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Generation of Xylose-Fermenting *Saccharomyces Cerevisiae* by Protein-Engineering

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1. Introduction

The utilization of ethanol produced from plant biomass (so-called “bioethanol”), which is derived from the fixation of atmospheric CO₂, as an industrial carbon source and car fuel is one of the most important research issues for the realization of a sustainable global environment. Currently, bioethanol is produced mainly from agricultural crop biomass, which, however, compete with food and animal feed. Alternatively, “lignocellulosic biomass”, such as woods and agricultural residues, is an attractive feedstock, and consists of cellulose, hemicellulose, and lignin. In agricultural crops, corn for example, starch, consisting of cellulose (hexose polymers), accounts for 70% of the mass, and its biological fermentation is easy. On the other hand, in lignocellulosic biomass, hemicellulose, accounting for 30% of the mass, is composed of pentoses as well as hexoses such as xylose (and L-arabinose), which cannot be fermented by natural microorganisms.

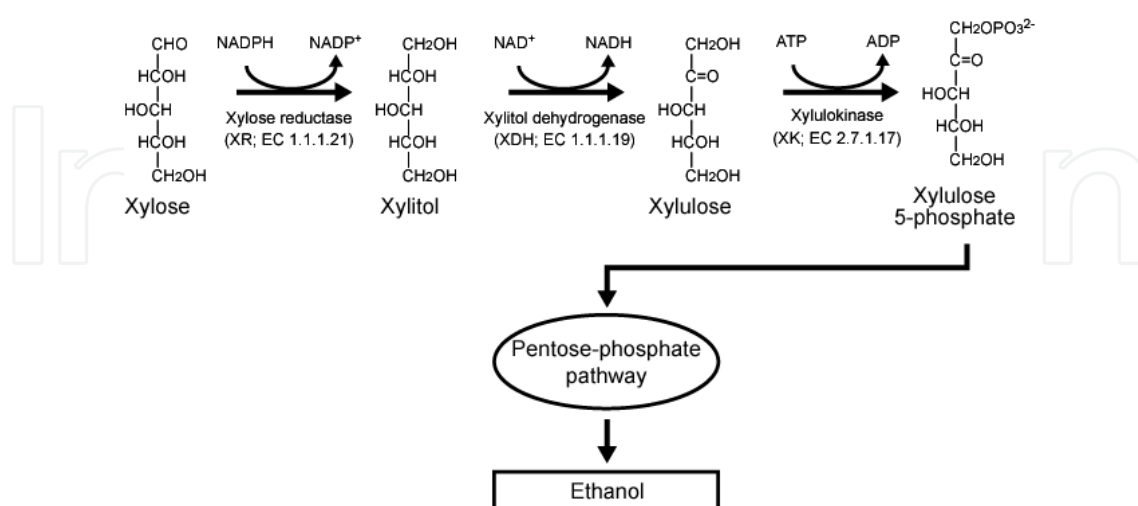


Fig. 1. Xylose metabolism in recombinant *S. cerevisiae*.

Yeasts, in particular, *Saccharomyces cerevisiae* have long been used for production of alcoholic beverages such as wine, beer and Japanese “sake”, but the native strains also cannot ferment

xylose as a carbon source. On the other hand, *S. cerevisiae* transforming with genes encoding to xylose reductase (XR; EC 1.1.1.21) and xylitol dehydrogenase (XDH; EC 1.1.1.9) (mainly from inherently xylose-metabolizing yeast, *Pichia stipitis*: PsXR and PsXDH) acquires the ability to ferment xylose to ethanol (Fig. 1), whereas produce the excretion of xylitol without the addition of a co-metabolizable carbon source. This is probably caused by several combined factors, and in particular, an intercellular redox imbalance due to a different coenzyme specificity of XR (with NADPH-preference) and XDH (with strict NAD⁺-dependence) has been thought to be one of the main factors (Jeffries & Jin, 2004). Therefore, generation of novel XR or XDH mutants with modified coenzyme specificity by protein-engineering approach can solve this bottle neck problem.

