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Construction of a *Candida utilis* strain with ratio-optimized expression of xylose-metabolizing enzyme genes by cocktail multicopy integration method

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We previously reported the construction of a recombinant *Candida utilis* strain expressing *mXYL1*, *XYL2* and *XYL3*, which encode mutated *Candida shehatae* xylose reductase K275R/N277D, *C. shehatae* xylitol dehydrogenase and *Pichia stipitis* xylulokinase to produce ethanol from xylose. However, its productivity was low. In this study, to breed a strain with higher productivity of ethanol from xylose, we used a cocktail multicopy integration method to attain optimized gene dosage of the three enzymes. Gene expression cassettes of the xylose-metabolizing enzymes were simultaneously integrated into *C. utilis* chromosomes in one step. Measurement of integrated gene copy number and xylose fermentability in all of the resulting integrant strains revealed that the copy number ratio of *XYL2*/*mXYL1* in strains with higher ethanol yield was higher than that in strains with lower ethanol yield, whereas the copy number ratio of *mXYL1*/*XYL3* was lower in strains with higher ethanol yield. The resultant strain CIS35, which was found to be the best producer of ethanol from xylose produced 29.2 g/L of ethanol, yielding 0.402 g ethanol/g xylose. This result provides that *C. utilis* may be a good candidate as a host for ethanol production from xylose.

[**Key words:** *Candida utilis*; Xylose; Ethanol fermentation; Cocktail multicopy integration; Xylose reductase; Xylitol dehydrogenase; Xylulokinase]

Lignocellulosic plant material is an abundant source of renewable biomass, which can be fermented to ethanol (1). However, the vast majority of fermentative yeasts, including *Saccharomyces cerevisiae*, are unable to grow on xylose as a sole carbon source, although some species of *Pichia*, *Candida* and *Pachysolen* can produce ethanol from xylose (2). Xylose-assimilating yeasts such as *Pichia stipitis* and *Candida shehatae* convert xylose to xylulose by a two-step redox reaction carried out by the NADPH-preferring xylose reductase (XR) (3), followed by the NAD^+-dependent xylitol dehydrogenase (XDH) (4). Xylulose is phosphorylated to xylulose 5-phosphate by a xylulokinase (XK) (5), and then further metabolized through the pentose-phosphate pathway.

The introduction of *XYL1* and *XYL2*, encoding XR and XDH from *P. stipitis*, facilitated xylose assimilation in *S. cerevisiae* (6). Additional introduction of *XYL3*, encoding *P. stipitis* XK, or overexpression of *XKS1*, encoding *S. cerevisiae* XK, significantly improved the rate and yield of xylose fermentation (7,8). However, the ethanol yield of these first-generation xylose-fermenting strains was much lower than the theoretical maximum yield of 0.51 g ethanol/g xylose, because a substantial amount of xylitol was produced from xylose.

A number of studies have focused on improving xylose-utilizing recombinant *S. cerevisiae* strains. One area for improvement is the redox imbalance caused by NADPH-preferring XR and NAD^+-dependent XDH. To overcome this imbalance, overexpression and disruption of endogenous genes involved in NADPH production and NADH reoxidation has been reported, as well as the introduction of XR and XDH enzymes with modified coenzyme specificity (9–14). In addition, overexpression of genes involved in the pentose-phosphate pathway and introduction of a heterologous xylose transporter improved xylose fermentability in an engineered *S. cerevisiae* strain (15–17).

As an alternative and simple strategy, optimization of *XYL1*, *XYL2* and *XYL3* expression levels has been reported to improve xylose assimilation in *S. cerevisiae* (18–21), whereby different types of expression vectors, such as YEp, YCp and YIp, or promoter engineering are used to control xylose-metabolizing enzyme expression levels. However, it is a lengthy process to obtain a recombinant strain with optimal expression levels by these methods.

Candida utilis, *C. shehatae* and *P. stipitis* belong to the Crabtree-negative group of yeasts. *C. utilis* is an industrially important microorganism, being used to produce several valuable materials including glutathione and RNA (22–24). Since the development of an efficient electroporation-based transformation method of *C. utilis* (25,26), this yeast has been used for the heterologous production of various biomaterials including monellin (27), α-amylase (28), L-lactic acid (29,30) and carotenoids such as lycopene (31,32). Unlike *S. cerevisiae*, *C. utilis* can grow vigorously under the strict aerobic condition, and high cell density fermentation under the anaerobic condition contributes to efficient production. Moreover, *C. utilis* has the highest respiration activity among Crabtree-negative yeasts (33), which is advantageous for the efficient production of biomaterials.

