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# Rapid and marker-free Cas9/CRISPR refactoring yields equivalent xylose-utilization performance in yeast†

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**Abstract**

Genomic integration of expression cassettes containing heterologous genes into yeast with traditional methods inevitably deposits undesirable genetic elements into yeast chromosomes, such as plasmid-borne multiple cloning sites, antibiotic resistance genes, *Escherichia coli* origins, and yeast auxotrophic markers. Specifically, drug resistance genes for selecting transformants could hamper further industrial usage of the resulting strains because of public health concerns. While we constructed an efficient and rapid xylose-fermenting *Saccharomyces cerevisiae*, the engineered strain (SR8) might not be readily used for a large-scale fermentation because the SR8 strain contained multiple copies of drug resistance genes. We utilized the Cas9/CRISPR-based technique to refactor an efficient xylose-fermenting yeast strain without depositing any undesirable genetic elements in resulting strains. In order to integrate genes (*XYL1*, *XYL2*, and *XYL3*) coding for xylose reductase, xylitol dehydrogenase, and xylulokinase from *Scheffersomyces stipitis*, and delete both *PHO13* and *ALD6*, a double-strand break formation by Cas9 and its repair by homologous recombination were exploited. Specifically, plasmids containing guide RNAs targeting *PHO13* and *ALD6* were sequentially co-transformed with linearized DNA fragments containing *XYL1*, *XYL2*, and *XYL3* into *S. cerevisiae* expressing Cas9. As a result, two copies of *XYL1*, *XYL2*, and *XYL3* were integrated into the loci of *PHO13* and *ALD6* for achieving overexpression of heterologous genes and knockout of endogenous genes simultaneously. With further prototrophic complementation, we were able to construct an engineered strain exhibiting comparable xylose fermentation capabilities with SR8 within three weeks. We report a detailed procedure for refactoring xylose-fermenting yeast using any host strains. The refactored strains using our procedure could be readily used for large-scale fermentations since they have no antibiotic resistant markers. This article is protected by copyright. All rights reserved

**Keywords: Cas9, marker-free engineered yeast, xylose fermentation**

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*Saccharomyces cerevisiae* has been employed as a host strain to produce value-added products (Borodina and Nielsen 2014). Numerous metabolic pathways to produce biofuels or value-added chemicals have been installed into *S. cerevisiae* as this yeast can be easily genetically manipulated. Traditionally, the incorporation of heterologous pathways into *S. cerevisiae* has been facilitated using episomal or integrative plasmids capable of restoring growth defects of host strains as well as harboring cloned genes under the control of selected promoters (Mumberg et al. 1995). Due to the availabilities of auxotrophic mutants, dominant drug resistant markers, and well-developed plasmid systems, *S. cerevisiae* became one of the most popular hosts for metabolic engineering. However, the plasmid-based genetic perturbation of *S. cerevisiae* faces several challenges for industrial applications. The procedure usually leaves unintended biological footprints inside the yeast genome, such as multiple cloning sites, bacterial drug resistance genes, and replicative origins that are necessary for cloning of target genes in *E. coli*. Among them, the antibiotics resistance genes definitely hamper the further use of the constructed strains in large-scale applications due to public health and safety concerns (Gootz 2010; Metcalf et al. 2014). Moreover, yeast genetic manipulations using the integrative plasmids usually suffer from the limited numbers of auxotrophic markers for integration. While some of those markers can be recycled (Gueldener et al. 2002), the repeated recycling procedure slows down strain improvement processes. Therefore, a seamless and fast procedure should be pursued for rapid and precise construction of engineered strains that can be utilized for commercial applications immediately.

Recently, the applications of the Cas9/CRISPR system to modify genomes from bacteria to human cells have revolutionized the method of rational genome engineering (Jinek et al. 2012). The Cas9 protein, found in the bacterial CRISPR system, is an RNA-guided DNA endonuclease which can create double-strand breaks (DSBs) on chromosomes *in vivo*. The DSB events could be further exploited to modify the nearby DNA fragments of the genome using homologous recombination (HR) and non-homologous end joining (NHEJ) activities in a host cell (Esvelt and Wang 2013). The Cas9 system has been introduced into baker's yeast for the introduction of targeted mutations. With yeast's incomparable homologous recombination abilities, precise engineering of genomes could be

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achieved with short synthesized DNA fragments along with expression of Cas9 and a guide RNA (DiCarlo et al. 2013). Moreover, we have demonstrated that the Cas9-based genome editing can be applied for introducing genetic perturbations into industrial polyploid yeast strains which have been reported to be difficult to introduce designed mutations (Zhang et al. 2014). Therefore, marker-less and scar-less integration of expression cassettes for introducing desired pathways could be achieved into both laboratory and industrial yeast strains without employing any unwanted drug resistance genes, auxotrophic markers, or bacterial origins in a fast way.

