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Integrative expression vectors for overexpression of xylitol dehydrogenase (*XYL2*) in Osmotolerant yeast, *Candida glycerinogenes* WL2002-5

Cheng Zhang · Hong Zong · Bin Zhuge · Xinyao Lu · Huiying Fang · Jian Zhuge

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Abstract Yeasts are excellent hosts for the production of recombinant proteins. *Candida glycerinogenes* WL2002-5, an osmotolerant yeast with extremely high glycerol productivity, provides an attractive eukaryotic expression platform. The integrative vectors PURGAP-*gfp* and PURGPD-*gfp* harbouring phleomycin-resistance coding sequence and GFP coding sequence with P*CgGAP*, P*CgGPD* promoter, respectively, were constructed. The recombinant plasmid PURPpGAP-*gfp* with the promoter P*PpGAP* based on the sequence of *Pichia pastoris* GAPDH gene and the plasmid PURScGAP-*gfp* with the promoter P*ScGAP* from *Saccharomyces cerevisiae* were constructed. After transformation, the copy number of *gfp* gene, which determined using fluorescent quantitative real-time polymerase chain reaction (FQ-RTPCR) in genome of *C. glycerinogenes* is 1. Expressions of *gfp* at different levels were conducted using different promoters by osmotic stress containing NaCl or glucose for the recombinant strains. In this study, *C. glycerinogenes* WL2002-5, expressing xylitol dehydrogenase (*XYL2*) gene from *Pichia stipitis*, has the ability to produce glycerol from xylose entered into pentose phosphate pathway. Two recombinant strains of PURGAPX, PURGPDX with *XYL2* overexpression were constructed to ferment a mixture of glucose and xylose simultaneously in batch fermentation. Compared to *C. glycerinogenes* WL2002-5 strain, glycerol production from xylose in strains PURGAPX, PURGPDX were increased by 95.9 and 121.1 %, respectively.

Keywords *Candida glycerinogenes* · Integrative vector · Phleomycin · Green fluorescent protein · Genetic transformation system · Xylitol dehydrogenase

C. Zhang · H. Zong · B. Zhuge (✉) · X. Lu · H. Fang · J. Zhuge
The Key Lab of Industrial Biotechnology of Ministry of Education, Research Centre of Industrial Microorganisms, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu 214122, People's Republic of China
e-mail: bzhuge@163.com

C. Zhang
e-mail: zc6704736@163.com

B. Zhuge
Zhejiang Condiments Industry Research Center, Zhejiang Zhengwei Food Co., Ltd, Yiwu 321000, China

Introduction

Yeasts are a group of lower eukaryotic microorganisms, and some of them are used in several industrial processes for production of various biochemical compounds [15]. Under extreme environmental circumstances, yeasts undergo rapid changes in cellular machinery, modulating metabolic pathways and gene expressions [27]. These response mechanisms aim to protect cells against detrimental effects and repair molecular damages [1].

In late 1980s, a new plasmid type was identified which provided stable integration in high copy numbers into the ribosomal DNA (rDNA) locus of *Saccharomyces cerevisiae*, forming clusters of tandemly repeated plasmid copies [21–23]. Since then, it has shown that integration into the rDNA can also be achieved in other yeast species. Many strategies based on integration into reiterated chromosomal DNA have been used to generate stable multi-copy integrants. At present, the best strategy in copy number and expression is to use integration into the rDNA cluster [10, 36]. The number and length of these rDNA repeats both vary considerably among species [24, 29, 44]. Enhanced recombinational activity and rDNA repeat expansion have been detected in the rDNA units of several organisms [29, 44].

Table 1 Plasmids and strains used in this study

Strains and plasmids	Genotype/properties	Source
Plasmids		
pUC18	Ampicillin, Invitrogen™	Laboratory stock
pGAPZb	Zeocin, Invitrogen™	Laboratory stock
pCAMBIA1302	GFP, Invitrogen™	Laboratory stock
PURGAP	5.8SrDNA: P*CgGAP*-2A-zeocin-Tzeocin	This work
PURGPD	5.8SrDNA: P*CgGPD*-2A-zeocin-Tzeocin	This work
PURGAP-*gfp*	5.8SrDNA: P*CgGAP-gfp*-2A-zeocin-Tzeocin	This work
PURGPD-*gfp*	5.8SrDNA: P*CgGPD-gfp*-2A-zeocin-Tzeocin	This work
PURScGAP-*gfp*	5.8SrDNA: P*ScGAP-gfp*-2A-zeocin-Tzeocin	This work
PURPpGAP-*gfp*	5.8SrDNA: P*PpGAP-gfp*-2A-zeocin-Tzeocin	This work
PURGAPX	5.8SrDNA: P*CgGAP-XYL2*-2A-zeocin-Tzeocin	This work
PURGPDX	5.8SrDNA: P*CgGPD-XYL2*-2A-zeocin-Tzeocin	This work
Strains		
C. glycerinogenes WL2002-5	Wild type	Laboratory stock
Pichia pastoris GS115	Wild type	Laboratory stock
Saccharomyces cerevisiae W303	Wild type	Laboratory stock
Pichia pastoris	Wild type	Laboratory stock

Candida glycerinogenes WL2002-5 (CCTCC: M93018) has been commercially exploited to produce glycerol [47]. Studies about *C. glycerinogenes* WL2002-5 focus on its physiological and fermentative properties, but rarely on glycerol biosynthesis or osmoregulation compared with other yeasts [6, 43]. Despite its industrial importance, the potential of *C. glycerinogenes* WL2002-5 as a host for heterologous gene expression has not been considered due to the lack of marker genes and a genetic transformation system [6]. The plasmids PURGAP and PURGPD harbored glyceraldehyde 3-phosphate dehydrogenase promoter [46] and the glycerol 3-phosphate dehydrogenase promoter [6], respectively. Plasmids PURGAP-*gfp* and PURGPD-*gfp* harboring a phleomycin-resistance coding sequence (GenBank: DQ124230.1) and a green fluorescent protein (GFP)-coding sequence (NCBI: NC_011521.1) were constructed. The recombinant plasmid PURPpGAP-*gfp* with the promoter P*PpGAP* based on the sequence of *Pichia pastoris* GAPDH gene, and the plasmid PURScGAP-*gfp* with the promoter P*ScGAP* from *S. cerevisiae* were also constructed. These vectors can be integrated into the 5.8SrDNA site of *C. glycerinogenes* WL2002-5. The vectors described in this study offer a convenient tool for creating a range of recombinant yeast strains based on various species and enable a comparative assessment of such yeasts for identification of the optimal host.

