Engineered *Saccharomyces cerevisiae* that produces 1,3-propanediol from d-glucose

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**Introduction**

1,3-Propanediol has been widely used in polymers, cosmetics, foods, lubricants and medicines, but its use is being restricted by its high cost. For example, one of the most successful applications for 1,3-propanediol has been in the synthesis of polytrimethylene terephthalate that is widely used in the manufacture of carpet and textile fibres (Biebl *et al.* 1999). As fine polymers, polytrimethylene terephthalate is rapidly extending its application in industry, but its production is restricted by the high cost and limited availability of 1,3-propanediol. 1,3-Propanediol has mainly been manufactured by high-cost chemical synthesis. There is an urgent need to develop a new and cost-efficient method to produce 1,3-propanediol.

Comparing with the chemical synthesis, fermentation method has the advantage of mass production at low cost, and has been used in the production of various industrial chemicals. The ideal fermentation production needs a safe micro-organism that can produce the desired chemicals from renewable resources. In nature, a few micro-organisms, including *Klebsiella* (Huang *et al.* 2002), *Citrobacters* (Boenigk *et al.* 1993) and *Clostridium* (Saint-amans *et al.* 2001) can produce 1,3-propanediol, but they all use an expensive resource glycerol. In *Klebsiella pneumoniae*, genes involved in the biosynthesis of 1,3-propanediol are *dhaB* and *dhaT* (Fig. 1b). DhaB is a B12-dependent dehydratase that converts glycerol into 3-hydroxypropionaldehyde, while DhaT is an NADH-dependent oxidoreductase that reduces 3-hydroxypropionaldehyde into 1,3-propanediol (Skraly *et al.* 1998). As *Escherichia coli* can produce glycerol from d-glucose, genes *dhaB* and *dhaT* had been cloned into *E. coli* to produce 1,3-propanediol from d-glucose, but the yield was low (Laffend *et al.* 1997). It turned out that the low yield of 1,3-propanediol was the result of the low activity of DhaT, which led to the accumulation...
and Gel Extraction kit were purchased from Promega Biotechnology Co. Ltd (Shiga, Japan). The Miniprep kit alkaline phosphatase were purchased from TaKaRa The PCR reagents, restriction enzymes and calf intestinal alkaline phosphatase. The ligation mixture was transformed into

Materials

Materials and methods

(a) *S. cerevisiae* W303-1A

D-glucose \(\rightarrow\) Glycerol

(b) *K. pneumoniae*

Glycerol \(\rightarrow\) 1,3-Propanediol

(c) *S. cerevisiae* W303-1A-ZR

D-glucose \(\rightarrow\) 1,3-Propanediol

**Figure 1** The proposed biosynthesis pathway of 1,3-propanediol in the engineered *Saccharomyces cerevisiae*. (a) Wild-type *S. cerevisiae* W303-1A can only synthesize glycerol from d-glucose. (b) *Klebsiella pneumoniae* can synthesize 1,3-propanediol only from glycerol. (c) The engineered *S. cerevisiae* W303-1A-ZR in this study can synthesize 1,3-propanediol from d-glucose. This might lead to a safe and cost-efficient method for industrial production of 1,3-propanediol.

of 3-hydroxypropionaldehyde in the cell. When the concentration of 3-hydroxypropionaldehyde reached 30 mmol l\(^{-1}\), the activity of DhaB was reduced by feedback inhibition (Barbirato *et al.* 1996). In 2002, Emptage *et al.* reported that gene *yqhD*, an alcohol dehydrogenase in *E. coli*, has high DhaT activity. By overexpressing *dhaB* and *yqhD* in *E. coli*, the high activity of *YqhD* in the production of 1,3-propanediol has been confirmed (Zhang *et al.* 2005; Wang *et al.* 2007).