*S. cerevisiae* has been used as a host strain of metabolic engineering to produce fuels and chemicals from abundant cellulosic sugars (Kim et al. 2014a; Kim et al. 2012b; Kim et al. 2013a; Nan et al. 2014; Tsai et al. 2014). To this end, one of the major foci was to engineer *S. cerevisiae* to assimilate xylose, a major sugar component from hemicellulose fractions of lignocellulosic biomass. Two major metabolic pathways have been employed for xylose fermentation by engineered *S. cerevisiae*. One is the xylose isomerases (XI) - xylulose kinase (XK) pathway (Brat et al. 2009; Kuyper et al. 2003). The other is xylose reductase (XR) - xylitol dehydrogenase (XDH) - xylulose kinase (XK) pathway(Jeffries 1983). Both pathways can direct xylose as a carbon source into the pentose phosphate pathway (PPP) (Jeffries 1983). A search in the NCBI Pubmed database as of December 2014 showed that there were almost 200 reports regarding introducing and engineering xylose metabolic pathways into yeast. Because all of them used the traditional plasmid-based gene integration method, the resulting engineered strains contained unwanted drug resistance genes, hampering large-scale fermentation. In this study, we refactored an efficient xylose-fermenting *S. cerevisiae* without introducing any markers. Our previously constructed yeast strain (SR8) was derived from the D452-2 yeast strain with multiple integrations of expression cassettes containing *XYL1*, *XYL2*, and *XYL3* in the background of *PHO13* and *ALD6* deletion. (Lee et al. 2012; Ni et al. 2007; Van Vleet et al. 2008). We reasoned that, starting from D452-2, with the Cas9 system we can replace the parental *PHO13* and *ALD6* genes with the XYL123 expression cassette containing *XYL1*, *XYL2*, and *XYL3*, hence refactoring a xylose-fermenting strain which can exhibit similar xylose fermentation capabilities of SR8. Because we can facilitate integration of the XYL123

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cassettes and disruptions of target genes simultaneously, only two gene-replacement events are necessary to refactor an efficient xylose-fermenting strain. It has been questioned whether Cas9 engineered strains are functionally equivalent to their siblings produced by traditional methods, since in some reports the off-target problem was emphasized (Wu et al. 2014). Here we have a chance to see whether this concern is dispensable.

In order to integrate the XYL123 cassette into the *PHO13* locus without using a selection marker, we transformed a parental strain (D452-2) with a plasmid carrying Cas9 (pCas9-NAT). A transformant with pCas9-NAT was then transformed with another plasmid bearing a guide RNA sequence targeting the *PHO13* locus along with DNA fragments containing *XYL1*, *XYL2*, and *XYL3*. It has been recently reported that deletion of *PHO13* coding for a HAD type IIA phosphatase can result in the up-regulation of the pentose phosphate pathway in yeast (Kim et al. 2014b). Therefore, the *PHO13* locus was selected for a target integration site to achieve the overexpression and deletion of target genes simultaneously. Because the size of the expression cassette with *XYL1* and *XYL2/XYL3* is more than 8 kb, instead of preparing one DNA fragment which is not easy by conventional PCR, we co-transformed two DNA fragments containing *XYL1*, and *XYL2* and *XYL3*, respectively. Specifically, we assembled two DNA fragments *in vivo* by two designs (Fig. 1). With Method A, we first amplified the *XYL1* cassette from pSR5-X1 (gift from S.R.K.), and the *XYL2/XYL3* cassette from pSR3-X23 (Kim et al. 2013b). At the 3' end of the XYL1 cassette and 5' of the XYL23 cassette, a 60 bp linker was added. The two DNA fragments were then concatenated with DNA assembler technique by the 60 bp linker *in vivo* (Kuijpers et al. 2013). In Method B, we amplified two DNA fragments covering the whole XYL123 with a 308 bp overlapping region inside the *XYL3* coding sequences from the existing plasmid pSR6-X123 (Kim et al. 2013b). The two DNA fragments with the overlapping region were also assembled with the DNA assembler technique (Fig. 1) (Shao and Zhao 2012).

