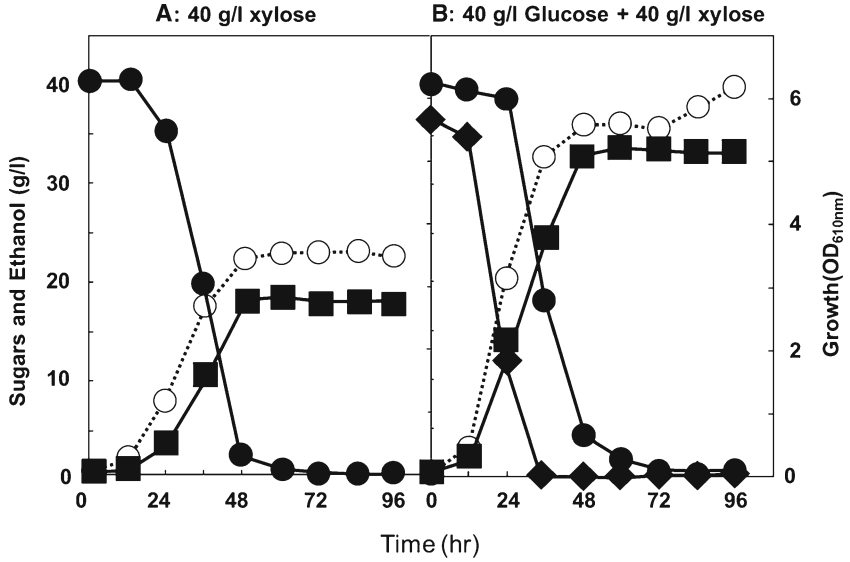

Fig. 5 Cell growth and fermentation of xylose or a mixture of glucose and xylose in *Z. mobilis* carrying pZA22-xtR. The recombinant strain was cultured statically in RM medium containing 40 g/l xylose (**a**) or a mixture of 40 g/l glucose and 40 g/l xylose (**b**) at 30 °C. *Open circles* indicate the growth of the strain, *closed circles* the residual xylose, *closed diamonds* the residual glucose, and *closed squares* the accumulation of ethanol

Fermentation of acid hydrolysate of wood biomass by the recombinant *Z. mobilis*

We next examined ethanol production by *Z. mobilis* [*sucZE2::manA*, pZA22-xtR] from actual acid hydrolysate of wood biomass from coniferous trees. After pre-culturing the recombinant strain for 48 h in RM medium supplemented with 20 g/l xylose, 100 ml of the pre-cultured broth was inoculated into 900 ml of 1:1 or 9:1 acid hydrolysate–RM medium in a 2-l jar fermentor without aeration. They were then cultured at 30 °C with gentle stirring at 50 rpm as shown in Fig. 7. In the case of 1:1 acid hydrolysate–RM medium, 28.6 g/l glucose was consumed perfectly within 24 h of cultivation. Thereafter, the 10.9 g/l mannose and 4.8 g/l xylose gradually disappeared from the culture medium over 72 h of cultivation. During fermentation, 18.9 g/l ethanol or 90.1 % of the theoretical yield was produced (Fig.7a). Furthermore, when 9:1 acid hydrolysate was used, 58.8 g/l glucose was consumed perfectly within 36 h; however, there was a 12-h delay in glucose consumption that was not seen with the 1:1 acid hydrolysate–RM medium (Fig. 7b). Utilization of the 19.2 g/l mannose and 10.8 g/l