The gene encoding xylose reductase (*XYL1*) and xylulokinase (*XKS1*) from *C. glycerinogenes* WL2002-5 was isolated under the help of high-flux sequencing technology and the chromosomal location of *XYL1*, *XKS1* was clearly detected on the *C. glycerinogenes* WL2002-5 chromosome. But the gene encoding xylitol dehydrogenase (*XYL2*) was not found from *C. glycerinogenes* WL2002-5 chromosome. In the present study, the *XYL2* genes from *P. stipitis* encoding for xylitol dehydrogenase under control of the glyceraldehyde 3-phosphate dehydrogenase promoter [46] and the glycerol 3-phosphate dehydrogenase promoter [6], respectively was integrated into the chromosome of *C. glycerinogenes* WL2002-5 strain. We studied the role of overexpression of *XYL2* in the conversion of xylose to glycerol and found that recombinant strains (PURGAPX and PURGPDX) improved xylose consumption rates significantly during the fermentation of a glucose–xylose mixture.

Materials and methods

Yeast strains, plasmids, growth and stress conditions

Candida glycerinogenes WL2002-5, from the Research and Design Center of Glycerol Fermentation, Jiangnan University [47], was used for engineering of xylose metabolic pathways. Zeocin (150 μg/mL) was added if necessary. *Escherichia coli* DH5 (*F-recA1 endA1 hsdR17* [*rK-mK+*] *supE44 thi-1 gyrA relA1*) (Invitrogen, Gaithersburg, MD) was used for gene cloning and manipulation. Strains and plasmids used in this work are described in Table 1.

C. glycerinogenes WL2002-5 was grown in YPD medium (yeast extract (1 % w/v), bactopeptone (2 % w/v), glucose (2 % w/v), and agar (1.5 % w/v) for solid medium). Liquid growth assays were performed by pregrowing the strains in YPD medium. Shake-flask cultivations were performed following Zhuge et al. [47]. YPD medium supplemented with 150 μg/mL phleomycin (YEPDS; CAYLA,

Table 2 Primers used in this study

Name	Sequence	Restriction
rdna1	ACG*GAGCTC*TGGAAGTTTGAGGCAAC	*Sac* I
rdna2	ACG*AAGCTT*AGTTCAGCGGGTATTC	*Hin*d III
pgpd1	ACG*CCGCGG*GAACAATCACGTGCAGTGTC	*Sac* II
pgpd2	ACG*CCATGG*TTTAATGTTTGATCTATTC	*Nco* I
pgap1	ACG*CCGCGG*CACCACAGCAGCACCAAC	*Sac* II
pgap2	ACG*CCATGG*TTTTTGTAATTGTGTTTG	*Nco* I
GFP1	ACG*CCATGG*ATGGTAGATCTGACTAG	*Nco* I
GFP2	ACG*GTCGAC*TCACACGTGGTGGTGGTG	*Sal* I
ZEO1	ACGaagattgtggcccctgtgaagcagaccctgaactttgacctgctga agctggctggcgatgtggagtccaaccctggcccc*GTCGAC*ATGGCCAAGTTGACCAG	*Sal* I
ZEO2	ACG*GCATGC*TCAGTCCTGCTCCTC	*Sph* I
B1	ACG*CCGCGG*GGATCCTTTTTTGTAG	*Sac* II
B2	ACG*CCATGG*TGTGTTTTGATAGTTGTTC	*Nco* I
N1	ACG*CCGCGG*AAAGTTAAAAGAAACTTTC	*Sac* II
N2	ACG*CCATGG*TTTTTATTTCTTTTTTTTG	*Nco* I
X2F	ACG*GGATCC*ATGA CTGCTAACCCTTCC	*Bam*H I
X2R	ACG*GTCGAC*TTACTCAGGGCCGTCAATG	*Sal* I

Toulouse, France) was used as a selective medium. *Escherichia coli* DH5a was cultured in Luria–Bertani (LB) medium supplemented with 100 μg/mL ampicillin or 25 μg/mL Zeocin and used for plasmid propagation.

Construction of reporter gene GFP vectors for transformation

The primers for construction of integrative expression plasmids are depicted in Table 2. To construct the expression cassette, the two primers rdna1 and rdna2 were designed on basis of the nucleotide sequence of the 5.8S ribosomal RNA (rRNA) gene of *C. glycerinogenes*. To facilitate the cloning, an additional restriction site of *Sac* I or *Hin*d III was incorporated into either primer. With genomic DNA of *C. glycerinogenes* as a template, a 0.7 kb polymerase chain reaction (PCR) product, 5.8SrDNA, was obtained and inserted into a pUC18 plasmid treated with the same restriction enzymes. Primers pgap1/pgap2 and pgpd1/pgpd2 were designed for characterization of the integration modes of PCg*GAP* [46], and PCg*GPD* [6], respectively on *C. glycerinogenes* genome DNA. Zeocin gene with 2A peptides [4, 8] was designed on basis of the nucleotide sequence of the plasmid pGAPZb. GFP gene was amplified with primers GFP1 and GFP2 by PCR with plasmid pCAMBIA1302 as a template. The *Nco* I- and *Sal* I-flanked GFP gene fragments were inserted into PURGAP and PURGPD, respectively, resulting in the recombinant plasmids PURGAP-*gfp* and PURGPD-*gfp* respectively.

The type of promoter used in the initiating vector affects the GFP gene expression and decides the role in the regulation of GFP expression. Primers B1 and B2 were designed for the promoter P*PpGAP* based on the sequence of *P. pastoris* GAPDH gene (U62648.1). Primers N1 and N2 were designed for the promoter P*ScGAP* based on the sequence of *S. cerevisiae* GAPDH gene (NC_001139.9). Then P*PpGAP* and P*ScGAP* were digested and inserted into plasmid PURGAP-*gfp* separately, resulting in the recombinant plasmids PURPpGAP-*gfp* and PURScGAP-*gfp* respectively. Yeast transformation was performed according to standard protocols [39].

Stability of the recombinant yeast

The stability of the recombinant *C. glycerinogenes* WL2002-5 was assayed according to Jayaram et al. [16], with phleomycin resistance [YPD containing 150 mg/l phleomycin (Zeocin™, Invitrogen)] as a selection marker.

