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Original Article

Construction of brewing-wine *Aspergillus oryzae pyrG*$^{-}$ mutant by *pyrG* gene deletion and its application in homology transformation

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***pyrG*$^{-}$ host cells are indispensable for *pyrG*$^{-}$ based transformation system. Isolations of *pyrG*$^{-}$ host cells by random mutations are limited by time-consuming, unclear genetic background and potential interferences of homogenous recombination. The purpose of this study was to construct brewing-wine *Aspergillus oryzae pyrG*$^{-}$ mutant by site-directed mutation of *pyrG* gene deletion which would be used as a host for further transformation. pMD-pyrGAB, a vector carrying *pyrG* deletion cassette, was used to construct *pyrG*$^{-}$ mutant of *A. oryzae*. Three stable *pyrG* deletion mutants of *A. oryzae* were isolated by resistant to 5-fluoroorotic acid and confirmed by polymerase chain reaction analysis, indicating that *pyrG* was completely excised. The Δ*pyrG* mutants were applied as *pyrG*$^{-}$ host cells to disrupt *xdh* gene encoding xylitol dehydrogenase, which involves in xylitol production of *A. oryzae*. The *xdh* disruption mutants were efficiently constructed by transforming a pMD-pyrG-xdh disruption plasmid carrying *pyrG*, and the produced xylitol concentration of the Δ*xdh* mutant was three times as much as that of the Δ*pyrG* recipient. Site-directed *pyrG* gene deletion is thus an effective way for the isolation of *pyrG*$^{-}$ host cells, and the established host-vector system could be applied in further functional genomics analysis and molecular breeding of *A. oryzae*.**

Keywords *Aspergillus oryzae*; *pyrG* gene; uridine auxotrophs; gene deletion

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Introduction

The filamentous fungus *Aspergillus oryzae* is an important industrial microorganism widely applied in food processing and fermentation industry due to its high production of proteases and carbohydrases. It has been safely used for more than 1000 years and its safety is affirmed as GRAS (Generally Recognized As Safe) by the US Food and Drug Administration [1]. *Aspergillus oryzae* is also an ideal host cell for heterologous gene expression, protein secretion, and metabolite productions [2]. The availability of large chromosome deletion technology [3,4] and the complete genome sequence of *A. oryzae* [5] have prompted studies on its functional genomics research, protein expression, and artificial modification of metabolic pathways.

Development of an efficient and stable transformation system is required for protein expression and molecular breeding in *A. oryzae*. Among various host-vector systems of filamentous fungi, one prominent system involves the use of *pyrG* gene encoding orotidine-5′-phosphate decarboxylase (EC 4.1.1.23; OMP decase) as a selective marker and its auxotrophic strain (*pyrG*$^{-}$) as a genetic transformation host [3,6]. The *pyrG* participates in uridine biosynthesis and is also a target for the antimetabolite 5-fluoroorotic acid (5-FOA). Cells lacking *pyrG* are uridine auxotrophs and resistant to 5-FOA. *pyrG*$^{-}$ host could be restored to a prototroph via ectopic expression of *pyrG* from the transformed heterologous gene, producing a positive selection. 5-Fluoroorotic acid was used to select *pyrG* deletion via a polymerase chain reaction (PCR)-mediated seamless gene deletion and marker recycling method, a negative selection for *pyrG* rescue [3,4,7]. Thus, the *pyrG* bidirectional selective marker can be reused unlimitedly by the rounds of gene knockout, a feature of considerable value for breeding of food-safe-grade microorganisms.

pyrG^{-} host cells are indispensable for *pyrG*$^{-}$ based transformation system. Isolations of *pyrG*$^{-}$ host cells have been carried out mainly by means of random mutation with ultraviolet (UV) [8–10], chemical mutagenesis [11–13], and spontanous mutation [14]. The last two steps in the biosynthesis of uridine are catalyzed by orotate phosphoribosyl transferase (EC 2.4.2.10; OPRTase) and OMPdecase. Random mutants lacking either OPRTase or OMPdecase are both uridine auxtotrophs and resistant to 5-FOA. Only those 5-FOA-resistant mutants keeping intact OPRTase could be used for *pyrG*$^{-}$ host cells, increasing difficulty and

complication of *pyrG*$^{-}$ mutant selection [8,10]. Random mutations might also lead to unclear genetic background, even disrupt some genes encoding essential catalytic steps of host strains. Moreover, the most part of homologous arms *pyrG* gene (~1.5-kb in *A. oryzae*) still remains in *pyrG*$^{-}$ mutant after random mutagenesis, which might cause the interferences of homologous integration of target genes carrying *pyrG* marker.