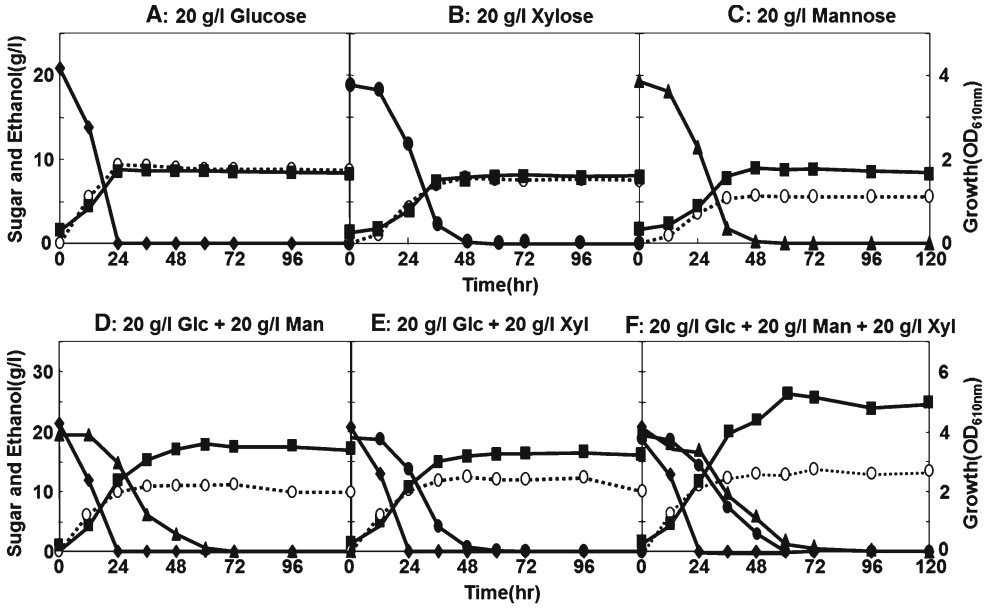

Fig. 6 Fermentation profiles for glucose, xylose, mannose, and their indicated mixtures in *Z. mobilis* harboring mannose and xylose catabolic enzyme genes. The recombinant strain was cultured statically in RM medium containing 20 g/l glucose (**a**), 20 g/l xylose (**b**), 20 g/l mannose (**c**), a mixture of 20 g/l glucose and 20 g/l mannose (**d**), a mixture of 20 g/l glucose and 20 g/l xylose (**e**), or a mixture of 20 g/l glucose, 20 g/l mannose, and 20 g/l xylose (**f**) at 30 °C. *Open circles* indicate the growth of the strain, *closed diamonds* the residual glucose, *closed open circles* the residual xylose, *closed triangles* the residual mannose, and *closed squares* the accumulation of ethanol

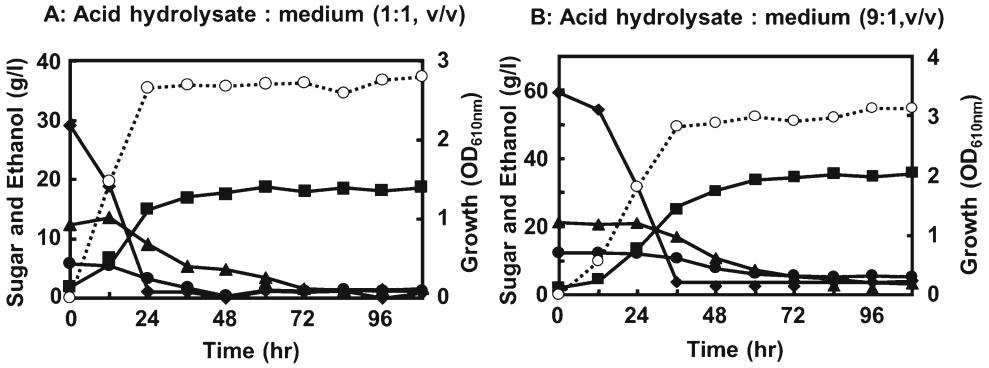

Fig. 7 Batch fermentation of acid hydrolysates of wood biomass by *Z. mobilis* [*sucZE2::manA*, pZA22-xtR]. One part acid hydrolysate of wood biomass was mixed with one part 2× RM medium without a carbon source (**a**) or nine parts acid hydrolysate was mixed with one part 10× RM medium without a carbon source (**b**). Both acid-hydrolyzed media were adjusted to a pH of 6.0 with NaOH and were cultivated statically at 30 °C. *Open circles* indicate the growth of the strain, *closed diamonds* the residual glucose, *closed circles* the residual xylose, *closed triangles* the residual mannose, and *closed squares* the accumulation of ethanol

xylose present in the medium followed the glucose consumption. Almost all of the glucose, mannose, and xylose contained in the acid hydrolysate of wood biomass was consumed to produce 35.1 g/l ethanol or 94.3 % of the theoretical yield.

Finally, on the basis of the results summarized above, we assessed continuous fermentation using 9:1 acid hydrolysate–RM medium in a reactor packed with immobilized *Z. mobilis* [*sucZE2::manA*, pZA22-xtR] cells at a dilution rate of 0.25 h^{-1}. After 48 h of continuous feeding with 9:1 acid hydrolysate–RM medium, we began analyzing the residual sugars and accumulated ethanol in the fermentation broth that overflowed from the top of the reactor every 12 h (Fig. 8). During fermentation, the residual sugars of glucose, mannose, and xylose were each less than 1 g/l in the overflowing broth. Ethanol productivity and yield (based on total sugars in 9:1 acid hydrolysate–RM medium) at 30 °C reached 10.27 g/l h with an average ethanol concentration of 37.3 g/l or 89.0 % of the theoretical yield. This continuous fermentation exhibited stable ethanol production throughout a 10-day test, indicating that the immobilized *Z. mobilis* [*sucZE2::manA*, pZA22-xtR] cells are suitable for the bioconversion of a saccharified material from wood biomass.