In a Cas9-based gene integration method, the yeast transformants expressing Cas9 and the guide RNA targeting a specific sequence can be killed because Cas9 cuts the targeted genome sequence efficiently. Nonetheless, we have observed that small numbers of colonies can survive

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without co-transformation of a repair DNA. This indicates that the suicidal expressions of both Cas9 and a guide RNA in yeast did not lead to 100% killing of the transformants due to NHEJ-based DNA repairs. While not all surviving colonies with co-transformation of a repair DNA might harbor the integration of a repair DNA into a target locus, we have observed decent frequencies (25~100%) of finding real integrants from the surviving colonies. Therefore, screening of about ten colonies through colony PCR was enough to identify the real integrants. Because we used two overlapping DNA fragments as repair DNA fragments, we used a phenotypic screening based on growth on xylose, and confirmed the integration of the XYL123 cassette by colony PCR. Of twenty-four colonies from the plates containing transformants with two DNA fragments with a short overlapping sequence (Method A: 60 bp linker between *XYL1* and *XYL2/XYL3*), six colonies grew well on xylose. Of twenty-four colonies from the plate containing transformants with two DNA fragments with a long overlapping sequence (Method B: 308 bp overlapping between *XYL1* and *XYL3/XYL2*), four colonies grew well on xylose. Colony PCR confirmed that all ten colonies contained the integrated copy of the XYL123 expression cassette at the *PHO13* locus, resulting in the deletion of *PHO13* as well as the overexpression of *XYL1, XYL2*, and *XYL3*. We selected one representative colony (CT1b-1) among the transformants growing on xylose best and performed a xylose fermentation experiment under oxygen-limited conditions to evaluate the efficiency of xylose fermentation. Compared with DX123, a control strain with the XYL123 cassette inserted into the *URA3* site of the parental strain D452-2, the CT1b-1 showed a higher xylose utilization rate and ethanol productivity (Fig. 2). Therefore, the CT1b-1 strain was selected for the second copy integration of the XYL123 expression cassette into the *ALD6* locus.

Beneficial effects of deleting *ALD6*, encoding a major aldehyde dehydrogenase, for xylose fermentation have been reported (Lee et al. 2012). The deletion of *ALD6* led to enhanced xylose fermentation as well as the elimination of acetate accumulation. Therefore, we selected *ALD6* locus as an integration site of the second copy of the XYL123 expression cassette. The second copy integration was performed with Method B mentioned above. Two PCR products containing a long overlapping region (308 bp) amplified from the plasmid pSR6-X123 were co-transformed with a

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plasmid (pCST154) capable of expressing a guide RNA targeting *ALD6* into the CT1b-1 strain expressing Cas9. *In vivo* homologous recombination of the two DNA fragments can generate a linearized DNA fragment containing *XYL1*, *XYL2*, and *XYL3* with flanking sequences homologous to the upstream and downstream regions of *ALD6*. Owed to this, transformants having a replacement of *ALD6* with the *in vivo* assembled XYL123 are highly possible to survive with the presence of Cas9 and the guide RNA targeting *ALD6*. As the parental strain (CT1b-1) is already capable of growing on xylose (Fig. 2), we performed colony PCR directly to identify transformants with the second copy of the XYL123 expression cassette at the *ALD6* locus. Of twenty-four colonies tested, twelve colonies contained expected integration of the XYL123 cassette into the *ALD6* locus, and they all showed similar xylose fermentation patterns. One representative colony was selected and named CT2-auxo. When we compared the xylose fermentation abilities of strains CT1b-1 and CT2-auxo (Fig. 2), CT2-auxo produced more ethanol (10.7 g/L vs. 9.7 g/L) and did not accumulate acetate (0 g/L vs. 1.3 g/L) as compared to CT1b-1.