2. Protein-engineering of PsXDH

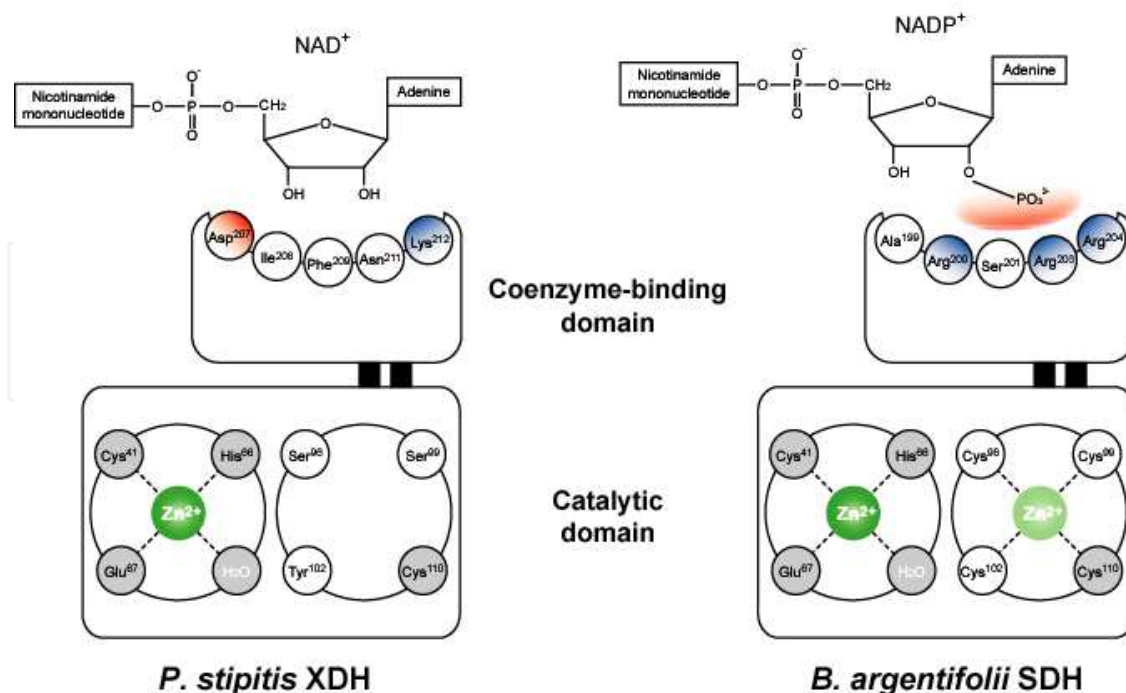
XDH belongs to polyol dehydrogenase (PDH) subfamily in a medium-chain dehydrogenase/reductase (MDR) superfamily (Nordling et al., 2002), in which also contains sorbitol dehydrogenase (SDH; EC 1.1.1.14) and L-arabinitol 4-dehydrogenase from several organisms. Most PDHs catalyze strict NAD⁺(H)-dependent interconversion between alcohols and their corresponding ketones or aldehydes. As well as other MDR enzymes, the coenzyme-binding mode is followed by a classical “Rossmann-fold”, and one zinc atom at the catalytic site is necessary for enzyme activity (referred to as “catalytic zinc”) (Fig. 2). Furthermore, some possess an additional second zinc atom, which is known as a “structural zinc”, although its role has not yet been clarified.

2.1 Modification of coenzyme specificity toward NADP⁺

2.1.1 Strategy of mutant design

Since NAD⁺(H) only differs from NADP⁺(H) in the phosphate group esterified at 2'-position of adenosine ribose, a limited number of amino acid residues interacting with these portions are the first candidates for protein engineering using site-directed mutagenesis. Most of these studies are Rossmann-fold type oxidoreductases, and “landmark” amino acid residues have already been proposed for the discrimination between NAD⁺(H) and NADP⁺(H) (Baker et al., 1992).

Generally, subunit structures in the MDR superfamily are comprised of two domains, namely a “coenzyme binding domain” within the intermediate segment and a “catalytic domain” consisting of N- and C-terminal segments (Fig. 2). The former possesses a similar β - α - β motif, centred around a highly conserved Gly-X-Gly-X-X-Gly sequence (where X is any amino acid; positions 9~11 in Table 1), in which several specific amino acid residues play a important role to discriminate between NAD⁺(H) and NADP⁺(H). The primary determinant of NAD⁺(H) specificity is the presence of an aspartate residue which forms double-hydrogen bonds to both the 2'- and 3'-hydroxyl groups in the ribosyl moiety of NAD⁺(H), and induces negative electrostatic potential to the binding site. Commonly, this residue in NADP⁺(H)-dependent dehydrogenases is replaced by a smaller and uncharged residue such as glycine, alanine and serine, accompanied by the concurrent presence of an arginine residue that forms a positive-binding pocket for the 2'-phosphate group of NADP⁺(H).



Colors of red and blue indicate positive- and negative-charge, respectively. Dark- and light-green zinc atoms correspond to catalytic and structural zinc, respectively. The gray-colored ligands are conserved between the two enzymes.

Fig. 2. Schematic diagram of PsXDH and BaSDH structures.

Bemisia argentifolii (whitefly) SDH (BaSDH) is the only NADP⁺(H)-dependent enzyme among the characterized PDHs (Banfield et al., 2001). Although the crystal structure of BaSDH-NADP⁺ complex has not yet been resolved, a phosphate ion from the crystallization buffer is found adjacent to Ala¹⁹⁹, Arg²⁰⁰ and Arg²⁰⁴, in the apo-form structure (Fig. 1). The Ala¹⁹⁹-Arg²⁰⁰ is obviously homologous to Asp²⁰⁷-Ile²⁰⁸ in PsXDH (positions 12 and 13 in Table 1) as described above. These aspartate and hydrophobic residues are conserved completely among other NAD⁺(H)-dependent PDHs.

2.1.2 D207A/I208R mutant

Therefore, D207A (Asp²⁰⁷→Ala), I208R and AR (D207A/I208R) mutants were first constructed, functionally expressed as (His)₆-tagged enzymes in *Escherichia coli* and purified by Ni²⁺-affinity chromatography, together with wild-type (WT) enzyme (Fig. 3) (Watanabe et al., 2005). Single substitutions produced a more positive effect on NADP⁺ kinetics than those of NAD⁺; their $k_{\text{cat}}/K_{\text{m}}^{\text{NADP}}$ values showed an approximately 5~48-fold increase (Fig. 4). However, the mutant enzymes still preferred NAD⁺ to NADP⁺. Double mutation, AR, led to a moderate increase of $k_{\text{cat}}/K_{\text{m}}^{\text{NADP}}$ compared to single mutations. In particular, there was a complete synergistic effect in the AR mutant; the increase in $k_{\text{cat}}/K_{\text{m}}$ for AR was 250-fold, the same as the product of the ratios of the increase in $k_{\text{cat}}/K_{\text{m}}$ for D207A and for I208R. On the other hand, $k_{\text{cat}}/K_{\text{m}}^{\text{NAD}}$ values did not significantly change in the mutants. Overall, the double mutants improved the catalysis for NADP⁺, although $k_{\text{cat}}/K_{\text{m}}^{\text{NADP}}$ (149 min⁻¹ mM⁻¹) did not reach the $k_{\text{cat}}/K_{\text{m}}^{\text{NAD}}$ of WT (2,760 min⁻¹ mM⁻¹).