Discussion

Numerous studies have been published on cellulosic bioethanol production from herbaceous biomass such as corncob, cornstalks, wheat straw, and sugarcane bagasse; this is not the case with ethanol production from wood biomass such as broadleaf and coniferous trees (Mohagheghi et al. 2004; Margeot et al. 2009; Li et al. 2010). The major sugar components of herbaceous biomass are glucose, xylose, and arabinose; those of wood biomass are glucose, xylose, and mannose. Because the sugar composition of lignocellulosic biomass varies among plants, it is necessary to engineer ethanologenic microorganisms according to the target hydrolysate. In the present study, generation of an ethanologenic bacterium able to produce ethanol from hydrolysates of wood biomass entailed introduction of both xylose and mannose catabolic enzyme genes and their efficient expression in *Z. mobilis*. The genetically engineered *Z. mobilis* was able to ferment the mixture of glucose, xylose, and mannose contained in hydrolysate from the coniferous Japanese cedar

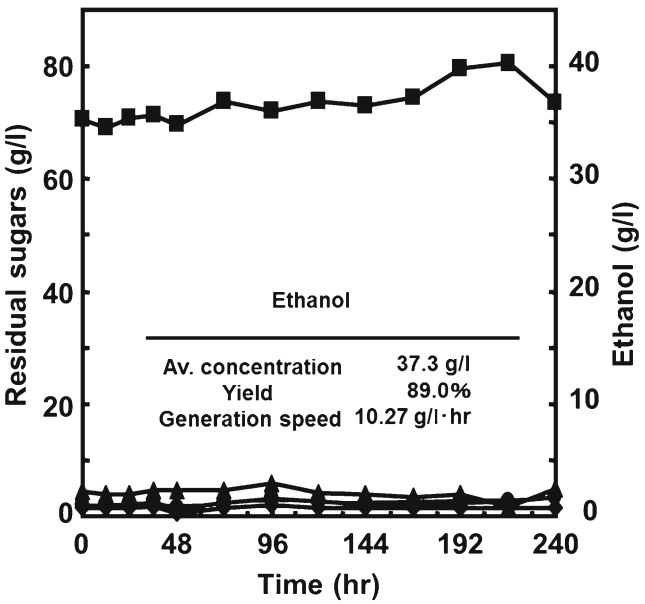

Fig. 8 Time courses of 25-ml-scale continuous fermentation using 9:1 acid hydrolysate medium at 30 °C and pH 6.0 with a dilution rate of 0.25 h^{-1}. After 48 h of continuous feeding of 9:1 acid hydrolysate medium at a dilution rate of 0.01 h^{-1}, feeding of 9:1 acid hydrolysate medium at a dilution rate of 0.25 h^{-1} was started. *Closed squares* indicate the accumulation of ethanol, *closed diamonds* the residual glucose, *closed triangles* the residual mannose, and *closed circles* the residual xylose

to produce ethanol in amounts roughly equivalent to the theoretical yield.

The introduction and expression of *E. coli manA* in *Z. mobilis* CP4 and the presence of endogenous *frk* were previously described by Weisser et al. (1996). The goal of those investigators was to confer the ability to assimilate mannose on *Z. mobilis* as an alternative selection marker for the genetic engineering of the strain. D-Mannose is incorporated into *Z. mobilis* cells through the action of a uniporter (glucose facilitator protein: GLF), after which the incorporated mannose is phosphorylated to mannose-6-phosphate by an endogenous FK. Because the phosphorylated mannose was not exhausted by the cells, they introduced *E. coli manA*, which catalyzed the isomerization of mannose-6-phosphate to fructose-6-phosphate. However, they did not address the issue of mannose fermentation at all. To enable *Z. mobilis* to efficiently ferment mannose, we initially introduced *frk* and *manA* into the cells, which caused a three- to fourfold increase in the activities of FK and PMI over those seen in wild-type *Z. mobilis* (Table 1). This enabled the recombinant strain to ferment 20 g/l mannose perfectly within 24 to 48 h of cultivation, producing ethanol at nearly the theoretical yield (Fig. 3a). By contrast, with the strain harboring *manA* alone, perfect consumption of mannose required more than 48 h of cultivation, indicating that enhancing the expression of *frk* improved mannose fermentation (Fig. 3a). We next evaluated co-fermentation of a mixture of glucose and mannose by a strain harboring *manA* or both *manA* and *frk*. As shown in Fig. 3b, we found little or no difference in the rates of sugar consumption and ethanol production between the two strains, which suggests that the large fraction of cells growing on glucose compensated for the slow rate of mannose consumption by the strain carrying *manA* alone. Furthermore, *Z. mobilis* with integrated *manA* also fermented 20 g/l mannose perfectly, producing the theoretical yield of ethanol within 48 h (Fig. 4). Thus, we had obtained a *Z. mobilis* strain capable of stably co-fermenting a mixture of glucose and mannose.