In order to compare the performances of xylose fermentation by the SR8 strain (constructed by traditional integration methods) and our refactored CT2-auxo, we needed to make CT2-auxo prototrophic because the SR8 strain is prototrophic due to the multiple integrations of expression cassettes with different auxotrophic markers (Kim et al. 2013b). CT2-auxo was sequentially transformed with PCR products containing wild-type *URA3*, *LEU2*, and *HIS3* genes from pRS plasmids (Mumberg et al. 1995). The resulting strain capable of growing on a minimal media lacking uracil, leucine, and histidine was named CT2-Pro. We thus compared the performances of xylose fermentation under oxygen-limited conditions by CT2-auxo, CT2-Pro, and the SR8 strains (Fig.2 and Fig. 3). When 40 g/L of xylose was used as a sole carbon source, CT2-Pro and SR8 showed almost identical xylose utilization and ethanol production rates. Both strains were able to complete the xylose fermentation within 30 hours, and ethanol yields were almost the same (SR8 ethanol yield = 0.298 ± 0.0018, CT2-Pro= 0.281± 0.008 g ethanol/g xylose). Both CT2-auxo and SR8 strains accumulated only negligible amounts of xylitol and acetate. In contrast, CT2-auxo produced less ethanol (yield = 0.263 ± 0.006 g ethanol/g xylose), suggesting that complementation of

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auxotrophic markers enhance xylose fermentation rates through indirect ways. No strain produced acetate, due to the successful deletion of *ALD6*. However, CT2-Pro produced more xylitol than CT2-auxo and SR8 at the time point of 24 hours (4.558 ± 0.3, 1.8 ± 0.1, and 3.249 ± 0.2 g/L respectively). We also compared the fermentation performances by CT2-Pro and the SR8 strain with mixed sugars (70 g/L of glucose and 40 g/L of xylose) under strict anaerobic conditions (Fig.3). Interestingly, the refactored CT2-Pro was able to consume both glucose and xylose slightly faster than the SR8 strain. Both strains exhibited similar ethanol yields (SR8 = 0.382 and CT2-Pro = 0.388 g ethanol/g sugars). The xylose utilization patterns by both strains were similar. Accumulated amounts of byproducts (xylitol and glycerol) were at comparable levels between the two strains.

Our fermentation results showed that the refactored CT2-Pro using Cas9 and *in vivo* DNA assembly can ferment both xylose and a mixture of glucose and xylose with comparable fermentation rates and yields as compared to the traditionally-constructed SR8 strain. This indicated the off-target effect is negligible and the replacement of traditional methods by Cas9 based technique is feasible. However, the ethanol yield by the CT2-Pro strain was slightly lower than the SR8 strain when xylose was used as a sole carbon source (Fig.3). The difference may be due to two possible reasons: the differences in the copy numbers and target integration sites of *XYL1*, *XYL2*, and *XYL3*. The SR8 strain was constructed through integrations of multiple copies of *XYL1* into the Ty3 loci, one copy of XYL123 in the *URA3* locus, and additional copies of XYL23 in the *HIS3* locus. Thus, the gene contents of *XYL2* and *XYL3* of CT2 and SR8 are the same but the copy number of *XYL1* in the SR8 strain is higher than that of CT2. Moreover, the XYL123 cassettes in SR8 were inserted in auxotrophic markers whereas these cassettes were inserted into *PHO13* and *ALD6* in the CT2-Pro strain. The difference of genome insertion may lead to the expression of genes behaving differently according to local chromosomal regulations. In addition, the lengthy laboratory evolution process employed for isolating the SR8 strain might have accumulated uncharacterized spontaneous mutations eliciting improved xylose fermentation. Therefore, further insertion of extra copies of *XYL1* into the CT2 strain and subsequent laboratory evolution may improve CT2-Pro's capabilities in fermenting xylose.

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From our results, CT2-auxo had a lower ethanol yield than CT2-Pro, indicating that auxotrophic strains had some drawbacks in fermenting xylose. Cakar et al. (Cakar et al. 1999) observed this phenotype before by finding that leucine auxotrophic strains had reduced growth and a lower final OD than that of wild-type strains. Even through plasmid complementation the behavior of auxotrophic strains was still different. Therefore, it is suggested to use prototrophic strains as a parental strain for Cas9-based quick XYL123 insertions to construct strains with a stronger background. During the strain construction, we have used the guide RNAs targeting *PHO13* and *ALD6* sequentially to replace the genes. The sequential integration process can be shortened into one transformation with a plasmid carrying both guide RNAs together. Multiplex maneuvers have been reported before (Sakuma et al. 2014) and this may speed up the construction process in the future, although this may not be feasible if too many fragments are transformed together.