Photomicrograph and fluorescence microscopy

The transformants were collected and suspended in a phosphate buffer solution (PBS) [20 mM Tris–HCl (pH 8.0), and 1.0 mM ethylene diamine tetraacetic acid (EDTA)] and then were observed by a fluorescence microscope (Olympus, Tokyo) at 600×. The excitation and emission filters were set at 485 and 520 nm, respectively.

Determination of copy number by fluorescent quantitative real-time PCR (FQ-RTPCR)

SYBR Green as the most widespread non-specific dye excited at 497 nm has the emission maximum at 520 nm and can be used on all RTPCR machines. SYBR Green

Table 3 Primers used for FQ-RTPCR

Name	Sequence(5′–3′)
GAP1	ACGATGGCTTTCACAGTTGG
GAP2	ACGTCAGTTAGAGAGAGCTGG
GFP1	ACGATGGTAGATCTGACTAG
GFP2	ACGTCACACGTGGTGGTGGTG
RTGAP1	ACGACCCAAAAGACTGTCGATG
RTGAP2	ACGATGGACATACCAGTCAAC
RTGFP1	ACGATCTTCTTCAAGGACG
RTGFP2	ACGTGTGGGAGTTGTAGTTG
RTXYLa	ACGACCAAACCCACCAGGTAC
RTXYLb	ACGAACGGAACCCAACTTAGAG

is used in various quantification applications [12, 13, 45, 30]. In this study, all samples were run in triplicate in a 25-μL reaction volume containing 2× SUPER SYBR Mix, 900 nM primers each, 23 μL of water, and 1 μL of the genomic DNA. The PCR was run in a Bio-Rad CFX96™ real-time System (Bio-Rad Biosystems) using the following parameters: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The RTPCR machines can test the presence of primer-dimers in the end products by melting curve analysis: after PCR, the fluorescence is recorded while the temperature ramps slowly from 60 to 95 °C.

To meet all these requirements, prior to the SYBR copy number assay, we plotted standard curves for the transgene (*gfp*) and the endogenous control gene (*CgGAP*) from *C. glycerinogenes* WL2002-5 genomic DNA with five serial dilutions from about 0.0001 to 10 pg of DNA template. After the data analysis, the C_t number and DeltaRn were exported for statistical analyses.

To design the endogenous control gene, both *gfp* and *CgGAP* underwent FQ-RTPCR with primers shown in Table 3.

GFP expression in *C. glycerinogenes* WL2002-5 transformants

The recombinant *C. glycerinogenes* WL2002-5 strains were cultured in the YPD medium under osmotic shock containing 2, 5, 10, 15 or 20 % NaCl for 36 h, and then green fluorescence was examined using fluorescent microscopy.

Also the recombinant strains were cultured in the YPD medium containing 2, 10, 20, 25, 30, 40, 50 or 60 % glucose for 36 h.

Construction of recombinant *XYL2* expressing vectors

For construction of integrative expression plasmids, two primers, X2F and X2R were designed on the basis of nucleotide sequence of *XYL2* gene of *P. stipitis*. To facilitate the cloning, an additional restriction site of *Bam*H I or *Sal* I (lowercase sequence) was incorporated into either primer, and inserted into a PURGAP or PURGPD plasmid treated with the same restriction enzymes. The constructed plasmid was designated as PURGAPX and PURGPDX.

Preparation of cell extracts and measurement of enzyme activity

Cell-free extracts for the assays of xylose metabolic enzymes were prepared as follows. The recombinant *C. glycerinogenes* WL2002-5 strains were cultivated in ferment medium overnight at 30 °C. The cells were harvested by centrifugation at 5,000 r/min for 5 min at 4 °C and washed with sterile water twice [9]. The cells were resuspended in TE buffer, and then disrupted at 4 °C with 1-s bursts of ultrasonic sound and 3-s intervals over a total period of 15 min and disruption was confirmed by microscopy [42]. The lysates were centrifuged at 4 °C, and the supernatants were then analyzed for the *XYL2* enzyme activity. Protein concentrations in the cell-free extracts were determined with a Bio-Rad protein assay using an Eppendorf Biophotometer (Eppendorf, Germany). The enzyme activities were then measured by following the reported procedures [26]. One unit of enzyme activity was defined as the amount of enzyme that reduced or oxidized 1 mmol $NAD(P)^+$ or NAD(P)H per minute. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12.5 % gel using the reported method [20], and the proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma).

Metabolite analysis and cell dry weight determination

The recombinant yeast strains were first cultivated in YPD medium overnight at 30 °C and 200 r/min. Cells were centrifuged at 5,000 r/min and 4 °C and then washed twice with sterile water. Yeast cells were transferred to 250 mL shake flasks with 30 mL of ferment medium (2 g/L urea and 5 g/L corn steep liquor with glucose and/or D-xylose mixture, the pH was not adjusted) to ferment at 30 °C and 220 r/min [47]. The initial inocula were 0.1 g/L biomass.

Glycerol content was determined by HPLC by using an HPX-87H Aminex ion exchange column (300 × 7.8 mm, Bio-Rad) at 60 °C. The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The eluate was monitored with a differential refractive-index detector (Shodex RI-101, Dionex, Japan). [6] Dry cell weight (DCW) was determined by drying cells at 80 °C until constant weight.

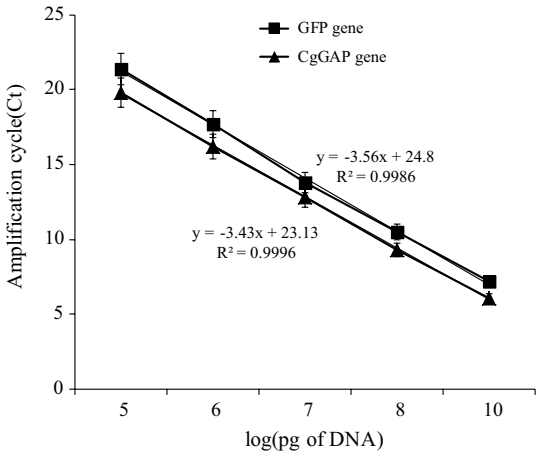


Fig. 1 Standard curve comparison for FQ-RTPCR. Standard curves for the *gfp* transgene and the *CgGAP* gene in serially diluted DNA samples from the *C. glycerinogenes* WL2002-5, which carries a single copy of the *CgGAP* gene. A very efficient amplification was obtained, as indicated by the *slopes* of the standards curves. C_t, cycle threshold

Results

Construction of integrative plasmid with reporter gene GFP for transformation

Integrative vectors PURGAP and PURGPD were constructed with the 5.8S rDNA gene as an integration target and with P*CgGAP* [46] and P*CgGPD* [6] as promoter, respectively. Also phleomycin-resistance coding sequence (zeocin) as a selectable marker and GFP as a reporter gene were cloned, resulting in the recombinant plasmids PURGAP-*gfp* and PURGPD-*gfp* (Table 1). A consecutive sequence containing *Pichia pastoris* GAPDH promoter P*PpGAP* and *S. cerevisiae* GAPDH promoter P*ScGAP* were introduced, resulting in the recombinant plasmids PURPpGAP-*gfp* and PURScGAP-*gfp* respectively (Table 1).

