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Engineering Transaldolase in *Pichia stipitis* to Improve Bioethanol Production

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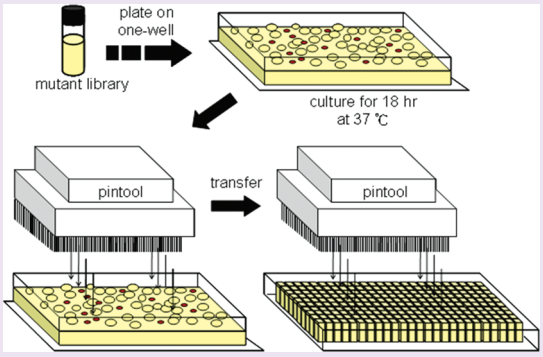
Supporting Information

ABSTRACT: In our effort to improve the efficiency and yield of xylose-to-ethanol bioconversion in *Pichia stipitis*, the transaldolase (TAL) in the pentose phosphate pathway was identified as a rate-limiting enzyme for improvement. A mutant containing the Q263R change was first obtained by directed evolution with 5-fold increase of activity, which was then incorporated into *P. stipites via* the pYDS vector to produce a genetically stable strain for fermentation on xylose. In comparison with the parental strain, TAL-Q263R(+) increases ethanol prodcution by 36% and 100% as measured by volumetric production rate and specific production rate, respectively. Thus improving the transaldolase activity in *P. stipitis* can significantly increase the rate and yield of xylose conversion to ethanol.



The bioconversion of lignocellulosic feedstocks to ethanol is an attractive route to create renewable fuels as well as to reduce carbon dioxide net emissions.[1] Hemicellulose and cellulose are the two major components in lignocellulosic materials, although the bioconversion of hemicellulose to ethanol is relatively less efficient than that from cellulose. Xylose is the most abundant sugar in hemicelluloses, so its efficient conversion to ethanol is key to the economical production of ethanol from lignocellulosic biomass.[2]

For ethanol production using xylose, *Pichia stipitis* has been known to have a great potential for industrial application, because it efficiently shunts most of its metabolic flux into ethanol.[3−5] *P. stipitis* can produce 22 g L^{-1} of ethanol from xylose with ethanol yields of 0.35−0.44 g_p g^{-1} of substrate.[4,6] *Saccharomyces cerevisiae* is currently the most efficient species for ethanol production from glucose,[7] although its utilization of xylose is extremely low.

In *P. stipitis*, xylose is first converted to xylitol by xylose reductase (XR) and then to xylulose by xylitol dehydrogenase (XDH); xylulose is subsequently phosphorylated by xylulokinase (XK) to form xylulose 5-phosphate (X5P). From xylose to X5P is the oxidative phase of the pentose phosphate pathway (PPP) (Figure 1a).[2,8,9] From here X5P is converted to both fructose 6-phosphate (F6P) and glyceraldehyde 3-phosphate (G3P), leading to the glycolytic pathway and ethanol fermentation. The X5P conversion occurs in the nonoxidative phase of PPP, involving ribulose 5-phosphate epimerase (RPE), ribose 5-phosphate ketol isomerase (RKI), transketolase (TKL), and transaldolase (TAL). In particular, transketolase (TKL) catalyzes the transfer of a C_2 unit from X5P to ribose-5-phosphate (R5P), yielding G3P and sedoheptulose-7-phosphate (S7P). Transaldolase then catalyzes the transfer of a C_3 unit from S7P to G3P, yielding erythorse-4-phosphate (E4P) and F6P. TKL comes in again, transferring a C_2 unit from a seond molecule of X5P to E4P to form G3P and another molecule of F6P. These enzmatic reactions are reversible (Figure 1b). Identification of the rate-limiting enzyme from xylose to ethanol is necessary for rational strain modification to improve the fermenting efficiency. The genome sequence of *P. stipitis*[10] allows identifying the gene related to the rate-determining enzyme controlling the xylose-to-ethanol conversion for further improvement. However, genetic manipulation in *P. stipitis* has been more challenging than in *S. cerevisiae*, because *P. stipitis* is less transformable, highly resistant to commonly used antibiotics, has unique codon usages, etc.[11,12] Efforts were then directed toward creating xylose-utilizable *S. cerevisiae* strains by incorporation of related genes from *P. stipitis*.[13−16] The engineered *S. cerevisiae*, however, still encountered many difficulties, such as accumulation of sedoheptulose 7-phosphate (S7P),[5,17] high xylitol secretion (as a result of NADH/NADPH imbalance),[18] and longer generation time,[19] suggesting that the PPP enzymes are regulated differently in *P. stipitis* than in *S. cerevisiae*, and genetic modification of *P. stipitis* to improve its ethanol production from xylose[11,12] is perhaps necessary toward this goal.