In this study, we applied the Cas9/CRISPR technique to refactor a xylose utilization strain SR8 by integrating the *Scheffersomyces stipitis* XYL123 cassette into two loci of the parental yeast strain D452-2, creating the strain CT2. The whole process could be finished in 2-3 weeks and CT2 performed almost the same as the model strain SR8. Moreover, CT2 is marker-free, hence carrying no bacterial genes such as origins and antibiotic resistance factors, enabling its use in industrial-scale bioethanol production. We described the detailed procedure of the refactoring process for constructing xylose-fermenting engineered yeast in the supplementary material. By changing the targeting sequence of the guide RNAs with appropriate repair DNA fragments, the method described in the study can be applied for constructing any engineered yeast strains that do not contain unnecessary genetic elements.

## Materials and Methods

### Strains, plasmids and culture conditions

All the strains and plasmids used and constructed from this study were listed in Table 1. Plasmids pCST154 and pCST155 were constructed by the FastCloning procedure with divergent overlapping primers (Li et al. 2011). Regular culturing of strains was performed in YPD20 medium containing

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1% (w/v) yeast extract, 2% (w/v) Bacto Peptone and 20 g/L of glucose and appropriate antibiotics. Regular concentrations of antibiotics are as follows: Nourseothricin,100 μg/mL; hygromycin B, 300 μg/mL for both liquid and solid media. Fermentations were done with YPX40, which has 40 g/L of xylose, or YPD70X40 which contains 70 g/L glucose and 40 g/L xylose.

**Cas9-based integration of an expression cassette into a target locus**

The ternary system of Cas9-directed genome engineering consists of the Cas9 protein and a guide RNA that conduct the site-specific double-strand breaking, and a donor DNA fragment supplemented to repair the DSB. A plasmid carrying the Cas9 gene was first transformed into the yeast strain D452-2, then another plasmid carrying *PHO13* or *ALD6* gRNAs was co-transformed with donor DNA fragments containing the XYL123 cassette with 40 bp flanking homologous sequences to the target genes. Transformants were screened with aerobic xylose utilization or PCR verification. The detailed procedure is described in the supplementary protocol.

**Fermentation and HPLC analysis**

For oxygen-limited fermentation conditions, yeast strains were pre-cultured in YPD20 medium for 36 hours and the cells were then diluted to have an initial cell density of OD 1 with a 25 mL working volume. Flasks were kept at 30 °C on a 100 rpm shaker so the micro-aerobic conditions were maintained. For anaerobic fermentation conditions, the media were prepared by nitrogen flushing. After the pre-culture, water washed cells were seeded into serum bottles containing the media and sealed with butyl rubber stoppers. 0.5 mL samples were taken every 12 hours to measure the concentrations of metabolites (glucose, xylose, xylitol, ethanol, acetate, and glycerol) with an Agilent 1200 series high-performance liquid chromatography system equipped with a Rezex ROA Organic Acid H+ (8%) column. The mobile phase was 0.005 N of $H_2SO_4$. The flow rate was kept at 0.6 mL/min and the operating temperature was set at 50 °C.

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**Figure Legends**

Figure 1. The construction of CT2-Pro, a xylose-utilizing strain refactoring SR8 using Cas9, guide RNAs, and expression cassettes containing *XYL1*, *XYL2*, and *XYL3*. The CT1 strain has an insertion of *XYL1*, *XYL2*, and *XYL3* at *PHO13*. The CT2-auxo strain contains an additional copy of *XYL1*, *XYL2*, and *XYL3* at *ALD6* in the back ground of the CT1. CT2Pro is a prototrophic strain of the CT2-auxo. UHS/DHS: Upstream/downstream homologous sequences, L: Linker.

Figure 2. Xylose fermentation profiles of (A) DX123, (B) CT1b-1, and (C) CT2-auxo strains under 40 g/L xylose (YPX40) oxygen-limited conditions. Open square: xylose; open circle: OD600; filled square: ethanol; open star: glycerol; filled triangle: xylitol; asterisk: acetate.

Figure 3. Fermentation profiles of SR8 (A) and CT2-Pro (B) fermenting 40 g/L xylose (YPX40) under oxygen-limited conditions. Fermentation profiles of SR8 (C) and CT2-Pro (D) when fermenting a mixture of 70 g/L glucose and 40 g/L xylose (YPD70X40) under anaerobic conditions. Closed circle: glucose; open square: xylose; open circle: biomass; filled square: ethanol; open star: glycerol; open triangle: xylitol; asterisk: acetate.