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Wild-type *C. utilis* can grow on xylose, but cannot ferment it into ethanol (34). Recently, we reported that a recombinant *C. utilis* strain, in which one copy of each of three genes encoding NADH-preferring mutated *C. shehatae* XR (K275R/N277D), native *C. shehatae* XDH and *P. stipitis* XK was integrated into the chromosome, could produce ethanol from xylose (35). However, the efficiency was too low to improve the rate of ethanol production or the yield.

Yamada *et al.* (36) reported the construction of an *S. cerevisiae* strain with optimized expression of three cellulolytic enzymes for the degradation of phosphoric acid swollen cellulose using the cocktail δ-integration method, which is a type of multicopy integration. By cocktail δ-integration, several kinds of gene expression cassettes were integrated into chromosomes simultaneously, and strains with the targeted phenotype were easily obtained. To our knowledge, a cocktail integration method has not been used in the optimization of metabolic engineering. In this study, we applied this method to the improvement of xylose fermentation by *C. utilis*, and thereby obtained *C. utilis* strains with an optimized gene dosage of xylose-metabolizing enzymes.

MATERIALS AND METHODS

Strains, media and culture conditions *Escherichia coli* strain DH5α (Toyobo, Osaka, Japan) transformants were grown in Luria-Bertani medium (10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl) containing 100 mg/L of ampicillin. The *C. utilis* strains used in this study are listed in Table 1. Cells were cultivated at 30°C in YPD2 medium (10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of glucose) unless stated otherwise. Solid media were made with 2% agar. Hygromycin B (HygB, Wako Pure Chemical Industries, Osaka, Japan) and cycloheximide (Wako Pure Chemical Industries) were added to YPD2 to a final concentration of 600 mg/L and 40 mg/L, respectively, to select transformants carrying the appropriate selection markers. To screen for cocktail multicopy integrant transformants, 60 putative transformants were aerobically precultivated in YPD2 medium for 24 h at 30°C. We inoculated each into 3 ml of YPX8 medium (10 g/L of yeast extract, 20 g/L of peptone, and 80 g/L of xylose) in 15-ml sterile culture tubes and incubated them with shaking at 100 rpm for 50 h at 30°C. For fermentation experiments, the cells were aerobically precultivated in YPD2 medium for 24 h at 30°C. Cells were washed with sterile water and inoculated at starting OD_{600} nm of 1, corresponding to approximately 0.46 g dry cells/L, in fermentation medium. Fermentation was carried out at 30°C in 300-mL spherical flat-bottom flasks containing 100 mL of YPX8 with gentle shaking (80 rpm).

TABLE 1. Strains and plasmids of *C. utilis* used in this study.

Plasmids and strains	Relevant features	Reference or source
Strains		
C. utilis		
NBRC0988	Wild type	NBRC
CUD1F	Single *URA3* disruptant derived from NBRC0988	26
CUD2F	Double *URA3* disruptant derived from NBRC0988	26
CUD3F	Triple *URA3* disruptant derived from NBRC0988	26
CUD4F	Quarduple *URA3* disruptant derived from NBRC0988	26
TMS174	NBRC0988 *URA3*::*GAP*p-*mXYL1-PGK1*t − *GAP*p-*XYL2-PGK1*t − *GAP*p-*XYL3-PGK1*t	35
CIS18	NBRC0988 *URA3*::cocktail multicopy integration of *mXYL1*, *XYL2* and *XYL3* Clone No. 18	This study
CIS35	NBRC0988 *URA3*::cocktail multicopy integration of *mXYL1*, *XYL2* and *XYL3* Clone No. 35	This study
Plasmids		
pVT148	*GAP*p-*mXYL1-PGK1*t, Hyg^R cassette	35
pVT150	*GAP*p-*XYL2-PGK1*t, Hyg^R cassette	35
pVT107	*GAP*p-*XYL3-PGK1*t, Hyg^R cassette	35
pCU155	*C. utilis* multicopy integration plasmid; *GAP*p-*GAP*t, Cyh^R marker, integration at *URA3* allele	27
pVT340	*GAP*p-*mXYL1-GAP*t, Cyh^R cassette	This study
pVT342	*GAP*p-*XYL2-GAP*t, Cyh^R cassette	This study
pVT344	*GAP*p-*XYL3-GAP*t, Cyh^R cassette	This study

Plasmid and strain construction Table 1 shows the genetic properties of all of the plasmids used in this study. The *mXYL1*, *XYL2* and *XYL3* genes encoding *C. shehatae* XR K275R/N277D, *C. shehatae* XDH and *P. stipitis* XK were isolated from pVT148, pVT150 and pVT107 by digestion with *Xba*I and *Bam*HI (35). pCU155 harboring the *C. utilis GAP* promoter, *C. utilis GAP* terminator and marker of cycloheximide resistance is a multicopy integration vector that targets the *URA3* loci (27). Each xylose-metabolizing enzyme gene was ligated into the *Xba*I-*Bam*HI site of pCU155 to construct pVT340, pVT342 and pVT344, respectively.