We sought to identify the key limiting enzyme in *P. stipitis*, improve its performance *via* directed evolution,[20−22] and develop an engineered *P. stipitis* strain to enhance xylose-to-ethanol

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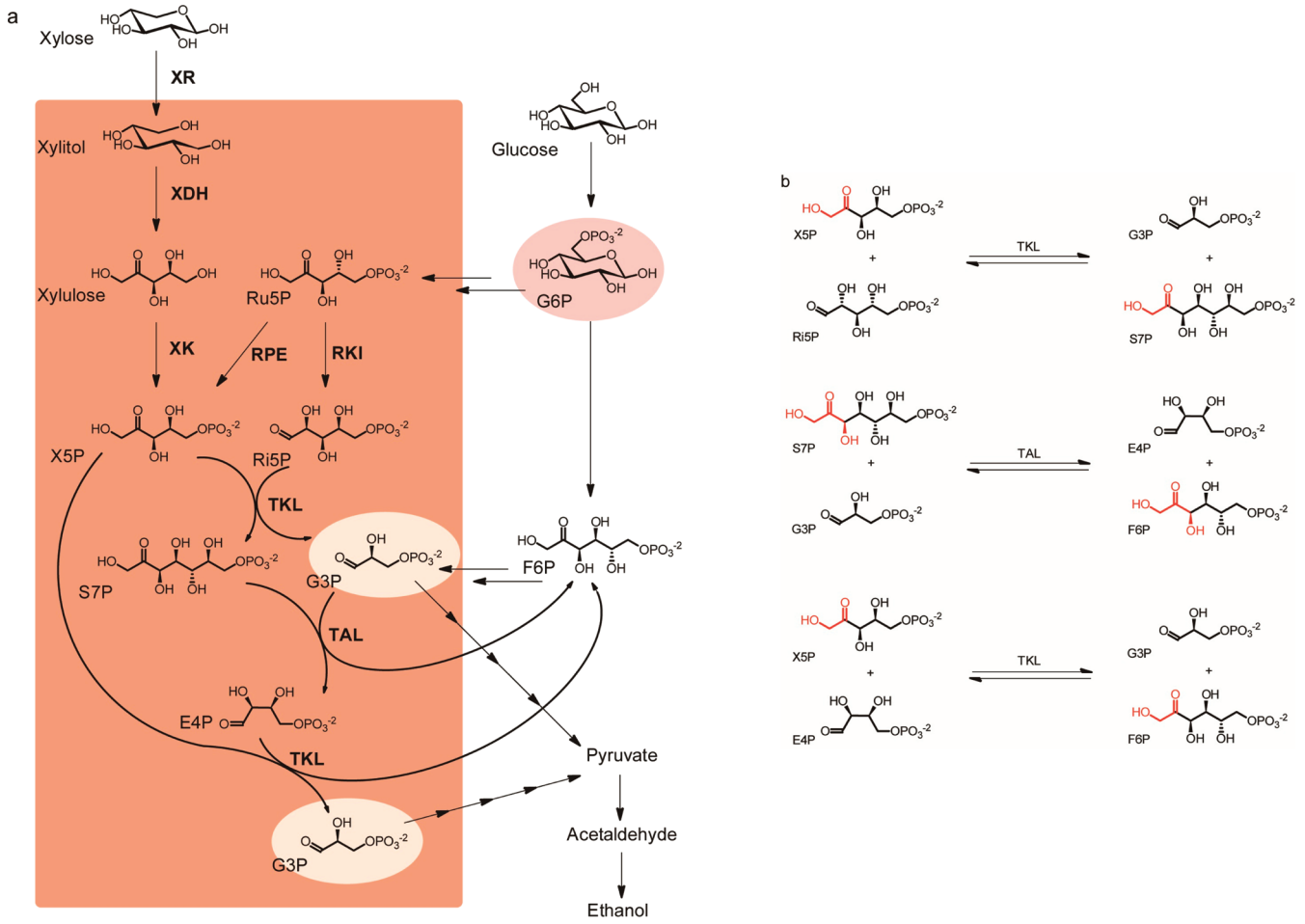