*Saccharomyces cerevisiae* has been used in fermentation industry for many years, mainly because it uses the low-cost feedstock d-glucose as the carbon and energy source. Furthermore, *S. cerevisiae* is considered much safer than *E. coli* because *E. coli* produces endotoxins that can cause diseases. Could *S. cerevisiae* be used to produce 1,3-propanediol? This was the question that led to this study. Wild-type *S. cerevisiae* can only produce glycerol, but has no capacity to produce 1,3-propanediol (Fig. 1a). In this study, we have integrated genes *dhaB* and *yqhD* into the chromosome of *S. cerevisiae* by *Agrobacterium tumefaciens*-mediated transformation, and constructed a new strain W303-1A-ZR. The engineered *S. cerevisiae* W303-1A-ZR can directly produce 1,3-propanediol from d-glucose (Fig. 1c). This study might lead to a safe and cost-efficient method for industrial production of 1,3-propanediol.

Media and growth conditions

Four media were used in this study. Luria–Bertani (LB) medium was used to grow *E. coli* and *A. tumefaciens* cells; it consisted of 1% tryptone, 0.5% yeast extract and 1% sodium chloride. Yeast strains were grown on yeast-peptone-dextrose (YPD) medium (1% bacto yeast extract, 2% bacto peptone extract, 2% glucose). B medium was used for *A. tumefaciens*-mediated transformation; they are minimal media supplemented with 20 \(\mu\)g ml\(^{-1}\) uracil, 30 \(\mu\)g ml\(^{-1}\) lysine, 40 \(\mu\)g ml\(^{-1}\) tryptophan and 40 \(\mu\)g ml\(^{-1}\) adenine (Bundock *et al.* 1995). The co-cultivation media (CM) was also used for *A. tumefaciens*-mediated transformation; they consisted of uracil, lysine, tryptophan and adenine at the concentration mentioned earlier for B medium, in addition to 50 \(\mu\)g ml\(^{-1}\) kanamycin. *Escherichia coli* JM109 was cultured in LB media supplemented with 100 \(\mu\)g ml\(^{-1}\) ampicillin or 25 \(\mu\)g ml\(^{-1}\) zeocin. *Agrobacterium tumefaciens* LBA4404 was grown in LB media supplemented with appropriate antibiotics to maintain the plasmid. The fermentations were carried out by shaking at 250 rev min\(^{-1}\) and at 30°C under aerobic conditions. Table 1 describes the various bacterial strains and plasmids used in this study. Cell growth was monitored by using the optical density at 650 nm and converted to dry cell weight (DCW).

PCR amplification of DNA fragments

Unless otherwise stated, PCR amplification was carried out in a 50-\(\mu\)l reaction mixture containing 1 ng of template, 200 \(\mu\)mol l\(^{-1}\) dNTP, 20 \(\mu\)mol l\(^{-1}\) primers and 1 unit ExTaq DNA polymerase. The reaction was started at 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 90 s, annealing at 52°C for 120 s, and extension at 72°C for 240 s. After the 35th cycle, a 10-min extension at 72°C was used. The reaction product was separated on a 0.8% agarose gel. The desired band was excised and gel purified. The PCR reaction was performed using an automated thermocycler (Whatman Biometra, Gottingen, Germany).

Construction of plasmids pZR1 and pZR2 harbouring genes *yqhD* and *dhaB*, respectively

Plasmid pZR1 harbouring *yqhD* was constructed as follows: the *yqhD* gene was amplified from *E. coli* genome DNA by using primers P1 and P2 (Table 1), digested with EcoRI and ligated into vector pGAPZB that had been similarly digested and treated with calf intestinal alkaline phosphatase. The ligation mixture was transformed into
Construction of the plasmids pZR4 harbouring both yqhD and dhaB

Genes yqhD and dhaB were integrated into vector pCAMBIA3300-zeocin by the following steps. First, the plasmid pZR1 was digested with BglII and BamHI. The DNA fragment pGAP-yqhD-AOX1TT containing the glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter, yqhD and terminator AOX1TT was purified from the digestion and ligated into pCAMBIA3300-zeocin which was digested with BamHI and dephosphorylated by calf intestinal alkaline phosphatase (Fig. 2). The ligation mixture was transformed into E. coli JM109 to form JM109-ZR3. The plasmid pZR3 was purified from JM109-ZR3 and confirmed by PCR amplification of yqhD using the primers P1 and P2 (Fig. 3).