Determination of *gfp* copy number by FQ-RTPCR

A very efficient amplification was obtained in standard curves, as indicated by the slope of the linear regression, with good correlation (Fig. 1). Slopes for *gfp* and *CgGAP* were −3.56 and −3.43 respectively, and correlation coefficients were 0.998 and 0.999, respectively. In design of internal reference gene, both *gfp* and *CgGAP* underwent RTPCR with primers shown in Table 3.

The RTPCR results in Table 4 show that the copy number in the recombinant strains was all the same single copy. There was only one product present peak in a melting curve graph.

Table 5 Stability of the recombinant plasmid

Generations	Phleomycin(+)	Phleomycin(-)
20	100	100
40	98	100
60	93	100
80	87	100
100	77	100

Stability of the integrative vector

Recombinant strain was unstable due to the environment, the host and the plasmid structure, and as a result, some plasmid fragments are lost and the expression product becomes unstable. By measurement of phleomycin-resistant cells, 77 % of recombinant strains cultivated in a non- selective medium were still phleomycin-resistant after 100 generations (Table 5). The plasmid is cultivated in a selective medium so as not to lose the plasmid.

Expression of *gfp* in *C. glycerinogenes* WL2002-5

The abundant tools make yeast a suitable model for integrated analysis of cellular osmoregulation. Integrative vectors PURGAP-*gfp*, PURGPD-*gfp*, PURPpGAP-*gfp* and PURScGAP-*gfp* were constructed and transformed into *C. glycerinogenes* WL2002-5. The recombinant *C. glycerinogenes* WL2002-5 strains were cultured in the YPD medium under osmotic stress containing NaCl, and then green fluorescence was examined using fluorescent microscopy (Fig. 2).

The results showed that *gfp* was functionally expressed under the control of P*CgGAP*, P*CgGPD,* P*ScGAP* and

Table 4 Copy numbers of *CgGAP* and *gfp* gene detected by FQ-RTPCR

Recombinant strain	Ct		log (gene copy number)		*gfp* gene copy number
	gfp gene	*CgGAP* gene	*gfp* gene	*CgGAP* gene	(*gfp*/*CgGAP* gene copy number)
PURGAP-*gfp*	17.86 ± 0.13	16.72 ± 0.21	1.54 ± 0.04	1.88 ± 0.07	0.82
PURGPD-*gfp*	16.67 ± 0.10	16.27 ± 0.08	1.89 ± 0.03	2.01 ± 0.02	0.94
PURPpGAP-*gfp*	18.68 ± 0.12	18.52 ± 0.29	1.29 ± 0.03	1.34 ± 0.09	0.96
PURScGAP-*gfp*	18.28 ± 0.19	17.31 ± 0.14	1.41 ± 0.05	1.7 ± 0.04	0.83

P*PpGAP* in *C. glycerinogenes* WL2002-5 (Fig. 2). The green fluorescence was less intense at low osmotic stress (2 % NaCl), but was much more intense when the osmotic stress increased (10–20 % NaCl). The expression efficiency differed when the recombinant strain was under varying osmotic stress (2–20 % NaCl), but P*CgGAP* and P*CgGPD* were induced remarkably by high osmotic stress. Functional analysis of P*CgGAP* and P*CgGPD* will facilitate further studies on the mechanism of glycerol synthesis from *C. glycerinogenes* WL2002-5 under osmotic stress.

As in most organisms, glycolysis and the tricarboxylic acid cycle in *C. glycerinogenes* WL2002-5 are the main pathways for sugar utilization and energy generation (ATP). Similarly, glucose is the most preferred sugar. Because of historical reasons, however, these pathways have been studied most extensively in *S. cerevisiae*. Therefore, integrative vectors with *gfp* gene were constructed and transformed into *C. glycerinogenes* WL2002-5. The recombinant *C. glycerinogenes* WL2002-5 strains were cultured in the YPD medium and then green fluorescence was examined using fluorescent microscopy (Fig. 3).

The results showed that the green fluorescence was less intense at low osmotic stress (2–20 % glucose), it became much more intense when the osmotic stress increased (20–30 % glucose), but it became less intense after that (40–60 % glucose). The expression efficiency differed when the recombinant strain was under varying osmotic stress. The

Fig. 3 Scanning microscopic images of the recombinant *C. glycerinogenes* WL2002-5 strains at different glucose concentrations. **a** PURGAP-*gfp*; **b** PURGPD-*gfp*; **c** PURScGAP-*gfp*; **d** PURPpGAP-*gfp* were cultured in the YPD medium containing 2, 10, 20, 25, 30, 40, 50, or 60 % glucose. Cells were harvested in exponential phase of growth and viewed under fluorescence microscope as described in "Materials and methods"