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Table 1 Strains and plasmids used in this study.

| Strains | Descriptions | Sources |
|---|---|---|
| D452-2 | *MATa, leu2, his3,ura3, can1* | (Mumberg et al. 1995) |
| SR8 | D452-2 integrated with multiple copies of *XYL1*, *XYL2*, and *XYL3*, evolutionary engineered in xylose-containing media, and *ALD6* deletion. | (Kim et al. 2013b) |
| DX123 | D452-2 integrated with 1 copy of *XYL1*, *XYL2*, and *XYL3* in the URA3 locus. | (Kim et al. 2012a) |
| CT1 | D452-2 *PHO13*::XYL123 *leu2, his3,ura3, can1* | This study |
| CT2-auxo | CT1 *ALD6*::XYL123 *leu2, his3,ura3, can1* | This study |
| CT2-Pro | CT1 *ALD6*::XYL123 | This study |
| | | |
| Plasmids | | |
| pCas9-NAT | Replacing the *TRP1* in p414-TEF1p-Cas9-CYC1t with *NAT1*. | (Zhang et al. 2014) |
| gRNA-leu-HYB | gRNA cassette targeting *LEU2* cloned inside pRS42H. | (Zhang et al. 2014) |
| pCST154 | 20 bp gRNA sequences targeting *ALD6* replacing the LEU2 targeting site in gRNA-leu-HYB | This study |
| pCST155 | 20 bp gRNA sequences targeting *PHO13* replacing the LEU2 targeting site in gRNA-leu-HYB | This study |
| pSR5-X1 | pRS305 harboring a XYL1 cassette | Gift from S.R.K. |
| pSR3-X23 | pRS403 *PGK1*P-*XYL2*-*PGK1*T *TDH3*P-*XYL3*-*TDH3*T | (Kim et al. 2013b) |

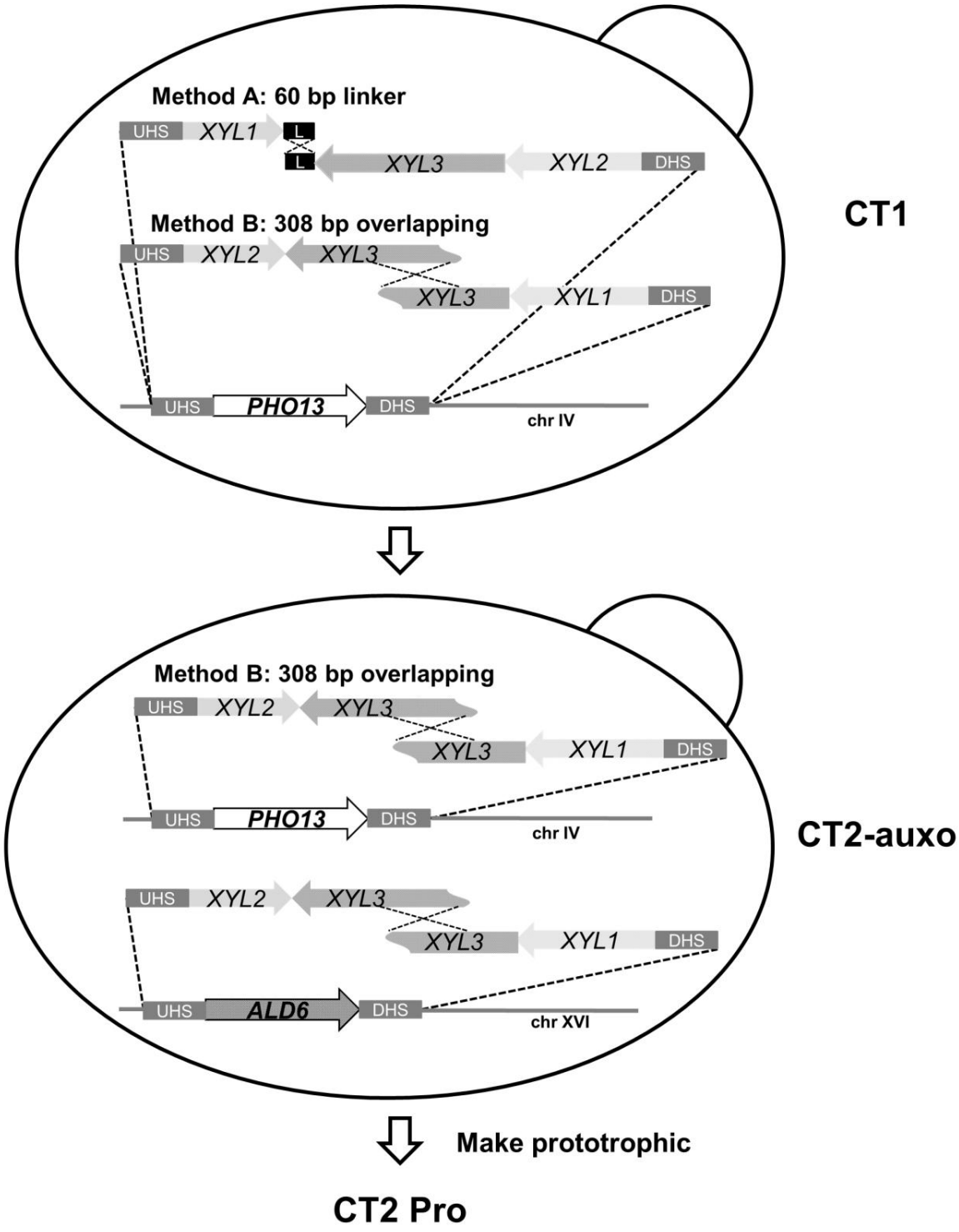
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| | | |
|---|---|---|
| pSR6-X123 | pRS306 *TDH3*P-*XYL1*-*TDH3*T *PGK1*P-*XYL2*-*PGK1*T *TDH3*P-*XYL3*-*TDH3*T | (Kim et al. 2013b) |

