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Ethanol production from cellulosic materials by genetically engineered *Zymomonas mobilis*

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Abstract

To confer the ability to ferment cello-oligosaccharides on the ethanol-producing bacterium, *Zymomonas mobilis*, the β-glucosidase gene from *Ruminococcus albus,* tagged at its *N*-terminal with the 53-amino acid Tat signal peptide from the periplasmic enzyme glucose–fructose oxidoreductase from *Z. mobilis,* was introduced into the strain. The tag enabled 61% of the β-glucosidase activity to be transported through the cytoplasmic membrane of the recombinant strain which then produced 0.49 g ethanol/g cellobiose.

Introduction

Ethanol-producing bacteria have attracted much attention because their growth rate is higher than that of the *Saccharomyces cerevisiae* presently used for commercial production of fuel alcohol and, with recent advances in biotechnology, are expected to make industrial ethanol production more economical. Among such ethanol-producing bacteria is *Zymomonas mobilis*, which has been used historically in tropical areas to make alcoholic beverages from plant sap (Swings & De Ley 1977). *Z. mobilis* is noteworthy for its high growth rate and high specific ethanol production, which would seem to make it an attractive candidate for industrial ethanol production, but its narrow spectrum of fermentable carbohydrates has limited its use, especially for fuel ethanol production from varied sources (Skotnicki *et al.* 1982). To overcome this limitation by using genetic manipulation, we previously developed a unique host-vector system and used it to expand the range of carbohydrate substrates utilized by *Z. mobilis* to include raffinose, melibiose, lactose and galactose (Yanase *et al.* 1988, 1990, 1991). Instead of using feedstocks such as starch crops, however, cellulosic biomass (cellulose and hemicellulose), as present in agricultural and forestry residues, waste paper and industrial wastes, could be used as an ideally inexpensive and abundantly available source of sugar for sustainable fermentation into transportation fuel.

A number of groups have taken on the challenge of breeding alcohol-producing microorganisms, including *S. cerevisiae*, *Z. mobilis*, *Escherichia coli*, *Klebsiella oxytoca* and *Erwinia harbicola* (Su *et al.* 1989, Dien *et al.* 2003, Fujita *et al.* 2004) but the production of ethanol from cellulosic materials by genetically engineered strains has not yet reached a level sufficient for commercial application. For this reason, we have focused on the efficient ethanol production of *Z. mobilis*. As the first step towards genetically engineering a strain that can produce ethanol directly from cellulosic materials, we have endeavoured to transfer the ability to ferment cellobiose to the strain by introducing a bacterial β-glucosidase gene.

Materials and methods

Bacterial strains, plasmids, and cultural conditions

E. coli JM 109 [*recA, supE, endA, hsdR, gyrA, relA, thi, (lac-proAB), F'tra, proAB, laclq, lacZdM15*] was used as the host strain for recombinant DNA manipulations; *Z. mobilis* ATCC 29191 was used as the breeding host strain for fermentation of cellobiose; and *Ruminococcus albus* ATCC 27210 was used as the source of the β-glucosidase gene (Ohmiya *et al.* 1988). *E. coli* was grown in Luria–Bertani medium containing 100 μg ampicillin ml^{-1}, while *Z. mobilis* was cultivated statically at 30 °C in RM medium (20 g glucose l^{-1}, 10 g yeast extract l^{-1}, 2 g potassium phosphate l^{-1}, pH 6). The *E. coli* vector pKK223-3 and the *Z. mobilis*/*E. coli* shuttle vector pZA22 (6.7 kbp, tetracycline and chloramphenicol resistances) was used for gene transfer (Misawa *et al.* 1986). Transformation of *Z. mobilis* was accomplished using the spheroplast transformation method described previously (Yanase *et al.* 1986).

Construction of fused β-*glucosidase gene*

The β-glucosidase gene was amplified by PCR from chr. DNA of *R. albus* using the primer set BGLn, 5′-GCGGAATTCATCAAGGTGTGAT GTTGATTATACC-3′ (5′-prime) and BGLc, 5′-CGCCTTAAGTCATGTTTGACAGCTTATCA TCGAT-3′ (3′-primer) (Ohmiya *et al.* 1988). The amplified DNA fragment (3150 bp), which included the 2841 bp of the β-glucosidase gene and its endogenous promoter, was digested with *Eco*RI and inserted into the *Eco*RI site within the tetracycline resistance gene in pZA22, yielding pZA22-βg (Figure 1).