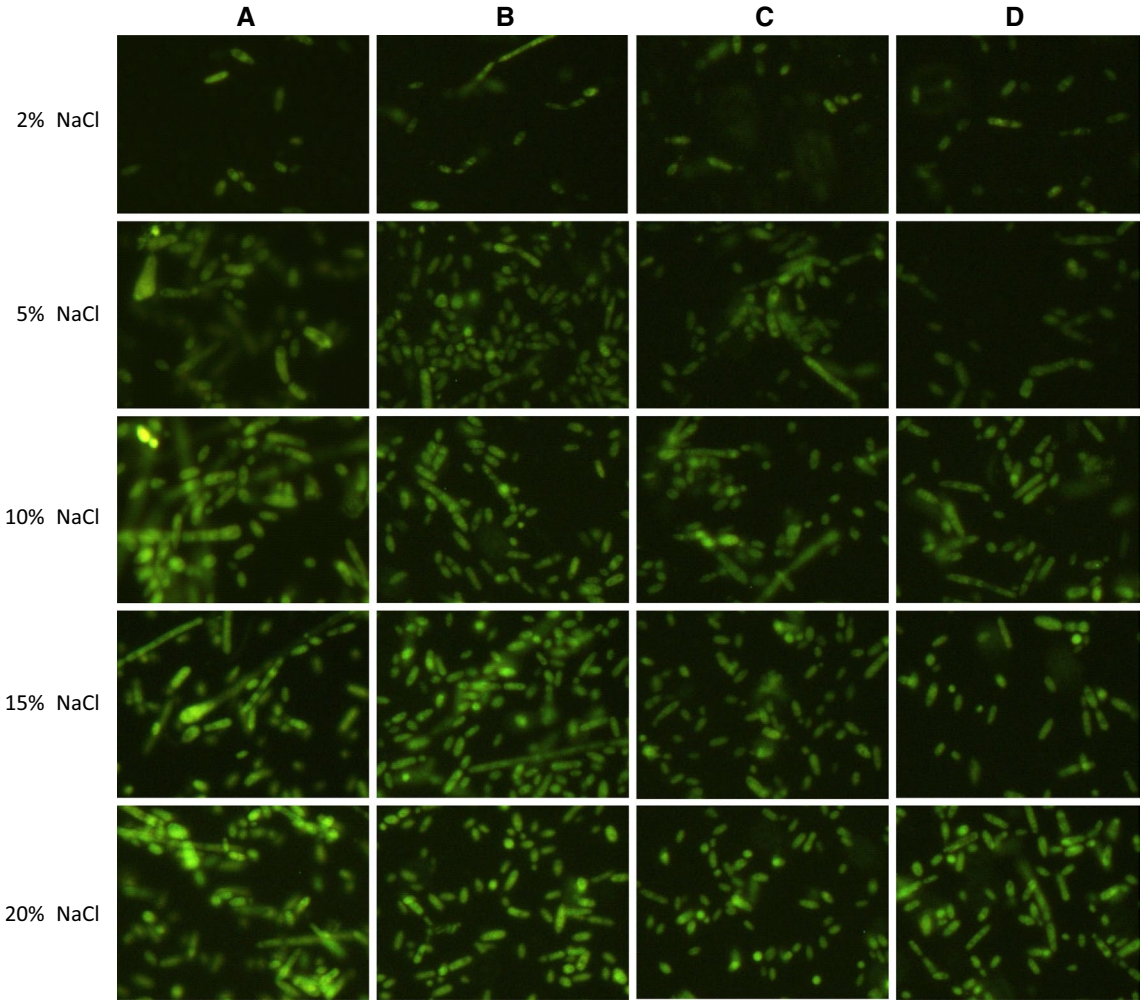
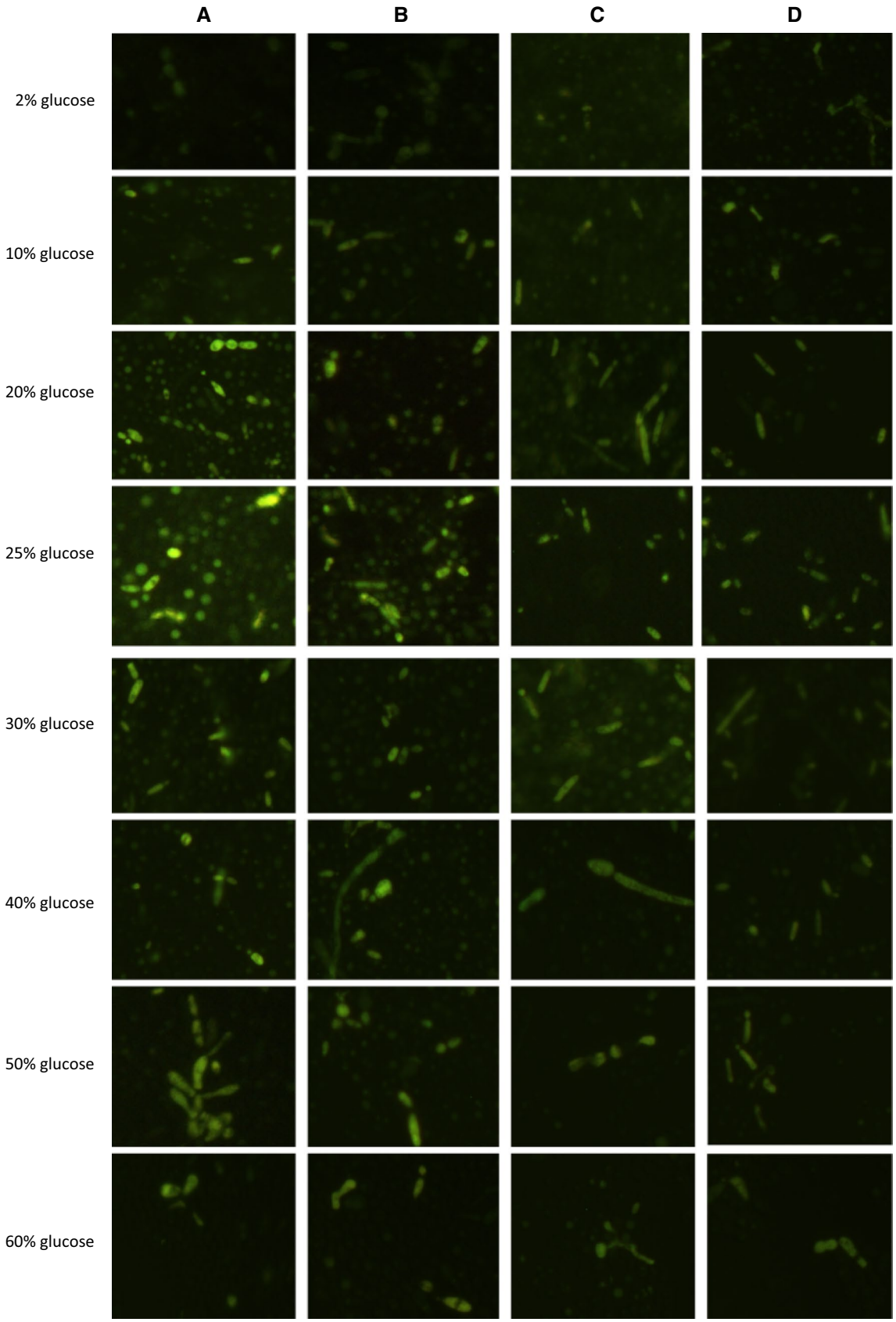