Figure 1. Xylose metabolism in the pentose phosphate pathway. (a) The colored box denotes xylose metabolism. Both F6P and G3P, which connects xylose assimilation to the glycolytic pathway for ethanol production, are highlighted. XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulokinase; RPE, ribulose 5-phosphate epimerase; RKI, ribose 5-phosphate ketol isomerase; TKL, transketolase; TAL, transaldolase; X5P, xylulose 5-phosphate; Ru5P, ribulose 5-phosphate; Ri5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; G3P, glyceraldehyde 3-phosphate; F6P, fructose 6-phosphate. (b) In the nonoxidative phase, TKL and TAL catalyze, respectively, a C_2- and a C_3-unit transfer.

production. Using both *in vitro* and *in vivo* analyses of RNA expression, enzymes kinetics, and ethanol production by addition of individual enzymes to *P. stipitis* strains, we concluded that TAL is a rate-limiting enzyme. Accordingly, we decided to improve the TAL activity by directed evolution, incorporate the evolved TAL with better activity in the parental TAL, and demonstrate the improved ethanol production of a TAL-engineered *P. stipitis* strain using xylose.

RESULTS AND DISCUSSION

Probing the Rate-Limiting Enzyme. We first set out to profile the enzymes involved in the xylose metabolism, wherein the mRNA level of each enzyme in the pathway was determined by quantitative PCR. Cells were cultured in a typical fermentation condition (oxygen-limited and in YPX medium (Bacto-yeast extract (10 g), Bacto-peptone (20 g), and 20% xylose), whereby the transcription levels for XR, XDH, XK, TKL, and TAL were up-regulated (Supporting Figure S2). On the basis of this measurement, the relative mRNA levels were ranked by their expression levels relative to actin and normalized by the highest one (Table 1; Supporting Figure S2).

However, the mRNA level may not directly reflect the enzymatic activity. Though XK was the lowest, a higher expression of XK was also known to provoke some adverse effects, for example, low growing rate and reduced ethanol production,[23] and whether increasing the expression and activities of TKL and TAL would improve the xylose flux toward a higher ethanol production remained to be tested (see below).[24−26]

We then decided to investigate the activity of individual enzymes involving in xylose metabolism. The genes of XR, XDH, XK, TKL, and TAL were amplified from *P. stipitis* CBS6054 genomic DNA and inserted into a cloning vector to express the enzyme in *E. coli* (Supporting Figure S3). Kinetics parameters of the enzymes were determined in a pseudo-first-order condition (Supporting Figure S4, Table S4). The turnover rate (k_{cat}) and the Michaelis constant (K_M) for each of these enzymes varied but within 2 orders of magnitude. Given the facts that the expression profiles vary from one condition to another and the mRNA transcription level is related to protein translation, the specificity constant (k_{cat}/K_M) was therefore modified by multiplying the normalized relative expression level to obtain relative specificity to approximate the activity *in vivo* for these selected enzymes. As a result, XK (1.1×10^2 M^{-1} s^{-1}) and TAL (3.7×10^2 M^{-1} s^{-1}) turned out to be