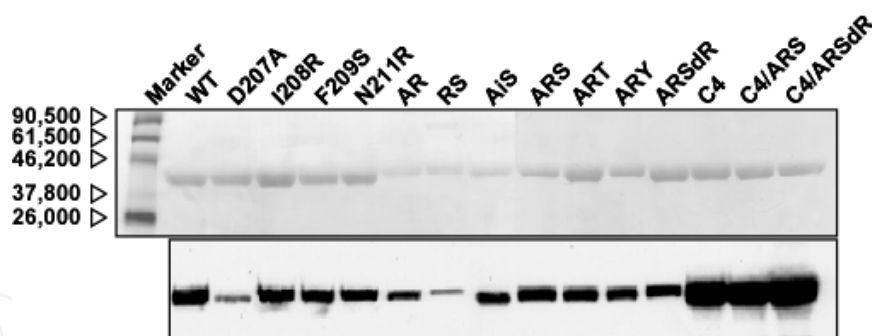


Fig. 3. Expression and purification of recombinant PsXDHs.

SDS-PAGE of purified enzyme (upper panel); intercellular expression level in *E. coli*, estimated by Western blot analysis (lower panel).

Enzyme	Source organisms	Coenzyme	Zn ²⁺	Structural zinc loop				Rossmann-fold motif				
				12	34	56	7	8	1	1	111	1
				9	0	1	234	5				
XDH	<i>P. stipitis</i>	NAD ⁺	1	91	EPGIPSRFSDEYKSGHYNLCPH	181	VFCAGPVG	GLLAAAVAKTFGAKGVIVVDIFDNKL				
XDH	<i>G. mastotermis</i>	NAD ⁺	1	93	EPGVPSRHSDEYKSGRYNLCPH	176	IYFCAGPVG	GLLVAAVASAFGAESVTIIDLVE SRL				
XDH	<i>S. cerevisiae</i>	NAD ⁺	1	94	EPGIPDRFSPEMKEGRYNLDPN	177	VFCAGPPIG	GLLAGKVASVFGAADVVFVLDLENKL				
XDH	<i>M. morgani</i>	NAD ⁺	1	90	EPGIPDLQSPQSRAGIYNLDPA	177	VFCAGPTIG	GIITQ SALAG-GCSDV IICDVFDEKL				
XDH	<i>A. oryzae</i>	NAD ⁺	2	97	EPGIPCRRCPECKEGRYNLCEK	179	VFCAGPVG	GLLCCAVARAFGSPKVI IAVDIQRGRL				
SDH	<i>B. argentifolii</i>	NADP ⁺	2	91	EPGVPCRRCEFCCKEGRYNLCPD	174	VFCAGPPIG	GLVSVLAAKAYGAFV-VCTARSPRRL				
SDH	<i>S. cerevisiae</i>	NAD ⁺	1	93	EPGVPSRYSDETKEGRYNLCPH	177	VFCAGPVG	GLLTGAVARAFGATDVI FVDVFDNKL				
SDH	Rat	NAD ⁺	1	113	EPGVPREVDEYCKIGRYNLTP	196	VFCAGPVG	GMVTL L VAKAMGAAQVVVTDLSASRL				
SDH	<i>Callithrix</i> sp.	NAD ⁺	1	95	EPGAPRETDEFCKTGRYNLSP	178	VFCAGPTIG	GLVTL L VAKAMGASQVVVTDLSAPRL				
SDH	<i>S. pombe</i>	NAD ⁺	2	92	EPGCVCRLCDYCRSGRYNLCPH	175	VMCCGTVG	GLLMMVAKAYGAIDIVAVDASPSRV				
SDH	Sheep	NAD ⁺	1	92	QPGAPROTDEFCKIGRYNLSP	175	VFCAGPTIG	GLVNL L AAKAMGAAQVVVTDLSASRL				
SDH	Silkworm	NAD ⁺	2	90	EPTQPCRSCELCRGRYNLQVE	173	ILFCAGPTIG	GLLCAMSAKAMGASKI I L T D V V Q S R L				
SDH	Peach	NAD ⁺	2	107	EPGISWCRCQCKGGRYNLCPD	190	VFCAGPTIG	GLVSVLSARAFGAARIVIVD V D D E R L				
SDH	Apple	NAD ⁺	1	113	EPGVPREVDEYCKIGRYNLTP	185	IYFCAGPTIG	GLVSVLAAARAFGAPRIVIVDMDRRL				
SDH	<i>E. japonica</i>	NAD ⁺	2	114	EPGISCKRCNLCKQGRYNLQK	197	VFCAGPTIG	GLVTL L AARAFGAPRIV I A D V N D E R L				
SDH	Human	NAD ⁺	1	94	EPGAPRENDEFCKMGRYNLSPS	177	VFCAGPTIG	GMVTL L VAKAMGAAQVVVTDLSATRL				
LAD1	<i>H. jecorina</i>	NAD ⁺	2	116	EPNIICNAPECLTGRYNGCEK	198	VFCAGPTIG	GLVSM L CAAAAGACPLVITDI SE SRL				

Table 1. Partial sequence alignment of the PDH subfamily enzymes including PsXDH.