Next, the DNA fragment pGAP-dhaB-AOX1TT containing the promoter GAP, dhaB and terminator AOX1TT was amplified by using primers P5 and P6 (Table 1) from plasmid pZR2, digested with BglII, purified, and ligated into pZR3 which was digested with BamHI and EcoRI. Then the ligation mixture was transformed into E. coli JM109 to form JM109-ZR4. The plasmid pZR4 was purified from JM109-ZR4 and confirmed by digesting with XbaI (Figs 2 and 3).

Agrobacterium tumefaciens-mediated transformation

Agrobacterium tumefaciens is a Gram-negative soil bacterium that can transfer a piece of its Ti plasmid DNA
(T-DNA) into the chromosome of plant cells or S. cerevisiae cells (Bundock et al. 1995; Piers et al. 1996; Sugui et al. 2005). In this study, genes yqhD and dhaB were integrated into the chromosome of S. cerevisiae by A. tumefaciens-mediated transformation.

First, plasmid pZR4 was transformed into A. tumefaciens LBA4404 by electroporation (Mark et al. 1990) to form LBA4404-ZR4. Agrobacterium tumefaciens LBA4404-ZR4 was grown overnight in B media containing zeocin, harvested, resuspended in B media (with or without 100 μmol l⁻¹ acetosyringone) at a final concentration of 1·10¹¹ cells ml⁻¹, and incubated for additional 6 h.

Meanwhile, S. cerevisiae W303-1A was grown overnight at 30°C with shaking, was diluted 20-fold into fresh YPD media and grown for additional 6 h. The cells were harvested by centrifugation, washed with B liquid media without acetosyringone (Bundock et al. 1995), and resuspended in the same media at a final concentration of 1·10⁹ cells ml⁻¹.

Subsequently, 50 μl of S. cerevisiae W303-1A and 50 μl of A. tumefaciens LBA4404-ZR4 prepared earlier were mixed together, deposited onto an autoclaved cellophane paper (25-mm diameter) on top of the solid CM media supplemented with acetosyringone, and incubated at 28°C for 72 h. Then, the cellophane paper was moved onto the solid YPD media supplemented with 150 μg ml⁻¹ zeocin, incubated at 30°C for 3–4 days. To prevent the growth of the donor A. tumefaciens, 200 μg ml⁻¹ of cefotaxime was included in the media. Recombinant S. cerevisiae, designated W303-1A-ZR, was further purified at 30°C in YEPD media supplemented with 150 μg ml⁻¹ zeocin.

PCR and Southern blot analysis of yqhD and dhaB in the chromosome of Saccharomyces cerevisiae

Genomic DNA was isolated from the recombinant S. cerevisiae W303-1A-ZR (Ausubel et al. 1987). The
existence of \textit{yqhD} in the chromosome was confirmed by PCR amplification of \textit{yqhD} gene using primers P1 and P2 (Table 1). PCR amplification was started at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and elongation at 72°C for 2 min. The presence of \textit{dhaB} in the chromosome was confirmed by PCR amplification of \textit{dhaB} using primers P3 and P4. PCR amplification was started at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min and elongation at 72°C for 4 min.

For Southern blot analysis, the genomic DNA was digested with \textit{XbaI} and \textit{EcoRI} and separated on 0.8% agarose gels by field-inversion gel electrophoresis. DNA was denatured with alkali and washed with the washing buffer, and transferred to positively charged nylon membrane (Santangelo et al. 1995). Dig-High labelled probes were synthesized from the \textit{dhaB} fragment in pZR2 and \textit{yqhD} fragment in pZR1, respectively. Southern hybridization was carried out by using DIG-High prime DNA labelling and Detection starter kit according to manufacturer’s instructions.