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Table 1. Expression Levels and Specific Coefficients for the Enzymes Involved in Xylose Metabolism

enzyme (variable)[a]	relative expression level	normalized relative expression level	k_{cat}/K_m (O_2-limited) (M^{-1} s^{-1})	k_{cat}/K_m × normalized relative expression level[b] (M^{-1} s^{-1})
XR[c] (NADPH)	19.7	1	$1.3 \times 10^6 \pm 7.5 \times 10^2$	1.3×10^6
(xylose)			$4.0 \times 10^2 \pm 1.9 \times 10$	4.0×10^2
XDH[c] (NAD)	9.6	0.48	$5.0 \times 10^4 \pm 8.7 \times 10^2$	2.4×10^4
(xylitol)			$1.8 \times 10^3 \pm 1.8 \times 10^2$	8.6×10^2
XK[c] (ATP)	2.3	0.11	$8.3 \times 10^3 \pm 2.4 \times 10$	9.1×10^2
(xylulose)			$1.0 \times 10^3 \pm 1.8 \times 10$	1.1×10^2
TAL[d] (F6P)	2.7	0.13	$2.4 \times 10^4 \pm 2.9 \times 10^3$	3.1×10^3
(E4P)			$2.9 \times 10^3 \pm 2.2 \times 10^2$	3.7×10^2
TKL[d] (X5P)	9.4	0.47	$1.4 \times 10^5 \pm 6.0 \times 10^3$	6.5×10^4
(R5P)			$9.4 \times 10^4 \pm 1.8 \times 10^4$	4.4×10^4

[a]F6P, fructose 6-phosphate; E4P, erythrose 4-phosphate; X5P, xylulose 5-phsphate; R5P, ribose 5-phosphate. [b]Adjusted k_{cat}/K_m by multiplying normalized relative expression levels (see text). [c]Enzymes in the oxidative phase of the PPP pathway. [d]Enzymes in the nonoxidative phase of the PPP pathway. (See Supporting Information Table S4 for detailed kinetic parameters).

the rate-limiting enzymes in the oxidative and oxygen-limited phases respectively in the PPP metabolic flux (Table 1).

Ethanol Production among Recombinant *P. stipitis*. We noted that the kinetics measured *in vitro* at individual enzyme levels may not accurately reflect its effect *in vivo*. Thus, to better understand the effect of each enzyme on ethanol production *in vivo*, TAL, TKL, RPE, RKI, XR, XDH, and XK as well as ADH1 were individually engineered and driven by the same promoter in the parent *P. stipitis* to obtain recombinant strains. The genetic transformation process began with constructing a new *P. stipitis* expression vector pYDS (Supporting Note) to successfully integrate each targeted gene into *P. stipitis* by homologous recombination and selection on YPD plates using Zeocin. In general, several hundreds of colonies appeared after incubation in 30 °C for 3 days, and colonies on the plate were picked and confirmed to express both endogenic and integrated enzymes by Western blotting with anti-6×His antibody (the positive rate is about 17%). The engineered *P. stipitis* strains identified were grown in YPD to saturation and transferred to a flask containing YPX (OD = 0.02) for xylose fermentation. In 48 h, the strain with TAL incorporation showed the most significant increase (30%) in ethanol production compared with the parental strain (PS) under the oxygen-limited condition (Supporting Figure S5). Of the other enzymes tested, TKL also improved ethanol production, perhaps due to the higher concentrations of S7P and G3P for TAL to drive more alcohol production. On the other hand, increasing the expression of XR or XK did not enhance ethanol production. Taken together, these findings suggest that TAL is a rate-limiting enzyme in PPP with activity close to TKL.[25,26] We then pursued directed evolution of TAL to improve its activity and then incorporate the gene of improved TAL in the parental strain with an attempt to increase the rate and yield of xylose fermentation to ethanol.