In the present study, in order to overcome the limitations of random mutation for screening *pyrG*$^{-}$ host cells, site-directed mutagenesis by *pyrG* gene deletion was used to construct *pyrG*$^{-}$ mutants and a host-vector system for brewing-wine *A. oryzae* CICC2012 was developed using *pyrG* as a selective marker. To verify the constructed transformation system, *xdh* gene encoding xylitol dehydrogenase of *A. oryzae* was disrupted targetly by homologous recombination under a selection based on complementation of uridine auxotrophy of the *pyrG*$^{-}$ mutant.

Materials and methods

Strains and culture media

Aspergillus oryzae CICC2012 was a wild-type strain (brewing-wine strain) obtained from China Center of Industry Culture Collection (Beijing, China) and used as an original strain to delete *pyrG* gene. Glucose polypeptone (GP; 2%) medium containing 2% glucose, 1% polypeptone, 0.5% KH_2PO_4, 0.1% $NaNO_3$, and 0.05% $MgSO_4 \cdot 7H_2O$ (1.5% agar added for solid medium) was used for cultivation of *A. oryzae* at 30°C and 0.2% 5-FOA, 0.15% uridine, and 0.07% uracil were added to select 5-FOA-resistant transformants. GP medium (0.5%) containing 0.5% glucose was used for preparation of mycelium. A double-layered osmotic plate (the sub-layer was 2% GP medium with 1.2 M NaCl and 1.5% agar while the upper-layer with 1.2 M sorbitol and 0.6% agar) was used for the regeneration of protoplast. *Escherichia coli* DH5α was used for the construction, propagation, and amplification of hybrid plasmids.

DNA techniques and PCR methods

Standard DNA techniques were used in this study [15]. Genomic DNA of *A. oryzae* was prepared using a previously described method [16]. PCR amplification was carried out with an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany). Oligonucleotide primers used in this study are shown in **Table 1**. Conventional PCRs were carried out with *Taq* plus DNA polymerase and *Pfu* DNA polymerase (Sangon, Shanghai, China). Plasmid pMD19-T (TaKaRa, Dalian, China) was used for the cloning of DNA fragments. DNA sequences were determined by Invitrogen (Shanghai, China).

Constructions of the *pyrG* gene deletion vector and *xdh* gene disruption vector

A *pyrG* deletion vector, pMD-pyrGAB, was constructed as shown in **Fig. 1A**. The location of fragment PA and PB in *A. oryzae* CICC2012 is shown in **Fig. 2A**. A 1.0-kb 5′-flanking region PA and a 1.5-kb 3′-flanking region PB of the *pyrG* gene (GenBank: GQ496621, DOGAN: AO090011000868) were amplified from *A. oryzae* genomic DNA with primer pairs A1/A2 and B1/B2, respectively. The two PCR products were mixed and combined via overlap extension PCR that was carried out using the primer pair A1/B2. The obtained

Table 1. Oligonucleotide primers used in this study

Primers	Sequences (5′–3′)	Restriction enzymes
A1	TAAGCCACGATCTCGATCATTAACTAAAGACCACGAGAGG	
A2	AGGTGTCTTATTCGTACGGATTGATATATGGAATGAAATG	
B1	CATTTCATTCCATATATCAATCCGTACGAATAAGACACCT	
B2	CTGTGACCTGTCTCAAATTATAGAACGAAAACTCAAAGCC	
A3	TAACTAAAGACCACGA	
B3	TAGAACGAAAACTCAA	
X1	GTCGAGCTCCCTGCTTTAATTTTCCGATTG	*Sac*I
X2	TAATACTTGGCTAGCGCTCCGTCGTAAGGT	*Aor*51HI
X3	ACCTTACGACGGAGCGCTAGCCAAGTATTA	*Aor*51HI
X4	CGCGAGCTCCCTAGTCATCTACTAATTTTCTCC	*Sac*I
P1	AAATATTCTAGACCCAAGCCGC	
P2	CTATCGTGTGGGAGCCTTATCAG	
X-F	AGCAGGTCACCAGGTAGG	
X-R	CGAGGCACTCCGTGTAAT	
P3	GACTTGAGCGAGCCGTAT	

2.5-kb fusion PCR product was cloned into the pMD19-T vector to generate plasmid pMD-pyrGAB.