A fused gene encoding β-glucosidase tagged at its *N*-terminal with the 53-amino acid secretion signal peptide from glucose–fructose oxidoreductase (GFOR) was constructed by recombinant PCR (Kanagasundaram & Scopes 1988). Initially, a DNA fragment containing the promoter region and GFOR signal sequence was amplified from *Z. mobilis* chr. DNA using primers GFORp-Bam, CGCGGATCCCAGAAATAATTATCTGACAG CGCTT (5′-prime), and GFBGnl, GATATTCG TTCCAATCAAGCTTTATCGCTGCCTGAAG ACCACTGGCTAAG (3′-prime), while the β-glucosidase gene, truncated at the initial codon, with the flanking region of *gfor* were amplified from pZA22-βg using primers GFBGnr, CTTA GCCAGTGGTCTTCAGGCAGCGATAAAGC TTGATTGGAACGAATATC (5′-prime), and BGLc-Sal, CGCGTCGACTCATTTTTGACAG

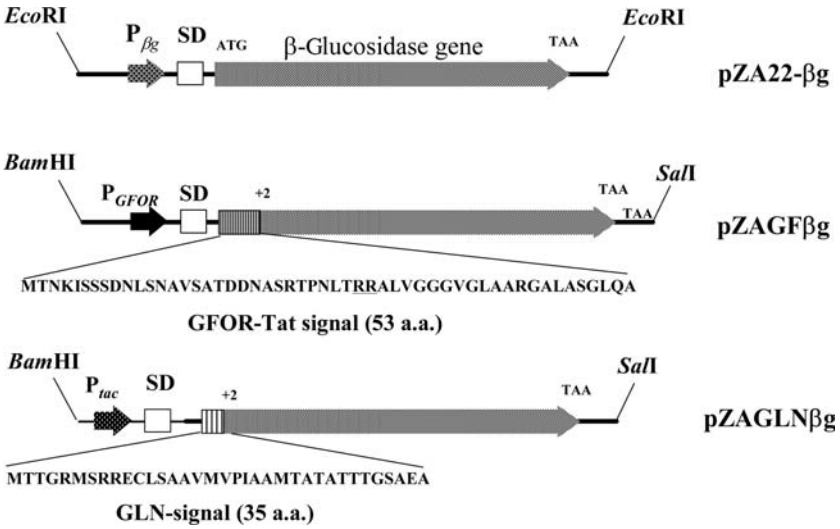

Fig. 1. Construction of pZA22-βg, pZAGFβg and pZAGLNβg. The β-glucosidase gene was amplified by PCR from chr. DNA of *R. albus* and then inserted into the *Eco*RI site of pZA22, yielding pZA22-βg. A fused β-glucosidase gene tagged at its *N*-terminal with the Tat secretion signal peptide from the GFOR gene was amplified by recombinant PCR and then inserted into the *Bam*HI and *Sal*I sites of pZA22, yielding pZAGFβg. A fused β-glucosidase gene tagged at its *N*-terminal with the secretion signal peptide from the GLN gene inserted into pKK223-3 was excised along with the *tac* promoter and SD and then inserted into the *Bam*HI and *Sal*I sites of pZA22, yielding pZAGLNβg.

CTTATCATCGAT (3′-prime). The two DNA fragments were then mixed, heated at 94 °C for 20 min, and incubated at 37 °C for 45 min to form a heteroduplex. Thereafter, the fused β-glucosidase gene was amplified from the heteroduplex using primers GFORp-Bam and BGLc-Sal. The 3403-bp fused β-glucosidase gene was then digested with *Bam*HI and *Sal*I and inserted into the tetracycline-resistance gene in pZA22, yielding pZAGFβg (Figure 1).

Similarly constructed was another fused gene encoding β-glucosidase tagged at its *N*-terminal with the 35-amino acid secretion signal peptide from gluconolactonase (GLN) (Kanagasundaram & Scopes 1986), which was then inserted into the tetracycline-resistance gene in pZA22, yielding pZAGLNβg (Figure 1).

Cellular fractionation

To determine the distribution of the expressed β-glucosidase in *Z. mobilis* cells, various cellular fractions, including the membrane, cytoplasmic and periplasmic fractions, were prepared using conventional methods previously used for *E. coli* (Choma & Yamazaki 1981).