Mutant Library Plating. To avoid conventional colony pickings and to increase screening throughput, we took advantage of a 1536-pin device (pintool) to transfer a well-distributed TAL mutant library to 1536-well formatted microtiter plates to screen for improved TAL by directed-evolution (Figure 2). Initially, pET23a-psTAL was used as a template for the creation of the TAL mutant library by standard error-prone PCR mutagenesis method[27] with the controlled mutation rate of 2−5 bases per gene by $MnCl_2$ and amplified in *E. coli* DH5. The library size was determined by serial dilutions as 10^8 to 10^9 mL^{-1}. We examined the diversity of the library constructs by transforming the mutagenesis plasmid library to BL21(DE3) cells followed by randomly picking 10 transformant colonies for sequence analysis. We found that most of the selected mutants contained two to five mutations. The TAL mutant library containing ~10^8 CFU mL^{-1} was plated on one-well LB+AMP-100 agar plates. After the tranformants were grown into colonies, they were inoculated by the pintool to 1536-well assay plates containing 4 μL $well^{-1}$ LB+AMP-100 medium. For each transfer, three copies were made for TAL activity assay, cell growth assay, and hit-picking. The transformed cells could express TAL without IPTG induction, and the highest TAL yield could be obtained from the cells cultured at 20−22 °C.

High-Throughput TAL Activity Screening. For high-throughput TAL activity screening, a sensitive fluorescence-based assay was established for the Viewlux (PerkinElmer, Inc.) reader installed on a robotic high-throughput screening (HTS) system. Briefly, we used glyceraldehyde phosphate dehydrogenase (G3PDH) to convert G3P from TAL reaction to GA3P and generate reduced nicotinamide adenine dinucleotide (NADH). The content of NADH was then monitored by the generation of fluorogenic resorufin (ex/em = 535 nm/595 nm) from resazurin by diaphorase catalysis (Supporting Figure S6). By using this assay, we could complete the activity evaluation of a large and diversified TAL mutant library cultured in 1536-well plates in less than 5 s per plate.

To demonstrate the screening method, we applied a mixed library of *E. coli* transformants (positive plasmid pET23a-TAL clone denoted TAL(+) and pET23a clone denoted TAL(−)) to one-well source plates (Supporting Figure S7a) to see if TAL(+) clones could show up as hits by the method. The cell growth in the 1536-well assay plates was monitored by addition of Alamar Blue reagent, and the successful rate of colony transfer was determined. In the present setting, greater than 83% of colonies successfully grown in the 1536-well assay plates after transfer were reached (Supporting Figure S7b), which rendered more than 1,300 mutants per assay plate. Cells with signals higher than four times the average signal were hit-picked for confirmation of TAL(+) clones by PCR (Supporting Figure S7c and d). Five each of the hits (4× above average signal; Z′ value = 0.90) and 15 nonhits (slightly higher or about background average signal) wells were selected from an assay plate and subjected to colony PCR. The confirmation rate was about 100%, which indicated that our protocols of activity assay and HTS screening were able to identify active TAL mutants from the background.

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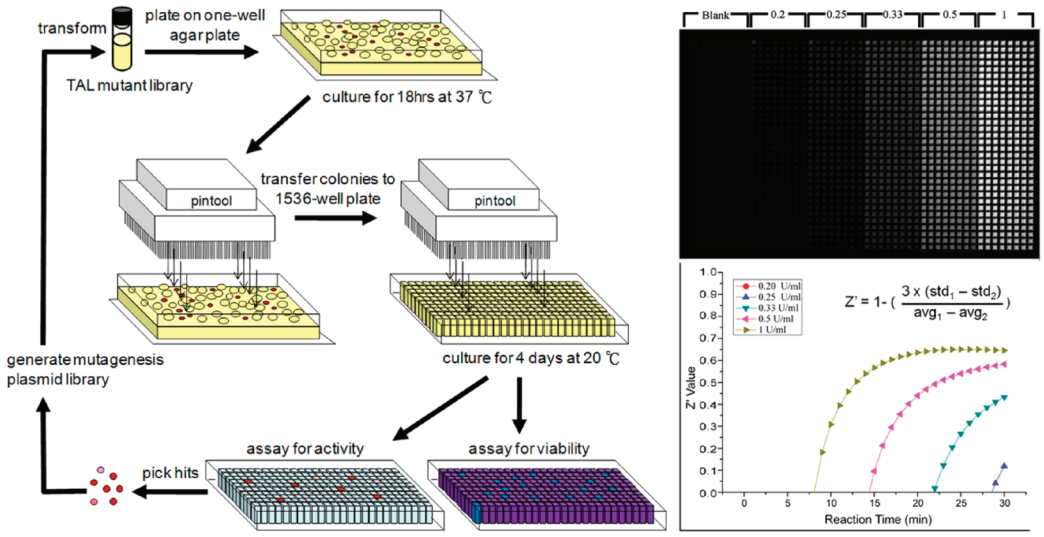
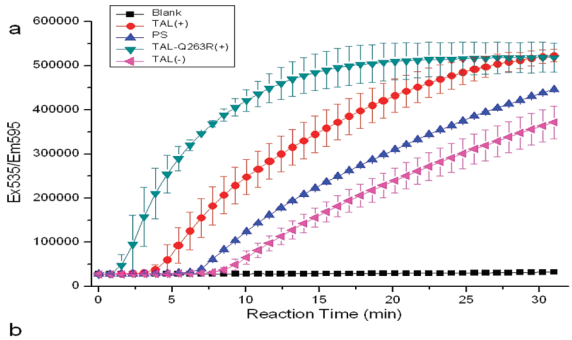