Enzyme assays

Crude cell extracts were prepared by sonication of cell pastes and subsequent centrifugation. Cell pastes were obtained by centrifugation of fermentation broth at 6000 rev min$^{-1}$ for 10 min at 4°C. The pastes were washed in 20-mmol l$^{-1}$ Tris-HCl buffer (pH 8.0) or 50 mmol l$^{-1}$ potassium phosphate buffer (pH 8.0), centrifuged as described earlier, and resuspended in a small amount of the appropriate assay resuspension buffer. The cells were then disrupted by sonication for 5 min on ice at a duty cycle of 60% with 1-s cycles. Cell debris was removed by centrifugation at 12 000 rev min$^{-1}$ for 10 min at 4°C in a microcentrifuge. The glycerol dehydratase activity was estimated by using the 3-methyl-2-benzothiazolinon method (Ahrens et al. 1998) with a correction factor of 1.41. The activity of 1,3-propanediol oxidoreductase isoenzyme was determined using the reverse reaction (conversion of 1,3-propanediol to 3-hydroxypropionaldehyde) owing to the instability of the 3-hydroxypropionaldehyde. The concentration of 1,3-propanediol oxidoreductase isoenzyme was determined...
by the method of Gerlind et al. (2004). All enzyme assays were performed at 30°C. One unit of enzyme activity is defined as the amount of enzyme needed to catalyse the conversion of 1 μmol of substrate per min at 30°C. Specific enzyme activity is indicated as unit per mg protein. All assays were performed in duplicate; the reported values are the average from two assays. The protein concentration of the cell extracts was determined by the method of Bradford.

Determination of 1,3-propanediol by gas chromatography

1,3-Propanediol and glycerol were determined using a gas chromatograph (Shimazu GC-14B, FID-detector). The column used in the study was 2 m column packed with Chromosorb 101. N₂ gas was used as a carrier at the flow rate of 40 ml min⁻¹. The detector temperature was set at 220°C and column temperature was set at 210°C.

Results

Construction of key plasmids needed for engineering Saccharomyces cerevisiae

To produce 1,3-propanediol from D-glucose in S. cerevisiae, we planned to integrate the heterogeneous genes yqhD and dhaB into the chromosome of S. cerevisiae by A. tumefaciens-mediated transformation (Piers et al. 1996). To this end, we had to integrate the two genes yqhD and dhaB into the vector pCAMBIA3300-zeocin which can transfer its T-DNA to S. cerevisiae. First, the gene yqhD encoding for 1,3-propanediol oxidoreductase isoenzyme was isolated from E. coli, and the gene dhaB encoding for glycerol dehydratase from K. pneumoniae. Both genes were cloned into the vector pGAPZB between the promoter GAP and AOX1TT terminator to form plasmids pZR1 and pZR2, respectively (Fig. 2). The GAP promoter and AOX1TT terminator would help the expression of heterogeneous gene in S. cerevisiae. The plasmid pZR1 containing the gene yqhD was digested with EcoRI, and the DNA fragments were analysed as shown in lane 3 of Fig. 3a. There were only two fragments in the digestion: one had the same size as empty plasmid pGAPZB digested with EcoRI (Fig. 3a, lane 1) and the other had the same size as the gene yqhD (Fig. 3a, lane 2). Digesting pZR1 with BglII yielded three DNA fragments (Fig. 3a, lane 4), which are consistent with the map of pZR1 (Fig. 2). Plasmid pZR1 contains four Bg I sites that could divide the plasmid into three major DNA fragments of 1960, 1211 and 897 bp, respectively, and a small DNA fragment of size 32 bp.

The plasmid pZR2 containing the gene dhaB was digested with XbaI, and the DNA fragments were analysed as shown in Fig. 3b. The size of empty plasmid pGAPZB (Fig. 3b, lane 1) is very close to the size of the gene dhaB (Fig. 3b, lane 2). Digesting pZR2 with XbaI yielded two DNA fragments with similar sizes around 2800 bp (Fig. 3a, lane 3). Digesting pZR2 with BamHI yielded two major DNA fragments which are consistent with the map of pZR2 (Fig. 2). Plasmid pZR2 contains three BamHI sites that can yield three major DNA fragments with sizes of 3000, 2300 and 300 bp.