Fig. 2 Scanning microscopic images of the recombinant *C. glycerinogenes* WL2002-5 strains at different NaCl concentrations. **a** PURGAP-*gfp*; **b** PURGPD-*gfp*; **c** PURScGAP-*gfp*; **d** PURPpGAP-*gfp* were cultured in the YPD medium containing 2, 5, 10, 15, or 20 % NaCl. Cells were harvested in exponential phase of growth and viewed under fluorescence microscope as described in "Materials and methods"


A
B
C
D
2% glucose
10% glucose
20% glucose
25% glucose
30% glucose
40% glucose
50% glucose
60% glucose

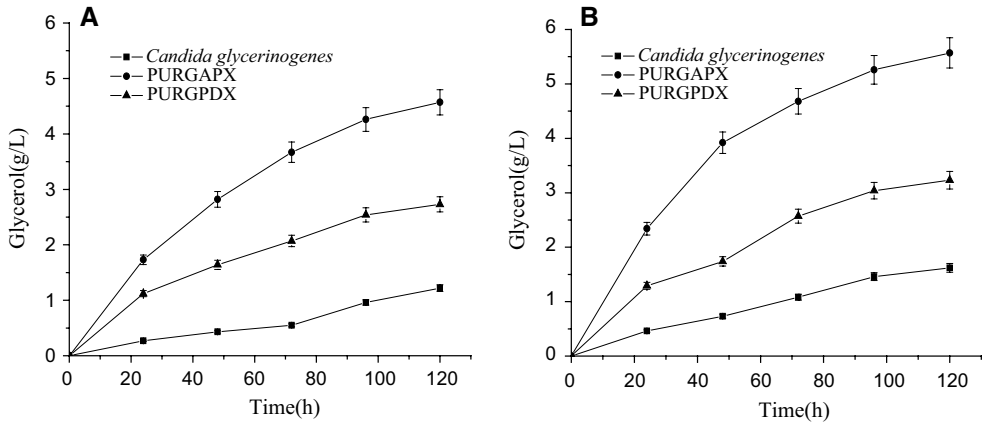


Fig. 4 Fermentation profile of *C. glycerinogenes* WL2002-5, PURGAPX and PURGPDX in ferment medium containing **a** 100 g/L of xylose and **b** 150 g/L of xylose

promoters P*CgGAP* and P*CgGPD* were induced remarkably by the high osmotic stress (20–30 % glucose). This may be the reason why *C. glycerinogenes* WL2002-5 has the high yield and recovery yield of glycerol. High concentrations of glucose (20–30 % glucose) on glycerol synthesis resulted in higher expression of GFP.

In response to the fluctuation in environmental osmolarity, yeast cells self-adjust by a series of molecular, physiological and morphological events known as the osmotic stress response [3, 14]. Adaptation to osmotic stress requires direct metabolic adjustments, and research on glycerol metabolism and the interaction of metabolic pathways is valuable and practical. Increasingly, GAPDH is a dynamic piece of the glycolytic pathway controlling the carbon flux course through the cell and also is an internal control factor for relative quantitation of gene expression [25, 33]. In glycolysis, GAPDH may function as a cellular switch in response to oxidative stress, and especially, GAPDH catalyzes a central and essential step in glycolysis. Catalysis by GAPDH pulls the carbon flux, and the impairment or deletion of GAPDH can change carbon flow [31].

Introduction of a xylose metabolic pathway into the *C. glycerinogenes* WL2002-5 strain

To introduce a xylose fermentation pathway into the *C. glycerinogenes* WL2002-5 strain, integrative vectors PURGAPX and PURGPDX were constructed. Therefore, an expression cassette harboring *XYL2* was introduced under control of the glyceraldehyde 3-phosphate dehydrogenase promoter and the glycerol 3-phosphate dehydrogenase promoter, respectively and integrated into 5.8SrDNA of the chromosome of *C. glycerinogenes* WL2002-5 strain through homologous recombination for xylose fermentation. After transformation, real-time fluorescent quantitative PCR is to be used to determine the copy number of *XYL2* gene in genome of *C. glycerinogenes* WL2002-5, and it was only one copy in the recombinant strains (data not shown). The xylose fermentation capabilities of the PURGAPX and PURGPDX strains were evaluated using ferment medium containing 100 and 150 g/L of xylose. The *C. glycerinogenes* WL2002-5 strain produced 1.2 and 1.6 g/L of glycerol within 120 h, respectively (Fig. 4). In contrast, the PURGAPX and PURGPDX strains consumed xylose efficiently and produced 4.6 and 2.7 g/L of glycerol with 100 g/L xylose as carbon source (Fig. 4a), and 5.7 and 3.3 g/L of glycerol with 150 g/L xylose (Fig. 4b) within 120 h, respectively.

XYL2 enzyme activity

Enzyme activities were measured under conditions used for preparation of inoculum. *XYL1* activities were 5.7 U/mg and similar in recombinant strains. *XYL2* activities were higher in PURGPDX strain than in PURGAPX strain (3.5 and 4.6 U/mg, respectively). The *XYL1* and *XYL2* activities were higher than previously reported, which may be due to the use of different growth media and conditions [11, 28, 38, 40]. SDS-PAGE analysis of the recombinant strains showed protein bands with apparent molecular masses of 40.0 kDa (Fig. 5), which showed *XYL2* were expressed in the recombinant strains.