Figure 2. High-throughput screening method for evaluating TAL activity. The TAL mutant library was plated on several one-well plates as sources. Each source plate grew 2,000−5,000 colonies. A pintool of 1536 floating pins was applied to inoculate 1536-well assay plates containing 4 μL well^{-1} culture medium. For each transfer, three assay plates were made for cell growth assay, activity assay, and hit picking (not shown). These assay plates were incubated for 4 days to reach growing saturation. Interested clones with expected TAL activity were picked from the third assay plate into the next directed-evolution cycle. The TAL activity to F6P was evaluated by the developed fluorescence-based assay in 1536-well plates. A plate image was taken by Viewlux (ex/em = 535 nm/595 nm) at 20 min after addition of assay reagent. The title bar indicates the TAL activity in U mL^{-1}. The Z′ values were calculated and plotted with the reaction time. The Z′ = 0.7 can be reached for 1 U mL^{-1} TAL at reaction time of 20 min, and thus a clone in the well should grow to generate at least 1 U mL^{-1} to be considered as an active hit.

In a practical directed-evolution of TAL, we obtained 39 hits. DNA sequencing indicated that 18 clones possessed valid mutations (Supporting Table S3). We finally screened almost 60,000 colonies (4 × 10 × 1536) and identified two TAL mutants, TAL-Q263R and TAL-K190M. Both mutants contained a single amino acid change from the parental enzyme. Interestingly, both mutation points were not in the catalytic site of TAL based on the X-ray crystal structure (Supporting Figure S8),[28] an observation similar to previous report.[21,29,30] Enzyme kinetics was conducted to obtain the turnover rates (k_{cat}) and binding affinities (K_M) of both TAL mutants, and their specificity constants (k_{cat}/K_M) were found to increase >5-fold, correspondingly (Figure 3).



b

Enzyme	V_{max} (μmol · min^{-1} mg^{-1})	K_M [mM]	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} · s^{-1})	Mutation
TAL-wild	16.15±1.180	0.56±0.046	9.50±0.695	1.7×10^4	---
TAL-Q263R	55.11±2.333	0.32±0.075	32.42±1.372	9.8×10^4	Gln263Arg
TAL-K	44.84±2.920	0.30±0.021	26.38±1.718	8.8×10^4	Lys190Met

Figure 3. Kinetics assay for TAL mutants. (a) TAL-Q263R(+) strain showed better TAL activity than the parental *P. stipitis* (PS). TAL(+) and TAL(−) denotes the recombinant strains with wild-type TAL, and vector only, respectively. (b) Two mutants, TAL-Q263R and TAL-K190M, had almost 6-fold enhancement of TAL activity.

Ethanol Production of TAL-Engineered *P. stipitis* Strains. Wild-type TAL and the mutant, TAL-Q263R, were genetically integrated to the *P. stipitis* genome by using the newly established expression vector pYDS through homologous recombination to obtain TAL-wild(+) and TAL-Q163R(+) strains. As expected, the engineered strains overexpressed not only endogenic but also the integrated TAL when cultured on xylose (Supporting Figure S9).