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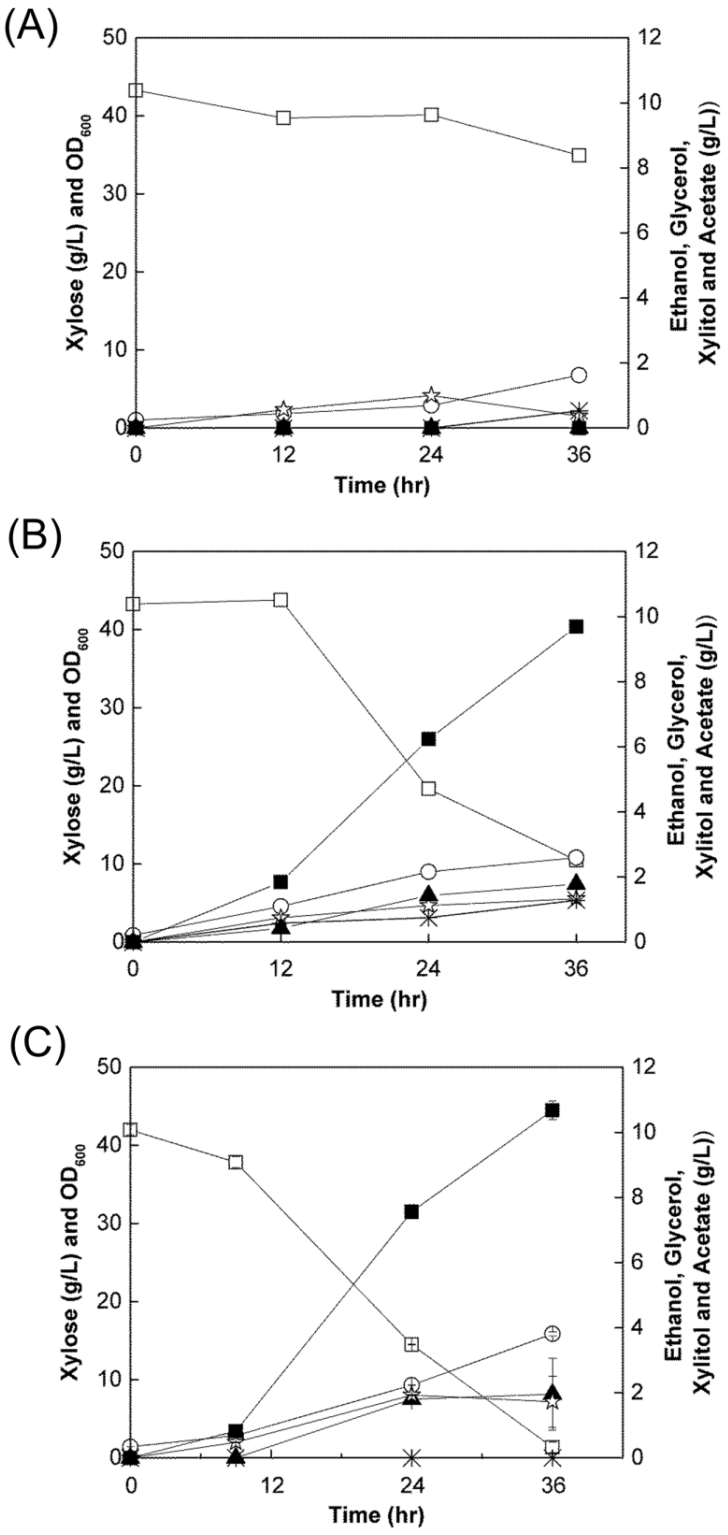
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Fig. 1 Tsai *et al.*