An *xdh* disruption vector, pMD-pyrG-xdh, was constructed as in **Fig. 1B**. Using the X1/X2 and X3/X4 primer sets, 1.0- and 0.9-kb regions of *xdh* (GenBank: GQ222265, DOGAN: AO090038000631) were amplified, respectively. Primers were designed including *Sac*I and *Aor*51HI restriction sites. Then, the X1/X4 primer pair was used to amplify a 1.9-kb region of *xdh* by overlap extension PCR. The obtained 1.9-kb fusion PCR product was cloned into the pMD19-T vector to obtain plasmid pMD-xdh. A 1.8-kb *pyrG* gene was amplified from *A. oryzae* genomic DNA using the primer pair P1/P2. After purification, *pyrG* blunt ends were created using T4 polynucleotide kinase. pMD-xdh was digested by *Aor*51HI. To prevent self-ligation, the digested pMD-xdh vector was dephosphorylated by incubation with calf intestine alkaline phosphatase. The blunting *pyrG* fragment was ligated into pMD-xdh using T4 DNA ligase to obtain the vector pMD-pyrG-xdh. Verification of the constructed plasmids was carried out by restriction analysis and subsequent sequencing.

Aspergillus oryzae protoplast preparation, transformation, and selection of stable transformants

A total of 0.8-ml *A. oryzae* CICC2012 spore suspension was plated out on 0.5% GP plate covered with cellophane [17] and cultivated at 30°C for 24 h until young mycelia overgrew. The mycelia was lysed with 40 ml of 10 mM PBS containing 2% cellulose (Kangdien, Weifang, China), 1% snailase (Sangon), 1.2 M $MgSO_4$ at 30°C for 3 h with gentle shaking to obtain *A. oryzae* protoplasts. Forty milliliters of STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, and 50 mM $CaCl_2$, pH 7.5) was added, and protoplasts were harvested by filtration mycelia through four-layer sterilized lens paper and the filtrate was centrifuged at 1000*g* at 4°C for 10 min. Pelleted protoplasts were washed three times in STC buffer and finally suspended in the above buffer at a concentration of $1-2\times10^{8}$/ml in 100 μl. DNA of the plasmid pMD-pyrGAB (7 μl; 1–2 μg/μl) was mixed with 100 μl of protoplasts. Transformation was performed by the PEG-$CaCl_2$ transformation method as described by Unkles *et al*. [18]. After transformation, the protoplasts were plated out on a double-layered regeneration agar medium with uridine, uracil, and 5-FOA and with 1.2 M sorbitol as osmotic stabilizer in order to select 5-FOA-resistant transformants. Control protoplasts were treated in the same way as above but without addition of DNA.

The colonies grown on the regeneration medium were transferred to both GP and GP with uridine and uracil to select the uridine auxotrophs. To test the mitotic stability of the *pyrG* deletion mutants, the spores of mutants were repeatedly inoculated on GP slants with uridine and uracil at least six times [8]. *pyrG* deletion mutants were confirmed by PCR analyses using the primer pair A3/B3. The detailed phenotypes, such as mycelial morphology, sporulation, and germination rate of *pyrG* mutants, were investigated and compared with wild-type strains.