In the aerobic condition, we found that both TAL-wild(+) strain and parental strain grew on xylose and produced ethanol (Supporting Figure S10 and S11), and the maximum ethanol yield was calculated to be 0.4 g_p g_s^{-1} (about 78% of theoretical conversion efficiency). When culturing under the oxygen-limited condition, we repeatedly obtained 30% increase in ethanol production for the TAL-wild(+) strain and 35−40% increase for the TAL-Q263R(+) strain on xylose (Supporting Figure S12a).

Both engineered strains produced about 2.2% (w/w) of ethanol (maximum yield = 0.44 g_p g_s^{-1}; theoretical conversion efficiency = 86%) in 48 h. Despite all strains reaching a saturation at 72 h (2.2% alcohol, w/w), the volumetric ethanol production rates (the slopes in the linear range of EtOH production at 12−36 h) for PS, TAL-wild(+), and TAL-Q263R(+) were determined to be 0.64, 0.69, and 0.87 g L^{-1} h^{-1}, respectively, which correlate well with the consumption of xylose (Table 2, Supporting Figure S12b, Figure 4a). Evidently, TAL-Q263R(+) is more productive than PS and TAL-wild(+) by 36% and 25%, respectively. It is noted that the improvement is less than that *in vitro*, perhaps limited by the activities of the other enzymes, likely the one with activity close to that of TAL, *i.e.*, TKL. However, the specific ethanol production rates[31] *in vivo* were determined to be 0.18, 0.31, and 0.36 g g^{-1}(dry-cell-wt) h^{-1} for PS, TAL-wild(+), and TAL-Q263R(+), respectively (Table 2, Figure 4b), corresponding to a 100% increase for TAL-Q263R(+). Given that the cell population of the parental *P. stipitis* grew denser than those of engineered strains (Supporting Figure S9c), the ethanol production efficiency per yeast cell of TAL-Q263R(+) indeed

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Table 2. Xylose Consumption and Ethanol Production Rates

strain	xylose (g L^{-1} h^{-1})	EtOH (g L^{-1} h^{-1})[a]	EtOH (g g DCW^{-1} h^{-1})[b,c]
PS	1.435 ± 0.0435	0.637 ± 0.0312	0.184 ± 0.0086
TAL(+)	1.446 ± 0.0562	0.691 ± 0.0461	0.307 ± 0.0025**
TAL-Q263R(+)	1.663 ± 0.0359	0.865 ± 0.0475	0.363 ± 0.0153**,##

[a]Volumetric ethanol production rate. [b]Specific ethanol production rate. [c]Calculated at 24 h. Double asterisk sign (**) stands for significant difference from the value of PS at the levels of $p < 0.01$ (both mutant strains vs the parental strain). Double pound sign (##) stands for significant difference between TAL-wild(+) and TAL-Q263R(+) at the levels of $p < 0.05$. DCW: dry cell weight.