Enzyme assay

β-Glucosidase activity was assayed using β-PNPG (*p*-nitrophenyl-β-D-glucopyranoside) as a substrate. The reaction was carried out in a mixture containing 100 μl 20 mM PNPG [in 100 mM potassium phosphate buffer (pH 7)], 900 μl 100 mM potassium phosphate buffer (pH 7), and 100 μl enzyme solution at 37 °C. The reaction was stopped by adding 2 ml 1% sodium carbonate. One unit of the enzyme activity is the amount of enzyme that liberates 1 μmol *p*-nitrophenol per min, which was calculated as $\varepsilon_{400\ nm} = 16 \times 10^3$. Alkaline phosphatase served as a marker enzyme for the periplasm of *E. coli*.

Analysis

Cellobiose and glucose levels in the culture broth were determined by HPLC using a Shodex Ionpak KS801 (4.6 × 300 mm) at 75 °C and a refractive index detector. The mobile phase was degassed water at 0.7 ml min^{-1}. An Aminex 8 column was used to assay metabolites such as lactate and acetate; 0.1 M H_2SO_4 served as the mobile phase at 40 °C. Ethanol was assayed using GC with a glass column (0.26 × 200 cm) filled with Porapak Type QS (80–100 mesh, waters, Milford, MA) at 180 °C and a FID detector. N_2 was the carrier gas (40 ml min^{-1}).

Results and discussion

Introduction of the β-*glucosidase gene from* R. albus

We selected the β-glucosidase gene from the rumen bacterium, *R. albus,* to introduce into *Z. mobilis* because its broad substrate range includes various cellooligosaccharides, as well as cellobiose (Ohmiya *et al.* 1985). Following transformation with pZA22-βg, *Z. mobilis* expressed β-glucosidase at a level comparable to that seen in *E. coli* (Table 1). On the other hand, because β-glucosidase could not be transported through the cytoplasmic membrane, 92% of its activity was located in the cytoplasm.

To secrete β-glucosidase into the periplasm, the enzyme was tagged with the secretion signal peptide of GFOR or GLN, two *Z. mobilis* enzymes that are translocated through the cytoplasmic membrane, so that the mature enzymes reside in the periplasm (Kanagasundaram & Scopes 1986, Wiegert *et al.* 1992). Because GFOR, which contains a tightly bound cofactor, NADP(H), catalyzes the dismutation reaction between glucose and fructose, the export system was classified into the Tat (twin arginine translocation)-dependent pathway. The secretion signal peptide (53 amino acid residues) is substantially longer than Sec signal peptides and contains an S/T-R-R-X-F-L-K consensus motif. On the other hand, GLN, which catalyzes the hydrolysis of gluconolactone to gluconate and is secreted via the Sec system, contains a typical 35-amino acid secretion signal.

Expression and cellular localization of fused β-*glucosidase in* Z. mobilis

β-Glucosidase tagged with the secretion signal from GFOR was efficiently expressed and then transported through the cytoplasmic membrane (Table 1). About 36% of the total activity was

Table 1. Localization of fused β-glucosidase expressed in *Z. mobilis*.

Cellular fraction	In *Z. mobilis*							In *E. coli* JM109			
	pZA22	pZA22-βg		pZAGFβg		pZAGLNβg		pZA22-βg			
	β-Glc	β-Glc		β-Glc		β-Glc		β-Glc		AP	
	U ml^{-1}	U ml^{-1}	(%)	U ml^{-1}	(%)	U ml^{-1}	(%)	U ml^{-1}	(%)	U ml^{-1}	(%)
Culture broth	0	< 0.1	< 0.1	1.1	4.7	1.5	11.2	0.1	0.5	0.4	1.8
Washing	0	1.2	9.4	8.4	35.7	1.2	9.0	< 0.1	< 0.1	0.1	0.5
Hypertonic	0	< 0.1	< 0.1	0.2	0.9	0.1	0.7	0.1	0.5	0.4	1.8
Osmotic shocked	0	0.3	2.4	4.7	20	1.4	10.4	1.7	8.7	18.2	82.4
Membrane fraction	0	0.4	3.1	1.1	4.7	1.9	14.2	1.1	5.6	1.1	5
Cytoplasm	0	10.8	85	8	34	7.3	54.5	16.6	84.7	1.9	8.6
Total activity	0	12.7		23.5		13.4		19.6		22.1	

One unit of β-glucosidase activity (β-Glc): 1 μmol *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucoside min^{-1}.
One unit of alkaline phosphatase activity (AP): 1 μmol *p*-nitrophenol released from *p*-nitrophenyl phosphate min^{-1}.