To identify OMP decase-deficient strains, *pyrG* deletion mutants were transformed with plasmid pMD-pyrG-xdh and positive transformants survived were obtained on the GP medium without uridine and uracil. To obtain stable transformants, colonies were selected and subcultured on GP slants. Conidia were plated on GP lacking uridine and uracil for at least three successive generations to test the stability of the

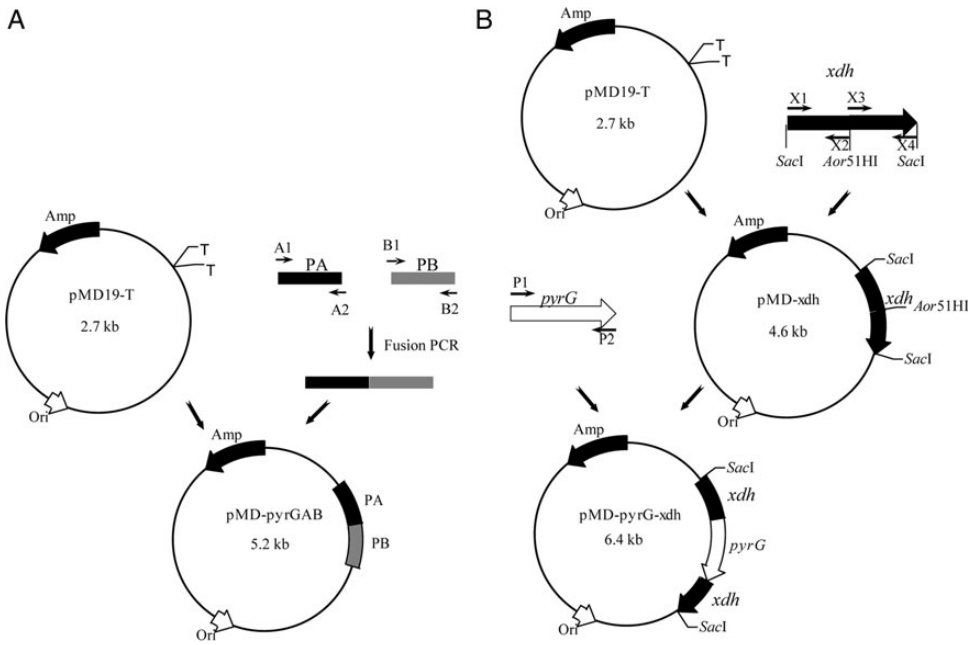

Figure 1. Flow diagrams of constructions (A) *pyrG* deletion targeting vector pMD-pyrGAB and (B) *xdh* gene disruption vector pMD-pyrG-xdh.

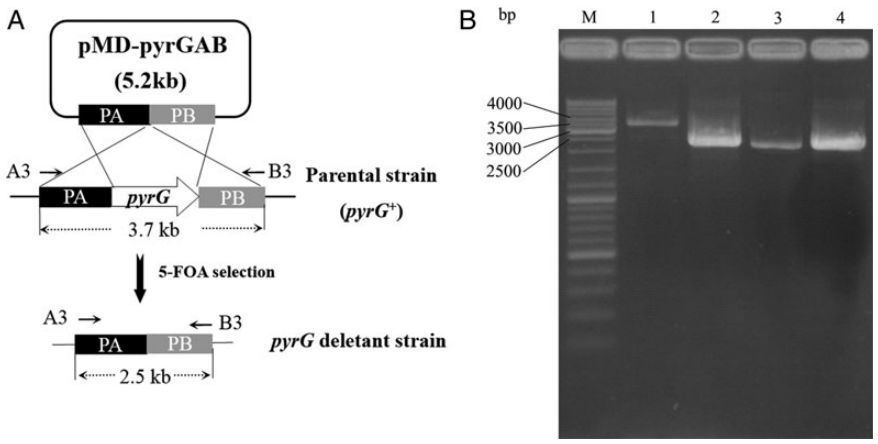

Figure 2. Construction scheme (A) and PCR confirmation (B) of the *pyrG* deletion mutants using primer pair A3/B3 M: DNA Ladder Mix; Lane 1: parental strain, *A. oryzae* CICC2012 (*pyr*G^{+}); Lanes 2–4: *A. oryzae* C2-1, C2-5, C2-13 (*pyr*G^{-}), respectively.

transformants. *xdh* disruption transformants were confirmed by PCR.