2.1.3 D207A/I208R/F209S mutant

It is difficult to judge whether the position of 209 should be involved in the mutation sites, because there is homologous Ser residue regardless of NAD⁺(H)- and NADP⁺(H)-dependent enzyme(s) (position 14 in Table 1). In fact, the homologous position has not been targeted by site-directed mutagenesis for the reversal of coenzyme specificity in other NAD(P)⁺-dependent dehydrogenases. Very surprisingly, an additional introduction of F209S mutation into AR mutant led dramatic striking effects in kinetic constants for both NAD⁺ and NADP⁺ (Fig. 4) The k_{cat}/K_m^{NAD} dropped 15-fold compared to WT by an increase of K_m and a decrease of k_{cat} . On the other hand, k_{cat}/K_m^{NADP} increased dramatically (up to 4,100-fold), caused by a large decrease of K_m value and increase of k_{cat} value. It is noteworthy that k_{cat}/K_m^{NADP} (2,790 min⁻¹ mM⁻¹) was almost identical to that of WT for NAD⁺.

F209S single mutation itself and/or double mutation with D207A or I209R (AiS and RS mutants, respectively) gave no significant effect, compared with ARS mutant. What is important factor(s) for the change of the kinetics constants for NADP⁺ between AR and ARS? ARY (D207A/I208R/F209Y) and ART (D207A/I208R/F209T) mutants were constructed for analysis of the volumetric effect of hydroxyl side chain (Fig. 4). k_{cat}/K_m^{NADP}

value of ART mutant was almost the same as that of ARS. Furthermore, the k_{cat} values for NADP^+ was reduced by an order of magnitude from $\text{ARS} > \text{ART} > \text{AR} > \text{ARY}$ in agreement with the order of the side-chain volume. These results indicate that not only the hydroxyl group but also the relatively small volume group is required for the side chain of the residue at this position to make hydrogen bonds with amino moiety(s) of the side chain of neighbour amino acid residues (probably Arg^{208} and Lys^{212} in the ARS mutant) similar to BaSDH. Rosell et al. (2003) reported a study about several $\text{NAD}^+(\text{H})$ -dependent mutants of a vertebrate $\text{NAD}^+(\text{H})$ -dependent alcohol dehydrogenase (ADH) isozyme 8. For complete reversal of the coenzyme specificity toward $\text{NAD}^+(\text{H})$, substitution of three amino acid residues at homologous positions of Asp^{207} - Ile^{208} - Phe^{209} in PsXDH was necessary. These results indicate that, in some cases of dehydrogenase, an amino acid residue close to the “landmark” amino acid residues would also be effective for coenzyme recognition.

2.1.4 D207A/I208R/F209S/N211R mutant

Although ARS mutant shows a satisfied ratio of $\text{NADP}^+/\text{NAD}^+$ preference in terms of k_{cat}/K_m , 15.4, the thermostability decreased significantly, as described below. BaSDH possess Arg^{203} , while the basic residue is only rarely seen among $\text{NAD}^+(\text{H})$ -PDHs involving PsXDH (position 15 in Table 1). A single mutation of N211R gave no effect on coenzyme specificity (Fig. 4). On the other hand, introduction of this mutation into ARS mutant (ARSdR) produced about a 2-fold decrease in the $k_{\text{cat}}/K_m^{\text{NAD}}$ value mostly because of a dramatic decrease of K_m value compared to WT, whereas no additional change in the kinetics constant for NADP^+ was observed. Finally, this best quadruple mutant showed over 4,500-fold higher $k_{\text{cat}}/K_m^{\text{NADP}}$ and over 32-fold lower $k_{\text{cat}}/K_m^{\text{NAD}}$ values, compared with WT; complete reversal coenzyme specificity was achieved. Furthermore, the N211R mutation compensated thermolability in ARS mutant (see below).

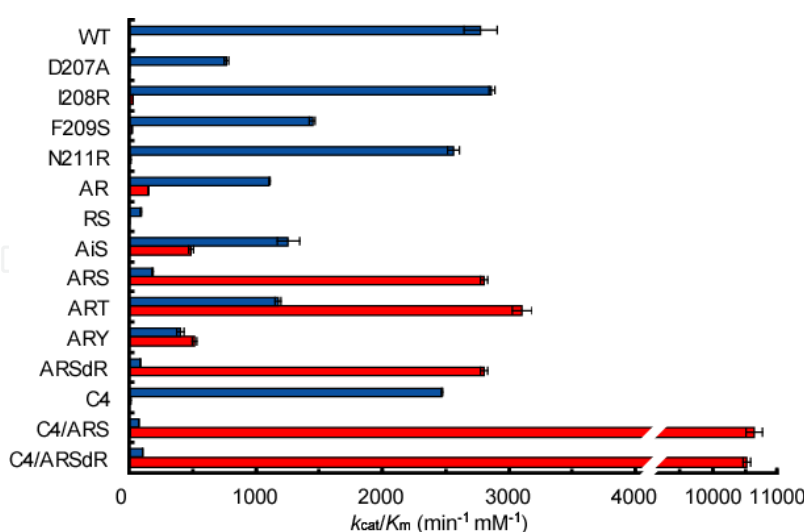


Fig. 4. Catalytic efficiency (k_{cat}/K_m) with NAD(P)^+ for WT and mutant PsXDH.

2.1.5 Importance of reference enzyme(s)

Metzger & Hollenberg (1995) also attempted to identify a set of amino acid residues in PsXDH for specificity toward NAD^+ . They introduced the potential $\text{NADP}^+(\text{H})$ -recognition

sequence of *E. coli* glutathione reductase (no homology with PsXDH) and thermophilic ADH (~30% homology) into the homologous sequence in PsXDH, Asp²⁰⁷-Ile²⁰⁸-Phe²⁰⁹-Asp²¹⁰-Asn²¹¹-Lys²¹². Among several mutants constructed, D207V/I208R/F209S/D210H mutant, which is very similar to the D207A/I208R/F209S (ARS) mutant, was not expressed in the host cell, in contrast of this study (see Fig. 3). Generally, there are a few sequence homologies among MDRs at the coenzyme recognition site except some specific residues, although the conformational fold is conserved. BaSDH, a reference enzyme in this study, is phylogenetically closer to PsXDH than glutathione reductase and/or ADH, supposing that the step-by-step acquirement of activity with NADP⁺ in PsXDH seems to mimic the natural evolutionary process.