Co-fermentation of glucose and xylose by the recombinant yeast (PURGAPX and PURGPDX)

It is a two-step process in the conversion of xylose catalyzed by NAD(P)H-dependent xylose reductase and NAD+-dependent xylitol dehydrogenase. Once xylulose is produced, it enters the pentose phosphate pathway. The ability to take up xylose by the use of the glucose transport system which has a broad specificity for pyranoses is well

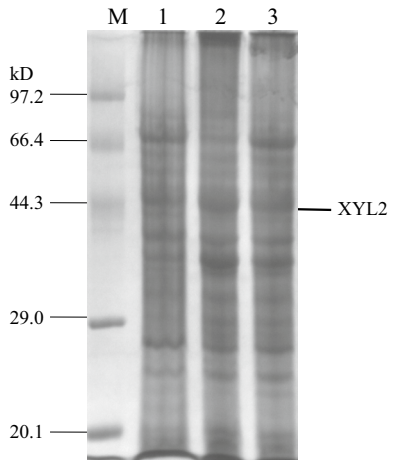


Fig. 5 SDS-PAGE analysis of the recombinant strain. *Lane M* standard protein marker, *lane 1 Candida glycerinogenes*, *lane 2* recombinant *C. glycerinogenes*/PURGAPX, *lane 3* recombinant *C.glycerinogenes*/PURGPDX

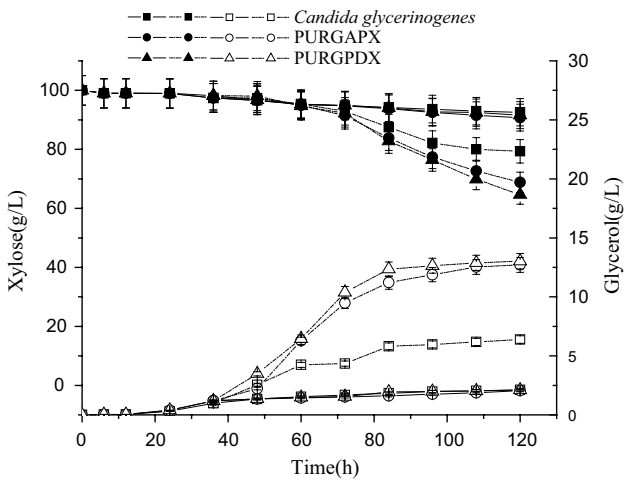


Fig. 6 Time courses of xylose consumption and glycerol production in ferment medium containing 100 g/L xylose by *C. glycerinogenes* WL2002-5, PURGAPX and PURGPDX. *Filled symbols* xylose; *empty symbols* glycerol production; *symbols with solid line* fermentation without glucose; *symbols with dash dot line* glucose was added at 36 h

known [7, 18, 19]. High- and low-affinity transport systems for glucose have been found in *S. cerevisiae* [2], and apparently both are used in xylose uptake [5, 32, 37]. However, the affinities of these systems for xylose are considerably lower than those for glucose, indicating that glucose is the primary substrate. The fermentation was carried out in 100 g/L xylose as carbon source without glucose. The cells were harvested by centrifugation at 5,000×*g* for 5 min at 4 °C and washed with sterile water twice and resuspended in sterile water for 3 h to extract the glucose and glycerol from the cells, there was almost no xylose consumption and glycerol production after 120 h fermentation even with 5 % NaCl. When 50 g/L glucose was added to the ferment culture at 36 h, the recombinant strains produced more glycerol than untransformed *C. glycerinogenes* WL2002-5 within 120 h (Fig. 6). These results indicated that the reasons for the inability of *C. glycerinogenes* WL2002-5 to utilize xylose except in the presence of other substrates such as glucose may be connected to metabolic regulation rather than limitations in the capacity of the metabolic steps involved. Glycerol synthesis, while in *S. cerevisiae* is ethanol synthesis, would be to select for the recombinant yeast possessing a xylose reductase and a xylitol dehydrogenase which are linked to the same coenzyme system, thus eliminating the formation of excess NADH in the process of glycolysis and pentose phosphate pathway.

Glucose fermentation to glycerol can be carried out efficiently by *C. glycerinogenes* WL2002-5 (Fig. 7a). In contrast, xylose fermentation is challenging because traditional glycerol-producing microorganisms cannot readily ferment xylose. We studied the role of overexpression of *XYL2* in the conversion of xylose to glycerol and found that recombinant strains of PURGAPX and PURGPDX improved glycerol production and xylose consumption rates during the fermentation of xylose and a glucose-xylose mixture. Glycerol fermentation was performed with 100 g/L glucose and 150 g/L xylose as carbon source with 2 g/L urea and 5 g/L corn steep liquor. The dry cell weight was not significantly different in these three strains after 36 h fermentation. The *C. glycerinogenes* WL2002-5 strain produced 16.1 g/L of glycerol from glucose (Fig. 7b, c). In contrast, the PURGAPX and PURGPDX strains consumed glucose and xylose efficiently and produced 31.7 and 35.6 g/L of glycerol within 120 h (Fig. 7b, c). All these three strains consumed total glucose after 36 h fermentation and produced 16.1, 16.9 and 18.7 g/L glycerol, respectively (Fig. 7b, c). These results suggested that the ability of glucose fermentation in these genetic strains did not differ significantly. At 48 h, PURGAPX consumed 16.7 % xylose and produced 18.7 g/L glycerol. PURGPDX consumed 20 % xylose and produced 21.7 g/L glycerol. Compared to *C. glycerinogenes* WL2002-5 strain, the glycerol production of PURGAPX and PURGPDX were increased by 12.5 and 34.2 % respectively at 48 h. At 120 h, the glycerol production with 31.7 and 35.6 g/L in PURGAPX and PURGPDX, respectively were increased significantly compared with 48 h. To calculate the glycerol production from xylose, the glycerol production at 36 h from glucose should be subtracted from the total amount of glycerol at 120 h. The actual glycerol production from xylose was 14.8 g/L glycerol produced from 65.2 g/L xylose by PURGAPX and the maximum 16.9 g/L glycerol produced from 70.7 g/L xylose by PURGPDX. The xylitol production was about 23.7, 18.4 and 12.9 g/L by *C. glycerinogenes* WL2002-5, PURGAPX and PURGPDX,