Culture conditions for fermentation and xylitol analysis

The *xdh* disruption strains were pre-cultured in 50 ml pre-culture media (g/l) (D-glucose: 10 yeast extract: 10, KH_2PO_4: 5, $NaNO_3$: 1, and $MgSO_4 \cdot 7H_2O$: 0.5) at 30°C with shaking at 150 rpm for 48 h using a 250-ml Erlenmeyer flask. Fungal mycelia were collected from the media and washed with 0.1 M potassium phosphate buffer (pH 6.0), and 2.5 g wet weight of mycelia was used to inoculate the fermentation media. Fermentation media was the same as pre-culture media except for 50 g/l xylose instead of glucose. Fermentation experiments were performed in 250 ml with 50 ml of fermentation media at 30°C and 150 rpm.

The concentrations of D-xylose and xylitol were analyzed by high-performance liquid chromatography (Shimadzu Corporation, Kyoto, Japan) using a Shimadzu RID-10A detector and Shodex SUGAR SZ5532 column. The 80% acetonitrile ($CH_3CN : H_2O = 80 : 20$) was used as the mobile phase with a flow rate of 1.0 ml/min and a column temperature of 60°C. Standard curves of D-xylose and xylitol were also obtained under the same conditions. Each sample in the experiment was measured in triplicate.

Results

Constructions of plasmid pMD-pyrGAB and pMD-pyrG-xdh

Plasmids pMD-pyrGAB and pMD-pyrG-xdh were used to construct the *pyrG* deletion mutant and the *xdh* disruption mutant, respectively. Plasmid pMD-pyrGAB was transformed into *E. coli* DH5α. Plasmid pMD-pyrGAB extracted from transformants was digested by *Sal*I and an expected 5.2-kb fragment was obtained (**Fig. 3A**, Lane 1). Determination of its nucleotide sequence and comparison with that of the

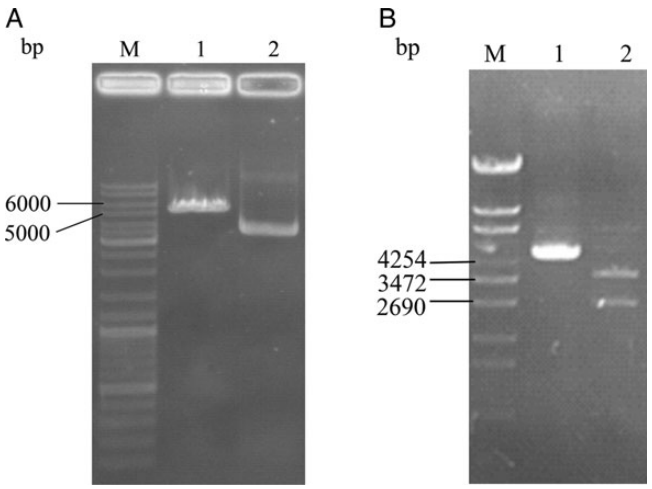

Figure 3. Targeting vectors digested by enzymes (A) *pyrG* gene deletion vector pMD-pyrGAB. M: DNA Ladder Mix; Lane 1: digested by *Sal*I; Lane 2: undigested. (B) *xdh* gene disruption vector pMD-pyrG-xdh. M: λ-*Eco*T14 I digest DNA marker; Lane 1: undigested; Lane 2: digested by *Sac*I.

A. oryzae RIB40 PA and PB revealed a 100% homology at both the nucleotide and amino acid levels. These results indicated that the *pyrG* deletion vector had indeed been cloned.

Similarly, plasmid pMD-pyrG-xdh was transformed into *E. coli* DH5α and digested by *Sac*I. Two expected 3.7- and 2.7-kb fragments were obtained (**Fig. 3B**, Lane 2). pMD-pyrG-xdh sequence analysis also revealed a 100% nucleotide homology compared with that of the *A. oryzae* RIB40 *xdh* gene and indicated that *xdh* disruption vector had been successfully cloned.