2.2 Thermostabilization

Protein stability is one of most important factors for application of industrial enzymes. Although XDH itself is not directly utilized for ethanol production, it is well known that there is significant relationship between the stability and intercellular expression level. Since there have been no reports about the XDH of thermophilic microorganisms, we cannot undertake site-directed mutagenesis based on the structural comparison of thermophilic XDH and mesophilic PsXDH and/or the random mutagenesis method. Alternative method for the thermostabilization should be developed.

2.2.1 Strategy of mutant design

In the BaSDH (Banfield et al., 2001), structural zinc atom is coordinated with four cysteine residues (Cys⁹⁶, Cys⁹⁹, Cys¹⁰² and Cys¹¹⁰) as well as many other structural zinc-containing ADHs (a pattern of C-C-C-C) (Fig. 1 and positions 2, 4, 6 and 7 in Table 1). There are some substitution patterns of these four cysteines in the PDHs. Among them, enzymes with the pattern of D-S-M-D, R-D-C-S, R-D-C-T have been shown to have no structural zinc. These analyses suggested that four fully conserved cysteine residues are necessary for binding to the structural zinc atom, and that PsXDH having a pattern of S-S-Y-C, has no structural zinc atom. On the other words, it is likely that the substitutions of Ser⁹⁶, Ser⁹⁹ and Tyr¹⁰² by cysteine residues may increase the thermostability due to introduction of the structural zinc; so-called C4 mutant (S96C/S99C/Y102C) (Watanabe et al., 2005).

2.2.2 Introduction of structural zinc

Atomic absorption spectroscopy analysis revealed that the WT and C4 mutant contain 1.0 and 1.9 mol zinc/mol subunit. Since the putative binding site for the catalytic zinc is conserved completely in PsXDHs (Cys⁴¹, His⁶⁶ and Glu⁶⁷), compared to other MDRs, this result should be interpreted as the acquirement of an additional zinc in C4 mutant. The C4 mutant displayed almost the same k_{cat}/K_m^{NAD} value; strict NAD⁺-dependent enzyme (Fig. 4). The thermostability of the C4 mutant was estimated from the T_m value by circular dichroism (CD) measurements (left panel in Fig. 5). The introduction of the structural zinc atom increased the thermostability of the C4 mutant (4.5°C increase of the T_m value compared to WT). Similar results were obtained by heat treatment of the enzyme; the enzyme activity of WT decreased to 15% after incubation for 10 min at 40°C, whereas no inactivation of the C4 mutant was detected by the same treatment (right panel in Fig. 5). The level of translational expression of the C4 mutant was approximately 3-fold higher than that of WT (Fig. 3), suggesting that the stability of the C4 mutant was also increased *in vivo*.

In cases of human ADHs (Jeloková et al., 1994) and bacterial phenylacetaldehyde reductase (Wang et al., 1999), it is impossible to remove structural zinc without loss of stable folding or enzyme activity. On the other hand, when ADHs from yeast (Magonet et al., 1992) and thermophilic archaea (Ammendola et al., 1992) are incubated with a chelating reagent, the structural zinc atom is selectively removed with no effect on the catalytic zinc or loss of enzyme activity, while the enzyme becomes very sensitive to treatment with heat and/or denaturant. These different results may reflect the role change of the structural zinc in the enzyme during evolution. Ancestral MDR enzyme had probably possessed this zinc by coordination of four cysteine ligands, which is absolutely necessary for both stability and enzyme activity (such as human ADH). Eventually, the role had been limited for maintaining the stability (such as yeast ADH), and subsequently no significant contribution, which led to a loss of the structural zinc and random substitution of four cysteine ligands (such as PsXDH). Therefore, C4 mutation of PsXDH also seems to mimic the natural evolutionary process "reversely", as well as modification of coenzyme specificity toward NADP⁺.

In case of random mutagenesis method, single (or double) mutation with moderate effects on thermostability are first isolated, and these combination and/or further randomization are subsequently carried out. However, C4 mutation don't have to be generated by this method, because single and/or double mutations of S96C, S99C and Y102C give no effect to stabilize PsXDH. Since it is easy to identify whether a MDR enzyme contains a structural zinc atom by simple analysis of the amino acid sequence alignment, this novel strategy for protein stabilization would be applicable for other MDRs that contain no structural zinc atom natively.

2.2.3 Thermostabilization of NADP⁺ dependent mutants

Modification of coenzyme specificity toward NADP⁺ in PsXDH resulted in a significant loss of thermostability (Fig. 5), which is also found in other several dehydrogenases/reductases. Therefore, the C4 mutation was combined with each ARS and ARSdR mutation, to construct the C4/ARS and C4/ARSdR mutant, respectively (Watanabe et al., 2005). Their translational expression levels in *E. coli* were almost the same as the C4 mutant (Fig. 3), estimating that the stability *in vivo* might increase compared to their parent enzymes, ARS and ARSdR. In fact, their T_m values were almost the same as that of the C4 mutant and, in particular, C4/ARS was dramatically stabilized by improving the T_m of 7.7°C compared to ARS (left panel in Fig. 5). Heat treatment of the enzymes provided similar results; after incubation for 10 min at 45°C, C4/ARS and C4/ARSdR maintained about half the activity, whereas no enzyme activity of ARS and ARSdR mutants was detected by the same treatment (right panel in Fig. 5). The zinc content of C4/ARS and C4/ARSdR mutants was 1.69 and 1.78 mol zinc/mol subunit, respectively, suggesting that their increased thermostability was due to the structural zinc atom introduced.