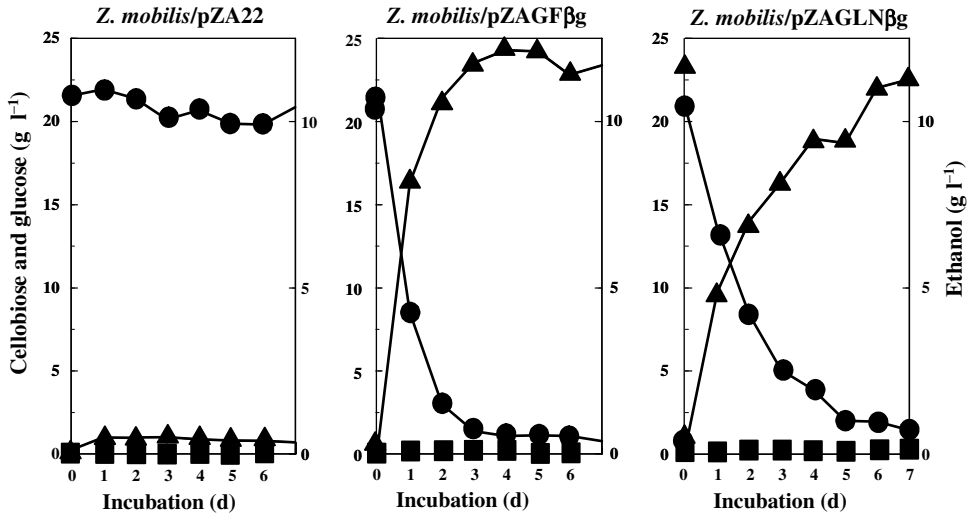

Fig. 2. Fermentation of cellobiose by the recombinant *Z. mobilis*. *Z. mobilis* carrying the indicated plasmids was cultured in 10% glucose for 24 h. The cells were then harvested, resuspended in RM medium containing 2% cellobiose, and incubated at 30 °C. Cellobiose, ethanol and its metabolites were measured by HPLC and GC, respectively. Residual cellobiose and glucose accumulated are indicated by the filled circles and by the filled squares, respectively; ethanol formed is indicated by the filled triangles.

localized in the cell-surface fraction, and 20% of the activity was in the periplasmic fraction. Moreover, when the strain was subjected to SDS-PAGE, a clear band corresponding to the fused β-glucosidase was observed in samples from the outer cytoplasmic fractions (data not shown), confirming that *R. albus* β-glucosidase could be translocated through the cytoplasmic membrane with the aid of the Tat secretion signal peptide from *Z. mobilis* GFOR.

In the case of the fused enzyme tagged with the signal peptide from GLN, 11% of the total activity was exported to the extracellular fraction, 9% to the cell surface and 10% to the periplasm.

Fermentation of cellobiose by recombinant Z. mobilis

After preculturing the recombinant *Z. mobilis* in RM medium containing 10% glucose for 48 h, the cells were washed, suspended to give an $OD_{610\ nm}$ of 2 (about 10^7 cells ml^{-1}) in RM medium supplemented with 20 g l^{-1} cellobiose, and cultivated anaerobically. As shown in Figure 2, whereas

Z. mobilis carrying pZA22 was unable to ferment cellobiose, the strain carrying pZAGFβg was able to utilize cellobiose to produce ethanol. After 2 d cultivation, the cellobiose in the culture medium was exhausted, and the amount of ethanol accumulated reached 10.7 g l^{-1}, which is more than 95% of the theoretical yield of ethanol from 22 g cellobiose l^{-1}. During the fermentation, none of the glucose liberated from cellobiose by β-glucosidase accumulated in the culture medium, suggesting that the initial hydrolysis by β-glucosidase was the rate-limiting step. Succinate, lactate and acetate, which are the assumed by-products, were also not detected in the culture medium during the fermentation.

Fermentation of cellobiose by the strain carrying pZAGLNβg was delayed, but the amount of ethanol accumulated reached the theoretical yield. Although Su *et al.* have reported the expression of *Xanthomonas albilineans* β-glucosidase in *Z. mobilis*, the ethanol productivity from cellobiose by the recombinant *Z. mobilis* was at a low level (Su *et al.* 1989). Thus, introduction of a fused β-glucosidase gene tagged with an endogenous secretion signal peptide also conferred the ability to ferment cellulosic materials to *Z. mobilis*.

Still, a more efficient secretion system will be essential for improving production of ethanol from cellobiose and cellooligosaccharides. In that regard, we have identified levansucrase and invertase on the cell-surface of *Z. mobilis*, and studies are now under way to develop a tool for displaying cellulases on the cell surface using the functional domains of these cell-surface enzymes.

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