Zhang et al. (1995) were the first to report the metabolic engineering of *Z. mobilis* to enable xylose fermentation. Since then, several groups have been involved in improving xylose fermentation by *Z. mobilis* (Kim et al 2000; Jeon et al. 2005; Agrawal et al. 2011). The basic strategy for xylose fermentation in bacterial cells entails introducing four genes encoding the xylose catabolic enzymes *xylA*, *xylB*, *tal*, and *tktA*. We also conferred the ability to ferment xylose on *Z. mobilis* by introducing *xylA/xylB* and *tal/tktA* under the control of the P*gap* promoter. The recombinant strain fermented perfectly 40 g/l xylose, producing nearly the theoretical yield of ethanol (Fig. 5a), while the strain exhibited diauxic fermentation of a mixture of 40 g/l glucose and 40 g/l xylose (Fig. 5b). This diauxic nature of fermentation likely reflects the fact that the affinity of xylose (Km=40 mM) for GLF is ten times lower than that of glucose (Km=4.1 mM) (Parker et al. 1995).

Although *Z. mobilis* harboring both xylose and mannose catabolic enzyme genes fermented perfectly a mixture of glucose, xylose, and mannose, producing 86 % of the theoretical yield of ethanol, the rates of fermentation showed first for glucose, then xylose, and finally mannose (Fig. 6d–f). Given the affinity of mannose for GLF (Km=8.4 mM) (Parker et al. 1995), this result is contrary to expectations. We therefore measured the enzyme activities in cell-free extracts prepared from cells grown in glucose, xylose, or mannose (data not shown). We found that PMI activity was markedly reduced in cells grown in glucose or xylose, which retarded mannose fermentation by the recombinant *Z. mobilis*. This suggests that the carbon source for growth can affect the expression of the genes encoding both the xylose and mannose catabolic enzymes. Moreover, cells grown in xylose exhibited higher enzyme activities, suggesting that the most suitable carbon source for pre-cultivation is xylose. At present, we are using qRT-PCR analysis to investigate the expression and stability of enzyme genes introduced into *Z. mobilis* cells.

We finally evaluated the performance of the recombinant *Z. mobilis* with actual hydrolysate prepared from wood biomass. Yamada et al. reported fermentation of a dilute acid hydrolysate (41.2 g/l glucose and 11.2 g/l xylose) prepared from Japanese Cedar by immobilized *Z. mobilis* 31821 carrying pZB5 (Yamada et al. 2002). The immobilized *Z. mobilis* could ferment both sugars perfectly to produce 20 g/l ethanol within 50 h. In addition, Jeon et al. (2010) reported a comparative evaluation of ethanol production from eight different hydrolysates of cellulosic raw materials by *Z. mobilis* CP4 carrying pZB5. The highest ethanol yields and productivities were obtained with wheat straw and bagasse hydrolysates; fermentation of wood hydrolysate, such as pine and eucalyptus, resulted in relatively low ethanol production; the yields from pine and eucalyptus hydrolysates were 25 and 71 %, respectively. Here we report high-performance cellulosic bioethanol production from wood hydrolysate by *Z. mobilis*. *Z. mobilis* [*sucZE2::manA*, pZA22-xt] fermented 58.8 g/l glucose, 19.2 g/l mannose, and 10.8 g/l xylose in acid hydrolysate from coniferous Japanese cedar perfectly to produce 35.1 g/l ethanol or 94.3 % of the theoretical yield within 72 h (Fig. 7b). Furthermore, using a tower-type reactor, continuous fermentation with high ethanol productivity (10.27 g/l h) without residual sugars was achieved at a dilution rate of 0.25 h^{-1} (Fig. 8). To our knowledge, this is the first report on the performance of bioethanol production from wood hydrolysate.

Z. mobilis [*sucZE2::manA*, pZA22-xt] thus appears to be favorable for the production of ethanol from wood biomass of coniferous trees. To enable stable co-fermentation of the major sugar components of wood biomass in a long period, attempts to integrate both xylose and mannose catabolic enzyme genes into the *Z. mobilis* genomic DNA are currently in progress.

Acknowledgments This work was financed by the New Energy and Industrial Technology Development Organization (NEDO), Tokyo, Japan.

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