Construction of the *pyrG* deletion mutant

Plasmid pMD-pyrGAB carrying *pyrG* deletion cassette was used to construct the *pyrG* deletion mutant (**Fig. 2A**). 5-Fluoroorotic acid-resistant colonies were obtained after incubation at 30°C for 3 days and transferred to GP plates with

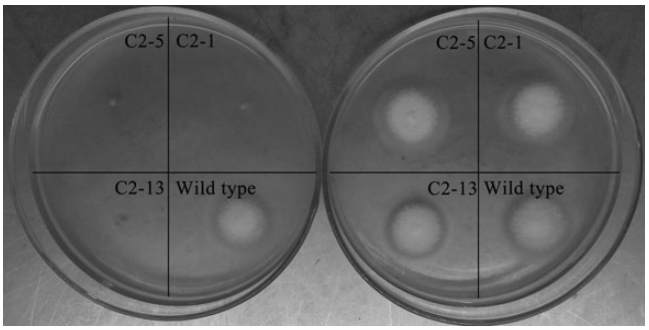

Figure 4. *pyrG* deletion mutants grown on GP medium with and without uridine and uracil Left: without uridine and uracil; right: with uridine and uracil.

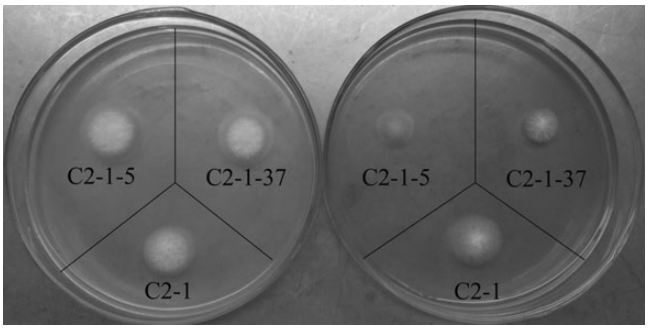

Figure 5. *xdh* disruption strains grown on glucose and xylose agar plates Left: glucose agar plate; right: xylose agar plate.

uridine and uracil. Then these strains were inoculated on both GP and GP with uridine and uracil medium to identify the uridine auxotrophs. Of the 32 colonies examined, all the strains could survive on the medium with uridine and uracil, but only three of them, named C2-1, C2-5, and C2-13, could not grow on G-P medium without uridine and uracil (**Fig. 4**). The spores of C2-1, C2-5, and C2-13 were repeatedly subcultured on GP slants with uridine and uracil to produce spores for at least six generations. The progenies of them still could not grow on the GP medium without uridine and uracil, indicating that the characteristics of the uridine auxotrophs were genetically stable.

Plate assay showed that the growth rate of the uridine auxotrophs was identical to that of the wild-type strain on GP with uridine and uracil (**Fig. 4**). No irregularities in mycelial morphology were observed except uridine auxotrophs, and their sporulation and spore germination rates were not affected either. These uridine auxotrophs were detected by further PCR to identify *pyrG* deletion.

For the three uridine auxotrophs, the predicted 2.5-kb fragments (**Fig. 2B**, lanes 2−4) were amplified from the genomic DNA of the transformants using the primer pair A3/B3 (**Fig. 2A**), while the wild-type *A. oryzae* CICC2012 had a band ~3.7-kb as revealed by PCR using the primer pair A3/B3 (**Fig. 2B**, Lane 1). The PCR result indicated that the three uridine auxotrophs completed double homogenous arms recombination, leading to the deletion of *pyrG*, and could be used for complementary transformation with *pyrG* as selective marker.

Complementary transformation of the *pyrG* deletion mutant

Aspergillus oryzae contains xylose-utilizing key enzymes such as xylose reductase and xylitol dehydrogenase [19]. Disruption of *xdh* by gene targeting would interrupt xylitol to D-xylulose and be helpful for xylitol accumulation. *xdh* disruptants (*Δxdh*) could display slower growth on the xylose agar plate than *xdh*$^+$ transformants, an easily detectable phenotype for primary screening of *xdh* disruptants [20]. One of the *pyrG*$^-$ mutants, *A. oryzae* C2-1, was transformed with the *xdh* disruption vector pMD-pyrG-xdh carrying *pyrG*. The regenerated *pyrG*$^+$ transformants were obtained and purified by selection on GP plates for at least three times, and then transferred to xylose agar plates. For two of the 50 *pyrG*$^+$ transformants, named C2-1-5 and C2-1-37, smaller colony diameters were formed on the xylose agar plates (**Fig. 5**). These two transformants were analyzed by PCR using the primer pair X-F/X-R and P3/X-R (**Fig. 6A**), and two predicted 4.2- and 2.6-kb fragments (**Fig. 6B**, Lanes 1 and 2) were amplified from the genomic DNA of the two *Δxdh* disruptants, confirming the replacement of wild-type *xdh* gene with the *xdh* disruption cassette. For the *A. oryzae* C2-1 and the *xdh*$^+$ transformants with larger colony diameters, the predicted 2.4-kb fragment was amplified using primer pair X-F/X-R (**Fig. 6B**, Lane 3), and no fragment was obtained using primer pair P3/X-R (**Fig. 6B**, Lane 4). These PCR results showed that the two *xdh*$^-$ transformants from C2-1 were *xdh* disruptants generated by homologous integration. The rest of *pyrG*$^-$ mutants, C2-5 and C2-13, were also successfully transformed to obtain *xdh* disruptants with *pyrG* prototroph (data not shown). Complementary transformation results identified that obtained *A. oryzae pyrG*$^-$ mutants kept intact OPRTase and were the suitable *pyrG*$^-$ hosts for heterologous gene transformation.