Unexpectedly, C4/ARS and C4/ARSdR mutants showed a further ~4-fold improved k_{cat}/K_m^{NADP} values, mainly caused by an increase in k_{cat}^{NADP} values (Fig. 4). Finally, their k_{cat}/K_m^{NADP} values reached much higher than the initial k_{cat}/K_m^{NAD} value of WT; ratio of NADP⁺/NAD⁺ preference in terms of k_{cat}/K_m , 142 and 101, respectively.

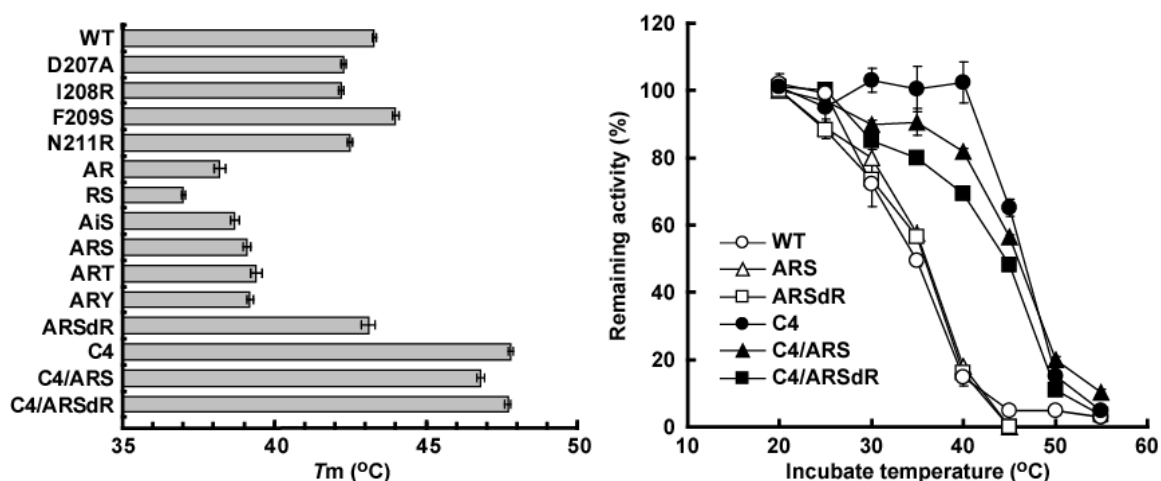


Fig. 5. Comparison of thermostability for WT and mutant PsXDH, estimated by CD analysis (left panel) and heat-inactivation (right panel).

Although protein stabilization generally confers rigidity on the protein and subsequently produces increased ligand affinity, there is no such change in the kinetic constants of C4/ARS and C4/ARSdR mutants. It is noteworthy that the C4 mutation site is separate from ARS/ARSdR sequentially and structurally (Fig. 1 and Table 1). These results suggest the possibility that thermostabilization can enhance the catalytic activity of enzymes, which modified the coenzyme specificity in other dehydrogenases/reductases.

2.2.4 Optimization of structural zinc-binding loop

If the hypothesis described in 2.2.2. is similar to evolutionary process, the effect might have been also appeared neighbor region of four cysteine ligands, suggesting one possibility that the optimization leads to further stabilize C4 mutant. The structural zinc is bound within a protruding loop from the catalytic domain, whose corresponding amino acid sequence among all PDHs is completely or highly conserved regardless of the existence of the zinc atom; Glu⁹¹, Gly⁹³, Lys¹⁰³, Gly¹⁰⁵, Tyr¹⁰⁷, Asn¹⁰⁸ and Leu¹⁰⁹ (numbering for PsXDH). Therefore, among amino acid residues except them, four positions of 95, 98, 101 and 112 in PsXDH C4 mutant (positions 1, 3, 5 and 8 in Table 1) were selected as site-directed mutation, to construct C4/P95S, C4/F98R, C4/E101F and C4/H112D mutants, respectively (Annaluru et al., 2007).

Among them, significant thermostabilization were found only in C4/F98R and C4/E101F mutants, compared with parent C4 mutant; higher T_m values of 4.2°C and 3.0°C, respectively. Similarly, after incubation for 10 min at 50°C, C4/F98R and C4/E101F maintained approximately 50% and 70% of the activity, whereas only 20% for C4 mutant. Therefore, based on the results, the C4/F98R/E101F mutant was constructed. Expectedly, the synergic effect of combination of two successive mutations on thermostabilization was observed in this mutant; the T_m value was 2.1°C and 3.3°C higher than C4/F98R and C4/E101F, respectively, and approximately 90% of activity remained after incubation for 10 min at 50°C. Finally, this quintuple mutation, S96C/S99C/Y102C/F98R/E101F, led to dramatically stabilize PsXDH WT by increase of 10.8°C in T_m .

The region of the structural zinc-binding loop is known to be involved in stabilizing the dimer-dimer interaction. In case of BaSDH, the arginine and phenylalanine residues at the

homologous positions with Phe⁹⁸ and Glu¹⁰¹ in PsXDH exist and participate in the interface interactions of two identical subunits. Since PsXDH is also homodimeric structure, thermostabilization from C4 to C4/F98R/E101F may be due to enhancement of intersubunit interaction rather than intrasubunit.