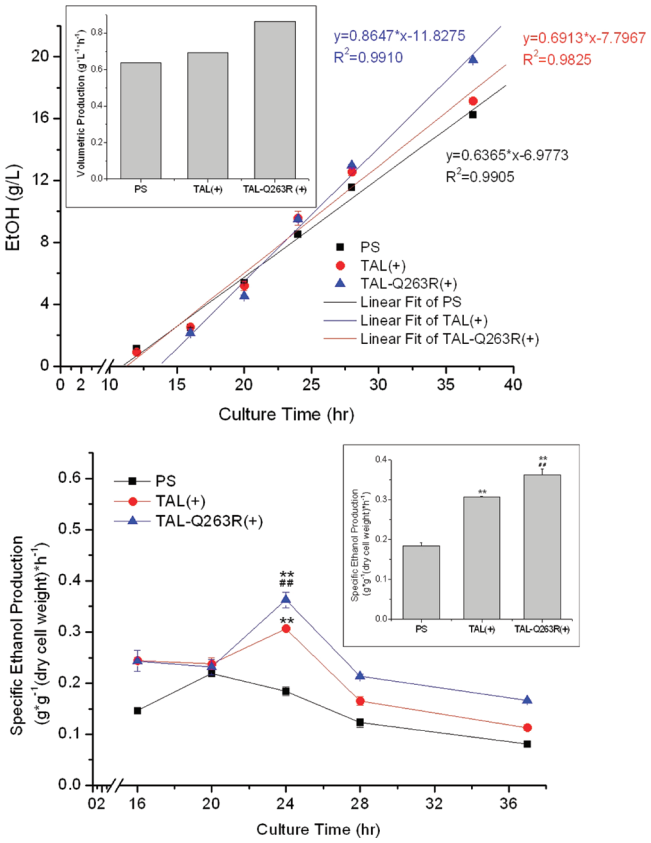

Figure 4. Rate determination for volumetric and specific ethanol production rates. (a) The volumetric ethanol production rates, and (b) the specific ethanol production rates. TAL-Q263R(+) showed better specific ethanol production than TAL(+) and the parental *P. stipitis* (PS). Double asterisk sign (**) stands for significant difference from the value of PS at the levels of $p < 0.01$ (both mutant strains *versus* the parental strain). Double pound sign (##) stands for significant difference between TAL-wild(+) and TAL-Q263R(+) at the levels of $p < 0.05$.

was significantly enhanced. Since the same cell density may not truly reflect the same quantities of TAL-wild(+) and TAL-Q263R(+), we then performed Western blotting to quantify both proteins under the same cell density, and it was found that the expressions of TAL-wild(+) and TAL-Q263R(+) made no difference at the protein level (Supporting Figure S7c). As a result, the TAL-Q263R(+) strain is better in xylose utilization and ethanol production than both the parental strain and the TAL-wild(+).

Conclusion. An *in vivo* comparison among *Pichia stipitis* strains engineered by introducing various PPP enzymes demonstrated that cells with overexpressed transaldolase (TAL) had the highest xylose utilization and ethanol production. This finding led us to conclude that TAL is a rate-limiting enzyme in *P. stipitis*. A sensitive fluorescence-based assay and a large-scale colony transfer method were established to facilitate the screening of TAL activity in a high-throughput manner. Several TAL mutants with higher activities were successfully identified from a vast mutant library. A new expression vector, pYDS, was developed for *P. stipitis* and applied to generate recombinant *P. stipitis* strains containing TAL mutants with improved activities by directed-evolution. These recombinant *P. stipitis* strains, when grown on xylose, expressed the engineered TAL mutant in addition to endogenic TAL. As a result, these cells used xylose more efficiently to produce ethanol than did the parental strain. The engineered strain, TAL-Q263R(+), is genetically stable; its TAL expression in the 10th generation was similar to that of the 5th generation (Supporting Figure S13). The cell density of TAL-Q263R(+) was lower than that of the parental *P. stipitis*, but the rate and yield of ethanol production were significantly improved. We reasoned that TAL-Q263R(+) has a higher capacity of channelling xylose metabolite to produce ethanol than to build biomass. Our next step is to improve TKL catalytic efficiency as well as to increase ethanol tolerance of *P. stipitis*.

METHODS

All experimental procedures were detailed in Supporting Information. In short, plasmid construction, recombinant protein expression, purification, and confirmation of purity were performed using standard protocols, wherein genes amplified from *P. stipitis* genomic DNA were cloned and expressed in *E. coli*. Cell cultrue, RNA purification, quantitative PCR, and enzyme kinetics were executed following standard methods. Enzyme evolution was carried out by the method described previously, in which the mutant library was created by means of error-prone PCR. A sensitive fluorescence-based assay was developed to facilitate selecting TAL variants in an modified high-throughput screening routine. Stable *P. stipites* mutants were made *via* the specific vector pYDS. Ethanol yield and xylose consumption were quantified and presented in conventional ways. Other related experimental procedures can be found in Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Supporting methods, supporting results, supporting references. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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