Next, the DNA fragment pGAP-yqhD-AOX1TT was digested from plasmid pZR1, purified, and ligated into pCAMBIA3300-zeocin to form pZR3. The plasmid pZR3 was digested with BamHI. As expected, the digested DNA fragment from pZR3 was bigger in size than the digested empty vector (Fig. 3c, lane 2 vs. lane 1). The integration of gene yqhD in the plasmid pZR3 was further confirmed by PCR amplification using primers P1 and P2 (Table 1). The PCR products had the same size as the gene yqhD (Fig. 3c, lane 3 vs. Fig. 3a, lane 2).

Finally, the DNA fragment pGAP-dhaB-AOX1TT was digested from plasmid pZR2, purified, and ligated into pZR3 to form pZR4. The plasmid pZR4 was digested with HindIII. As expected, the digested DNA fragment from pZR4 was bigger in size than the digested pZR3 (Fig. 3d, lane 2 vs. lane 1). The integration of both genes yqhD and dhaB in the plasmid pZR4 was further confirmed by digesting with XbaI. The digested pZR3 contained two DNA fragments, which is consistent with the two XbaI sites in pZR3 (Fig. 3d, lane 3), while the digested pZR4 contained four DNA fragments, consistent with the four XbaI sites in pZR4 (Fig. 3d, lane 4). Thus, the key plasmid pZR4 needed for engineering S. cerevisiae was constructed.

Integration of genes yqhD and dhaB into the chromosome of Saccharomyces cerevisiae

Plasmid pZR4 containing both genes yqhD and dhaB was transformed into A. tumefaciens LBA4404 to form LBA44404-ZR. Incubation of S. cerevisiae W303-1A cells with A. tumefaciens LBA44404-ZR led to the formation of zeocin-resistant colonies on media containing acetosyringone, indicating that the T-DNA of pZR4 in A. tumefaciens has transferred into the chromosome of S. cerevisiae W303-1A. The engineered S. cerevisiae strain was designated W303-1A-ZR. Stability of the zeocin-resistant phenotype of W303-1A-ZR was confirmed by growing the cells on fresh YPD media containing 150 μg ml⁻¹ zeocin (Fig. 4).

To confirm the presence of the yqhD and dhaB in the chromosome, gene yqhD was amplified from genomic
DNA isolated from W303-1A-ZR by using primers P1 and P2, and the gene dhaB was amplified using primers P3 and P4. Genomic DNA isolated from W303-1A was used as an empty control. As shown in Fig. 5a, genes yqhD and dhaB could be amplified from W303-1A-ZR (Fig. 5, lanes 4 and 6), but not from W303-1A (Fig. 5, lanes 3 and 5), indicating that both genes yqhD and dhaB were present in the chromosome of W303-1A-ZR, but not in W303-1A. The integration of both genes yqhD and dhaB in the chromosome of W303-1A-ZR was further confirmed by Southern blot analysis (Fig. 5b). Except for genes yqhD and dhaB, another DNA fragment with higher molecular weight could also be observed on the Southern blot, indicating that the T-DNA had integrated into the chromosome of S. cerevisiae.

The engineered *Saccharomyces cerevisiae* W303-1A-ZR can produce 1,3-propanediol from D-glucose

The engineered W303-1A-ZR grew normally as the wild-type strain W303-1A (Fig. 6). Both W303-1A and W303-1A-ZR were grown to log phase, and the cell extracts were prepared. The activity of glycerol dehydratase and 1,3-propanediol oxidoreductase isoenzyme in the cell extracts were measured by the standard assay conditions. Both specific activities were detected in the cell extracts of *S. cerevisiae* W303-1A-ZR, but not in *S. cerevisiae* W303-1A (Table 2). This indicates that genes dhaB and yqhD were expressed in W303-1A-ZR.

To test whether the engineered *S. cerevisiae* W303-1A-ZR could produce 1,3-propanediol from D-glucose, both W303-1A and W303-1A-ZR were grown aerobically, and the concentration of 1,3-propanediol was measured. As we expected, W303-1A-ZR could produce 1,3-propanediol, but wild-type W303-1A could not (Table 2). This suggests that genes dhaB and yqhD not only can be expressed, but also function in the engineered *S. cerevisiae* W303-1A-ZR.