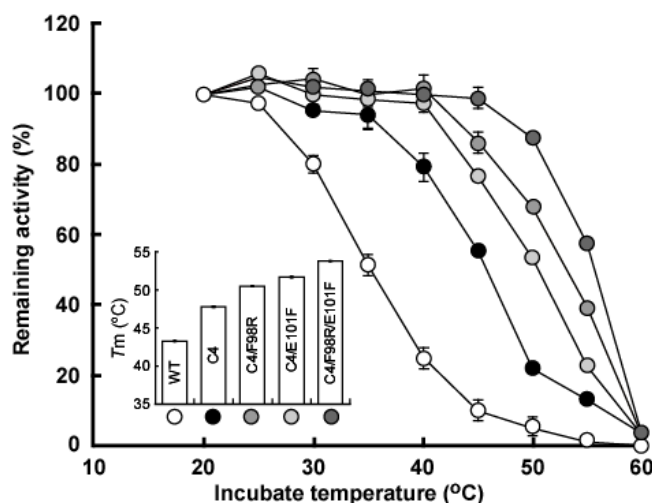


Fig. 6. Heat-inactivation curve of C4-derivative mutant and the T_m (inset).

3. Protein-engineering of PsXR

XR belongs to the aldo-keto reductase (AKR) superfamily, which is made up of 14 different families and approximately 120 members (Ellis, 2002). Their three-dimensional structure share a common $(\alpha/\beta)_8$ barrel fold, with a highly conserved coenzyme binding pocket at the C-terminus; no Rossmann-fold type binding mode, in contrast of XDH. Although most members of this superfamily show strong dependence on NADPH, a few members including XR (AKR2B5), 3 α -hydroxysteroid dehydrogenase (AKR1C9) and 3-dehydroecdysone 3 β -reductase (AKR2E1) can utilize both NADH and NADPH. To relax an intercellular redox imbalance between XR and XDH in *S. cerevisiae* cells, generation of XR mutant(s) with modified coenzyme specificity is alternative approach.

3.1 Modification of coenzyme specificity toward NADH

3.1.1 Strategy of mutant design

NADH-preferring PsXR mutants were designed by different three strategies based on the following observations (Watanabe et al., 2007).

1. In contrast of most XR including PsXR, XR from *Candida parapsilosis* (CpXR) shows 100-fold higher utilization of NADH than NADPH in terms of catalytic efficiency; it possesses a unique arginine residue in the coenzyme-binding pocket instead of Lys²⁷⁰ in the PsXR (Table 2) (Lee et al., 2003).
2. Several site-directed mutagenetic and crystallographic studies for modifying coenzyme specificity toward NADH, using NADPH-preferring NAD(P)H-preferring XR from *Candida tenuis* (CtXR), were reported (Fig. 7) (Petschacher et al., 2005; Petschacher & Nidetzky, 2005). This fungal XR shows ~77 % sequential similarity to PsXR. The K274R/N276D mutations lead the highest ratio of NADH/NADPH preference in terms

of k_{cat}/K_m among the series of CtXR mutants investigated, and corresponds to K270R/R272D mutations in PsXR.

- 2,5-Diketo-D-gluconic acid reductase, a member of the AKR superfamily (AKR5C1), also shows inherent NADPH specificity. However, mutations equivalent to K270G and R276H in PsXR leads to efficient NADH preference (Banta et al., 2002).

Enzyme	Source organisms	Coenzyme preference	Sequence						
			2	2	2	2	2	2	2
XR	<i>P. stipitis</i>	NADPH	V	E	L	K	S	N	R
XR	<i>C. tropicalis</i>	NADPH	L	E	L	K	S	N	R
XR	<i>P. guilliermondii</i>	NADPH	V	E	L	K	S	N	R
XR	<i>P. tannophilus</i>	NADPH	L	E	L	K	S	T	T
XR	<i>K. lactis</i>	NADPH	L	E	L	K	S	S	R
XR	<i>A. niger</i>	NADPH	L	E	L	K	S	N	R
XR	<i>C. parapsilosis</i>	NADH	L	E	M	K	S	S	R
XR	<i>C. tenuis</i>	NADPH	V	E	M	K	S	L	R
XR(M)	<i>C. tenuis</i>	NADH	V	E	M	R	D	L	R
2,5-DKGRA	<i>Corynebacterium</i> sp.	NADPH	Y	D	L	K	S	V	R
2,5-DKGRA(M)	<i>Corynebacterium</i> sp.	NADH	Y	D	L	G	S	V	R
2,5-DKGRA(M)	<i>Corynebacterium</i> sp.	NADH	Y	D	L	K	S	V	H

“M” is a mutant enzyme.

Table 2. Partial sequence alignment of the AKR superfamily.

3.1.2 Characterization of mutants

Specific activities of the recombinant PsXR WT enzyme in the presence of NADH and NADPH were 7.2 and 15.7 U mg⁻¹, respectively; NADPH-preference specificity. Among several constructed single and/or multiple mutants, the high ratio of NADH/NADPH in k_{cat}/K_m was found in R276H and K270R/N272D mutants: 2.62 and 7.29, respectively. Their kinetic constants for NADH were almost same as those of WT, although K270R/N272D mutant showed approximately 5-fold higher K_m value with NADH. On the other hand, the k_{cat}/K_m^{NADP} values of R276H and K270R/N272D mutants decreased 28-fold and 370-fold, compared with WT, which were mainly due to 40-fold decrease of k_{cat} value and 1,120-fold increase K_m value, respectively. When the R276H mutation was introduced in the K270R/N272D mutant, no further improvement of NADH preference appeared. This may be due to antagonistic effect by the introduction of two arginine residues, which may also account for a similar result found in the corresponding triple mutant of CtXR, K274R/N276D/R280H (Petschacher et al., 2005; Petschacher & Nidetzky, 2005): the effect of steric hindrance on the 2'-phosphate group of NADP⁺, generated by the introduction of the side chain of Arg²⁷⁰, could be strongly reduced when side chain of Arg²⁷⁶ is substituted to the smaller side chain of a histidine.