Transformation of *C. utilis* was carried out by electroporation as previously described (29). *Bgl*II-digested plasmids harboring the heterologous genes encoding the xylose-metabolizing enzymes were used to transform yeast cells. In order to construct a cocktail integrant strain, the three linearized plasmids (pVT340, pVT342 and pVT344) were mixed at a DNA molar ratio of 1:1:1 and used to transform the wild-type NBRC0988 cells. The transformed cells were grown on YPD2 plates containing cycloheximide.

Analytical methods Xylose, xylitol, acetate, glycerol and ethanol were analyzed essentially as described previously (35). The supernatant was subjected to HPLC analysis using an ICSep-ION-300 column (Tokyo Chemical Industry, Tokyo). The column was operated at 60°C at a flow rate of 0.4 ml/min, with 0.01 N sulfuric acid as the solvent. The ethanol yield was determined by the ratio of ethanol concentration to xylose consumption (g ethanol produced/g xylose consumed).

Quantification of the copy number of the integrated genes The copy number of the integrated individual genes in the strains was quantified using real-time PCR. The primers used are listed in Table S1. Template genomic DNA of the yeast cells cultivated in YPD2 medium for 24 h at 30°C was isolated using a Qiagen Genomic tip 500/G (anion exchange column, Qiagen, Hilden, Germany) and Qiagen Genomic DNA Buffer Set (Qiagen). The following seven sets of PCR primers were used to detect the *C. utilis ACT1*, *GAP* promoter, *PGK1* terminator, *URA3*, *mXYL1*, *XYL2* and *XYL3*: ACT1f/ACT1r, GAPpf/GAPpr, PGK1tf/PGK1tr, URA3f/URA3r, XYL1f/XYL1r, XYL2f/XYL2r and XYL3f/XYL3r. Quantitative real-time PCR was performed using a LightCycler 480II instrument (Roche Diagnostics Corp., Indianapolis, IN, USA) with an SYBR Premix Ex Taq Perfect real-time kit (Takara Bio, Ohtsu, Japan). The normalized gene copy number was calculated by the standard curve method with the *ACT1* gene as the standard gene (36). The gene copy number was normalized on the basis that the copy number of each xylose-metabolizing enzyme gene in TMS174 was one.

Enzyme assays Yeast cells were grown to stationary phase at 30°C in YPD2 medium. Cells were harvested by centrifugation at 3000 ×*g* for 5 min. The cell pellet was washed and suspended in buffer (50 mM phosphate buffer, pH 7.0). The suspended cells were then mixed with glass beads (Sigma, St. Louis, MO, USA) and vortexed using Mix Tower A-14 (Taiyo Instruments, Tokyo) at 4°C for 15 min. Cell debris was removed by centrifugation at 13,000 ×*g* for 10 min. Xylose reductase, xylitol dehydrogenase, and xylulokinase activities were measured in the cell extracts as previously described (35). One unit of enzyme activity was defined as the amount of enzyme that reduces or oxidizes 1 μmol of NAD^+ or NADH per min. Protein concentration was assayed by the Bradford method, using bovine serum albumin as a standard. Specific activities were expressed as U/mg of protein.

Nucleotide sequence accession numbers The DNA sequences of *C. shehatae XYL1*, *C. shehatae XYL2*, *P. stipitis XYL3*, *C. utilis ACT1* and *URA3* are in the DDBJ/EMBL/GenBank nucleotide database with accession nos. DM015263, AF278715, AF127802, AB682755 and E11619, respectively.

RESULTS

Construction and selection of recombinant *C. utilis* strains by cocktail multicopy integration A cocktail multicopy integration method based on a δ-integrative plasmid has been reported in *S. cerevisiae* (36), although the δ sequence of *C. utilis* has not been found. It has been reported that up to 90 copies of the multicopy integrative expression vector pCU155 have been integrated in tandem at the *URA3* loci of *C. utilis*, and no notable decrease of the heterologous protein production was observed in the transformants with the plasmid after 50 generations of nonselective growth (27). The *C. utilis* NBRC0988 strain is proposed to be a tetraploid and has four *URA3* genes, and has a single copy of the *URA3* gene on each chromosome (26). Therefore, we speculated that pCU155 might be used as a cocktail multicopy integrative plasmid in *C. utilis*.

Expression cassettes of three xylose-metabolizing enzyme genes, *mXYL1*, *XYL2* and *XYL3*, were introduced into yeast chromosomes simultaneously. By transformation, we obtained 300–400 cycloheximide-resistant colonies. As a result, a pool of recombinant *C. utilis* transformants with various genes present in