To check the stability of genes dhaB and yqhD in the chromosome, the aforementioned assays were performed with samples taken from several continuous fermentations of W303-1A-ZR. All the assays showed similar specific activities of the dehydratase and the oxidoreductase (data not shown), suggesting the stable integration of both genes in the chromosome of W303-1A-ZR.
1,3-Propanediol production in S. cerevisiae

Dendrocalymna (D) and W303-1A-ZR showed that the engineered W303-1A-ZR grows normally.

Fermentation method has the advantage of mass production at low cost, but the few naturally available micro-organisms producing 1,3-propanediol could only use a high-cost feedstock glycerol for fermentation. There is an urgent desire to produce 1,3-propanediol from low-cost D-glucose in a single micro-organism (Nakamura and Whited 2003). By expressing K. pneumoniae dha regulon genes, Emptage et al. (2002) have patented a recombinant E. coli that can convert D-glucose to 1,3-propanediol with high titre.

In this study, we constructed a recombinant S. cerevisiae strain W303-1A-ZR that can convert D-glucose to 1,3-propanediol. We cloned genes yqhD and dhaB required for the production of 1,3-propanediol from glycerol, integrated them in the chromosome of S. cerevisiae W303-1A and demonstrated that the engineered S. cerevisiae could produce 1,3-propanediol from the low-cost feedstock D-glucose. This study also confirmed that A. tumefaciens genetic transfer system is a valuable tool in yeast biotechnology. To our knowledge, this is the first report on engineering S. cerevisiae for the production of 1,3-propanediol by using ATMT (Agrobacterium tumefaciens-mediated transformation) method.

The yield of 1,3-propanediol in our engineered S. cerevisiae was about 0.4 g l⁻¹. This is reasonable because the wild-type S. cerevisiae W303-1A can only produce 5–6 g glycerol in 1 l of YPD media containing 10% D-glucose. The importance of this work is that it opened a new way to produce 1,3-propanediol from low-cost feedstock. To further increase the yield of 1,3-propanediol in S. cerevisiae, we can either modify the surrounding genes in the glycerol pathway in S. cerevisiae W303-1A-ZR to produce more glycerol, or integrate genes yqhD and dhaB into some yeast strains that can produce high levels of glycerol. For example, we can delete the genes dhaK encoding glycerol kinase and dhaD encoding glycerol dehydrogenase in S. cerevisiae W303-1A-ZR to prevent glycerol from re-entering central carbon metabolism. We can integrate genes yqhD and dhaB in Candida glycerinogenes strain WL2002-5 that can produce 120 g glycerol in 1 l media containing 25% D-glucose (Zhuge et al. 2001). These studies might lead to a safe and cost-efficient method for industrial production of 1,3-propanediol in S. cerevisiae.

Discussion

Metabolic engineering is an emerging technique that genetically modifies or designs biochemical pathways in micro-organisms, and has a potential importance in fermentation industry. In this study, we used metabolic engineering to construct S. cerevisiae that can produce 1,3-propanediol. 1,3-Propanediol is a very important chemical that could improve the quality of polymers, food and medicines. However, because it has been synthesized chemically by expensive enzymes at high pressure and high temperature, 1,3-propanediol is expensive. Fermentation method has the advantage of mass production at low cost, but the few naturally available micro-organisms producing 1,3-propanediol could only use a

Table 2 Expression of glycerol dehydratase (DhaB) and 1,3-propanediol oxidoreductase isoenzyme (YqhD) and production of 1,3-propanediol in engineered Saccharomyces cerevisiae W303-1A-ZR. The different strains were all cultivated at 30°C for 72 h in media containing 100 g l⁻¹ D-glucose

<table>
<thead>
<tr>
<th>Strains</th>
<th>DhaB (U mg⁻¹ protein)</th>
<th>YqhD (U mg⁻¹ protein)</th>
<th>1,3-Propanediol (g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W303-1A-zeocin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W303-1A-yqhD</td>
<td>2.9 ± 0.2</td>
<td>3.0 ± 0.3</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td>W303-1A-ZR</td>
<td>4.1 ± 0.2</td>
<td>3.0 ± 0.3</td>
<td>0.4 ± 0.05</td>
</tr>
</tbody>
</table>

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