Xylitol production of the *xdh* disruption mutants

To investigate the effect of *xdh* gene disrupted on xylitol production, the Δ*xdh* mutants C2-1-5 were tested for xylitol production from D-xylose media. Consumption of D-xylose for the C2-1-5 was similar to that of C2-1. In terms of xylitol production, C2-1-5 displayed the xylitol productivity of 0.133 g/(l h) at a concentration of 12.8 g/l xylitol (**Fig. 7**), whereas the xylitol productivity of C2-1 was 0.044 g/(l h) at a concentration of 4.2 g/l xylitol (**Fig. 7**). The xylitol productivity and concentration of Δ*xdh* mutants were three times as much as that of the C2-1 strain. These results indicated that the xylitol productivity could increase prominently by *xdh* gene disruption.

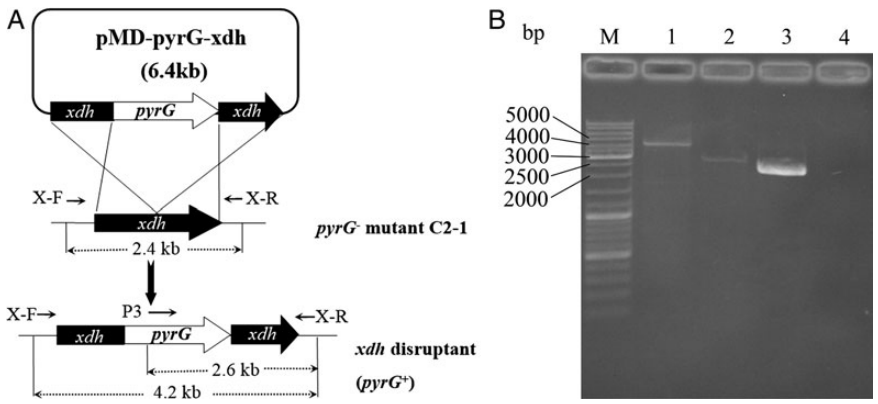

Figure 6. Construction scheme (A) and PCR confirmation (B) of the *xdh* disruptants M: DNA Ladder Mix; Lanes 1 and 2: *xdh* disruptant *A. oryzae* C2-1-5 (Δ*pyrG*, Δ*xdh*) PCR assay using primer pair X-F/X-R and P3/X-R, respectively; Lanes 3 and 4: *A. oryzae* C2-1 (Δ*pyrG*) PCR assay using primer pair X-F/X-R and P3/X-R, respectively.