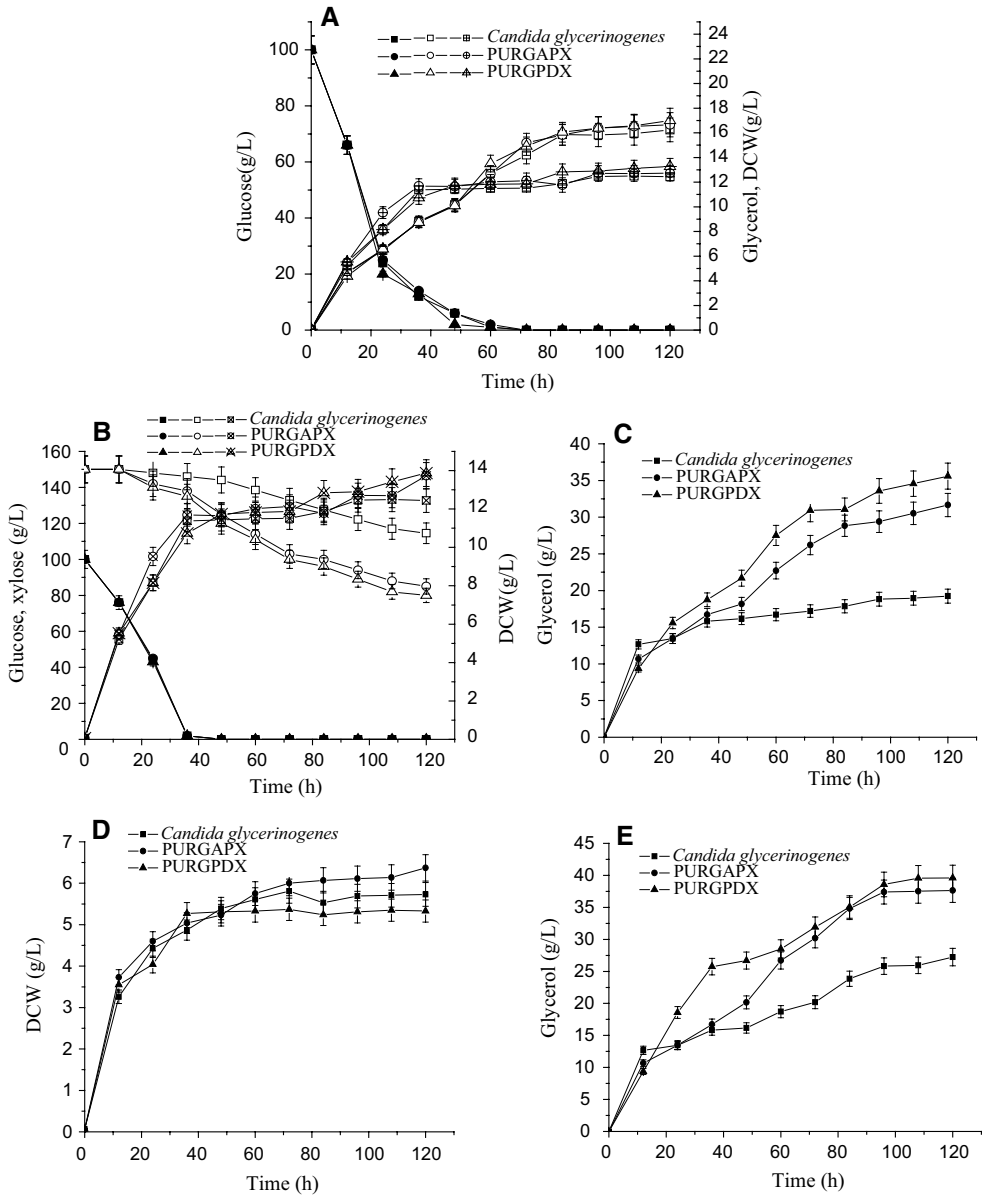


Fig. 7 Time courses of dry cell weight, glucose, xylose consumption and glycerol production in ferment medium containing 100 g/L of glucose and 150 g/L of xylose mixture with or without 5 % NaCl by C. glycerinogenes WL2002-5, PURGAPX and PURGPDX. **a** Ferment medium containing 100 g/L of glucose only. *Filled symbols* glucose, *empty symbols* glycerol production, *crossed empty symbols* dry cell weight; **b** *filled symbols* glucose, *empty symbols* xylose, *crossed empty symbols* dry cell weight; **c** glycerol production without 5 % NaCl; **d** dry cell weight and **e** glycerol production with 5 % NaCl

respectively. The by-product ethanol, produced mainly from glucose fermentation, was 1.3 g/L by *C. glycerinogenes* WL2002-5, 1.4 g/L by PURGAPX, 1.7 g/L by PURGPDX. During the glucose fermentation phase, there was less than 1 g/L lactic acid produced in these three strains. In addition, no acetic acid was produced during the glucose consumption phase. Compared to xylose fermentation, there was much more glycerol production in mixture fermentation and glycerol yield was increased significantly, because much more biomass was produced in the latter condition.

As the promoters were regulated by osmotic, fermentation was performed with 100 g/L glucose and 150 g/L xylose as carbon source with 5 % NaCl as osmoregulater. The dry cell weight was not significantly different but much slower than without 5 % NaCl in these three strains (Fig. 7d) after 36 h fermentation. All these three strains consumed total glucose after 36 h fermentation. Both PURGAPX and PURGPDX strain produced more glycerol (6.1 and 4.3 g/L glycerol) than without 5 % NaCl after 120 h fermentation (Fig. 7e). These results suggested that glycerol production

of osmotic fermentation in these genetic strains differ significantly. The promoters used in the recombinant strains were up-regulated by high osmotic pressure.

Discussion

Yeast systems differ in their capability to produce a given foreign protein. Thus, it is desirable to pre-select the most suitable expression platform from a range of candidates in a particular strain development. An integrative DNA transformation vector of PURGAP and PURGPD for *C. glycerinogenes* WL2002-5 was developed. The *C. glycerinogenes* WL2002-5-derived 5.8S rDNA fragment for integration in heterologous systems was assessed. The transformation system developed in this study can be applied to transform wild-type strains of *C. glycerinogenes* WL2002-5, and provides detailed genetic analysis of *C. glycerinogenes* WL2002-5. It is possible to explore the potential of *C. glycerinogenes* WL2002-5 as a new host for the production of foreign polypeptides and to improve the productivity of useful materials such as enzymes and metabolites. To increase glycerol production from the lignocellulosic substrates, we constructed genetically engineered yeast *C. glycerinogenes* WL2002-5 with heterologous expression of *XYL2*. Although the resulting strains PURGPAX, PURGPDX successfully fermented xylose, the activities of xylose-metabolizing enzymes were still low. Several metabolic engineering strategies were explored to improve xylose fermentation by recombinant XR-and XDH-expressing *S. cerevisiae* strains [17, 34, 35, 41]. However, no strain capable of efficiently converting xylose to glycerol has been successfully developed with high xylose fermentation rate compared to glucose. Recombinant strains of PURGAPX and PURGPDX produced much higher glycerol than *C. glycerinogenes* WL2002-5 strain during xylose and glucose–xylose mixture medium. Global optimization of an engineered *C. glycerinogenes* WL2002-5 system will be necessary for metabolizing xylose both at a cellular level and at a process scale. This study expands the number of available characterized integration sites and demonstrates that such sites provide a virtually inexhaustible pool of integration targets for stable multi-gene expression.

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