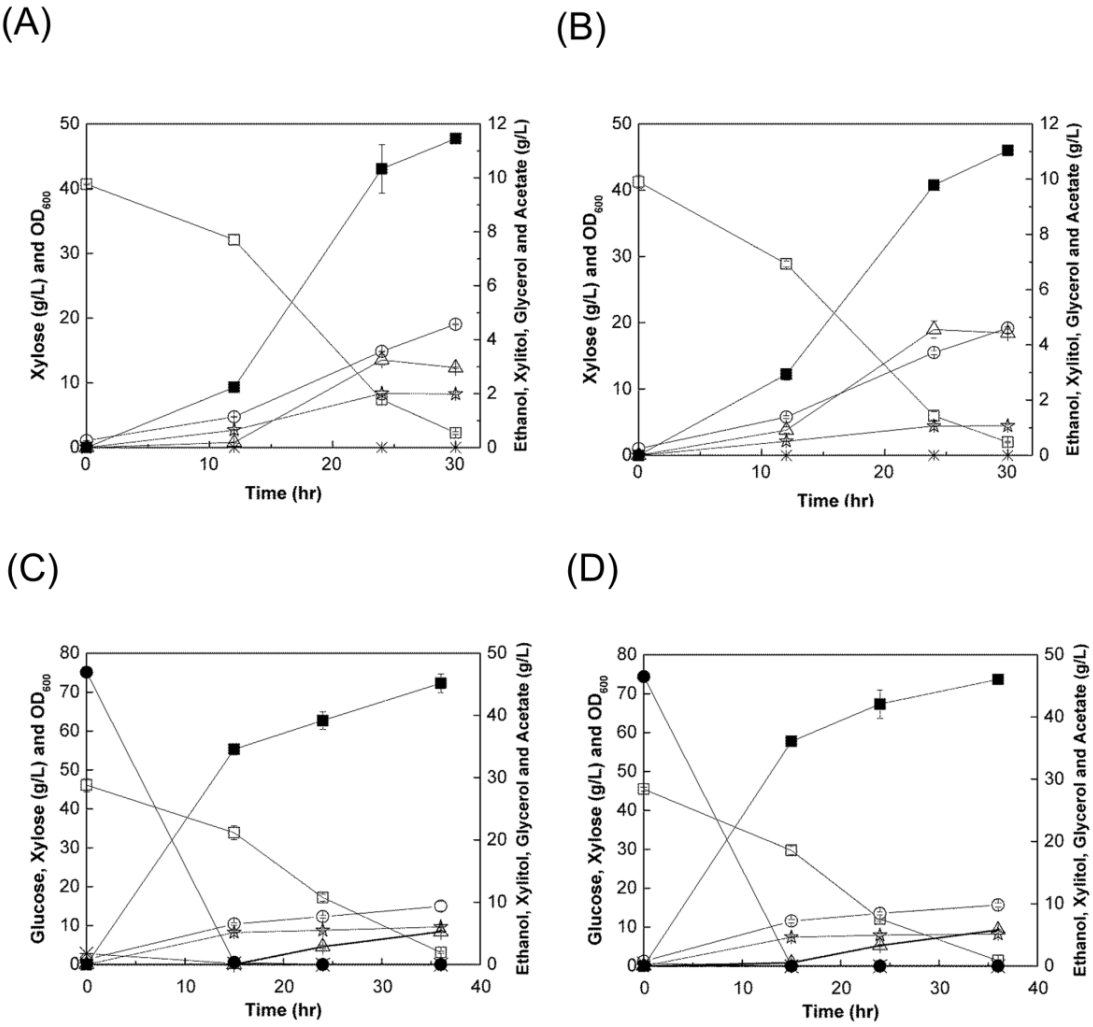

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Fig. 2 Tsai *et al.*




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Fig. 3




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