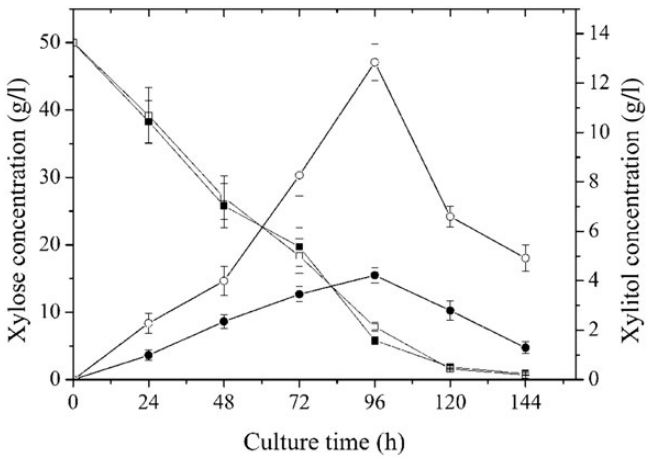

Figure 7. Effects of *xdh* disruption strains on xylitol production from 50 g/l xylose Symbols for *A. oryzae* C2-1(Δ*pyrG*), xylose (■), xylitol (•); for *A. oryzae* C2-1-5(Δ*pyrG*, Δ*xdh*), xylose (□), xylitol (○).

Discussion

In this study, the *pyrG*$^{-}$ mutants were successfully obtained by *pyrG* gene deletion and were characterized with phenotype identification, PCR confirmation and genetic complementation. But in case of random mutants, isolation of *pyrG*$^{-}$ mutants became more complicated and time-consuming. According to the uridine biosynthetic pathway, the uridine auxotrophic strains could be blocked at OMPdease (*pyrG*) or OPRTase (*pyrF*) level. Random mutants by UV or chemical mutagenesis were either OMPdease-deficient or OPRTase-deficient [6,8,21]. In order to determine the deficient step in uridine biosynthesis, the OMPdease and OPRTase activity assays or their gene sequencings were required before complementary transformation. It was reported that the ratio of OPRTase-deficient mutants was higher than that of OMPdease-deficient in many strains [6,10,22,23]. In our experiments, the 5-FOA-resistant cells could also be isolated after incubation at 30°C for ~2 weeks in control plates without DNA transformation, suggesting the occurrence of spontaneous mutants during strain cultivation. The *pyrG* gene sequencing results showed that the obtained 20 random mutants all kept intact *pyrG* sequences; hence, uridine auxotrophs selected by spontaneous mutation here were all OPRTase-deficient, unsuitable as *pyrG*$^{-}$ hosts. A similar tendency by random mutations had been observed in other strains, such as *Mortierella alpina* [10], *Phanerochaete chrysosporium* [21], *Acremonium cellulolyticus* [24], and *Pyrococcus abyssi* [25], but the reason for this phenomenon remains unclear. Moreover, in the present study, three Δ*pyrG* mutants were obtained on the regeneration plate only after incubation for 3 days which was <2 weeks of spontaneous mutants. The time of occurrence of homologous integration might be earlier than that of spontaneous mutation [14,26]. And all these Δ*pyrG* mutants keep OPRTase intact, indicating more effective and timesaving using *pyrG* gene deletion for isolations of *pyrG*$^{-}$ mutants.

Using *A. oryza*e Δ*pyrG* strain as a host, of the 50 regenerated *pyrG*$^{+}$ transformants, two *xdh* disruptants were obtained (ratio was 4%). While in our previous result using *A. oryza*e *pyrG*$^{-}$ strain obtained by UV mutagenesis as a host, two *xdh* disruptants were obtained from 220 regenerated *pyrG*$^{+}$ transformants (ratio was 0.91%; data not shown), suggesting that Δ*pyrG* strain was a superior host for gene disruption with the *pyrG* marker. *pyrG*$^{-}$ random mutant had an endogenous mutated sequence in its chromosome; hence, recombination at the *pyrG* locus was expected to be predominant compared with that of the targeted locus [27], leading to additional ectopic integration and lower gene targeting frequency. The 1.5-kb region necessary for the function of the *pyrG* gene was removed in Δ*pyrG* strain, preventing homologous recombination at the *pyrG* locus. Furthermore, mutations induced by UV or chemical mutagenesis are often unstable and liable to reverse mutation and may include undesirable mutations [26,28]. These results indicated that the current system based on site-directed *pyrG*

gene deletion is stable and extremely helpful for obtaining targeted transformants without additional ectopic integrations.

In conclusion, isolations of *pyrG*$^{-}$ host cells by *pyrG* gene deletion could be more effective and labor-saving compared with the random mutation. The established host-vector system could be used for performing multiple genes targeting without additional ectopic integrations in brewing-wine *A. oryzae*. The present system is compatible with food production and is applicable for further functional genomics analysis and molecular breeding of *A. oryzae*.

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