Surprisingly, the greatest thermostabilization was found in the R276H single mutant (increase of 8.8°C in T_m). Similar results were obtained by heat treatment of the enzyme: the activity of the WT enzyme decreased to only 4.1% after incubation for 10 min at 30°C, whereas mutants containing the R276H mutation maintained 92% of activity after the same treatment. These results were unexpected because, as observed in PsXDH (see above), the modification(s) of coenzyme specificity leads to a significant loss of thermostability.

3.2 Modification of coenzyme specificity toward NADPH

In CtXR, several limited numbers of amino acid residues commonly play a role to bind both to NADH and NADPH; Glu²²³, Lys²⁷⁰, Ser²⁷¹, Asn²⁷² and Arg²⁷⁶ in PsXR (Fig. 7) (Kavanagh et al., 2002, 2003). Therefore, it is very surprisingly that the substitution of Glu²²³→Ala in PsXR led to eliminate NADH-dependent activity completely, although the k_{cat}/K_m^{NADPH} value was ~25% of WT (Khattab et al., 2011). Furthermore, since only Ser²⁷¹ is specific for NADPH-binding, the substitution to alanine may change the coenzyme specificity toward NADH over NADPH. However, the additional S271A mutation in E223A mutant compensated the decreased NADPH-dependent activity, and the k_{cat}/K_m^{NADPH} value (32.4 min⁻¹ μM⁻¹) was almost comparable with that of WT (38.6 min⁻¹ μM⁻¹).

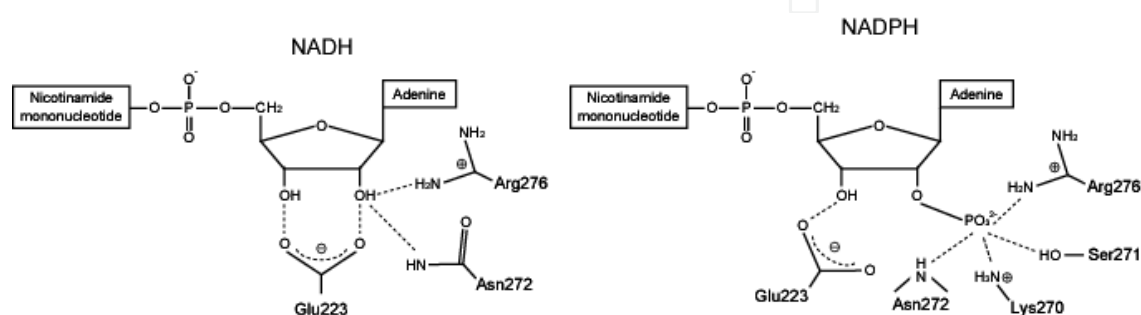


Fig. 7. Schematic diagram of putative interactions between PsXR and NAD(P)H.

4. Xylose-fermentation by *S. cerevisiae* using mutated XDH and/or XR

Although the native *S. cerevisiae* cannot ferment xylose, the *S. cerevisiae* transformed with the native genes encoding PsXR and PsXDH, a most potent recombinant strain, acquires the ability to ferment xylose to ethanol.

4.1 Screening of XDH and XR mutants

Each gene encoding PsXDH WT, ARS, ARSdR, C4, C4/ARS or C4/ARSdR was constitutively expressed in a laboratory *S. cerevisiae* strain together with PsXR WT gene (Watanabe et al., 2007b), whereas each gene encoding PsXR WT, K270R, R276H or K270R/N272D together with PsXDH WT gene (Watanabe et al., 2007ac); referred to as Y-X ("X" is a enzyme name). Fermentation by these recombinant yeasts was started with 5 g L⁻¹ glucose and 15 g L⁻¹ xylose in a minimal medium. Y-WT produced ethanol at 2.22 g L⁻¹ and excluded xylitol at 1.42 g L⁻¹. Among the XDH mutants, Y-ARSdR produced the highest amount of ethanol at 2.97 g L⁻¹ and excreted the lowest amount of xylitol at 0.16 g L⁻¹, whereas among the XR mutants, Y-R276H produced the highest amount of ethanol at 2.30 g L⁻¹ and excreted the lowest amount of xylitol at 0.97 g L⁻¹. These results indicate that these protein-engineering approach are useful for the fermentation of xylose to ethanol using *S. cerevisiae*.

4.2 Which of modification between XDH and XR is better?

The fermentation by Y-ARSdR and Y-R276H was further subjected using a bioreactor under the same conditions as the preliminary experiment and compared with that by Y-WT. Y-

ARSdR produced ethanol at 7.02 g L^{-1} with a yield of 0.46 g of ethanol g^{-1} of total consumed sugars, whereas 5.94 g L^{-1} with a yield of 0.43 g of ethanol g^{-1} of total consumed sugars for Y-R276H (Fig. 2). When compared with Y-WT, Y-ARSdR and Y-R276H showed 86% and 52% decrease of unfavorable xylitol excretion with 41% and 20% increased ethanol production, respectively. Measurement of the intracellular coenzymes concentrations that the ratios of NADH/NAD^+ and $\text{NADPH}/\text{NADP}^+$ are more similar to those of strain harboring empty vectors (Y-vector) by an order of magnitude from Y-ARSdR>Y-R276H>Y-WT (Fig. 9). These results clearly indicate that utilization of $\text{NADP}^+(\text{H})$ by XDH and XR gives more positive effect to prevent xylitol accumulation than that of $\text{NAD}^+(\text{H})$, due to maintenance of redox balance in yeast cells.

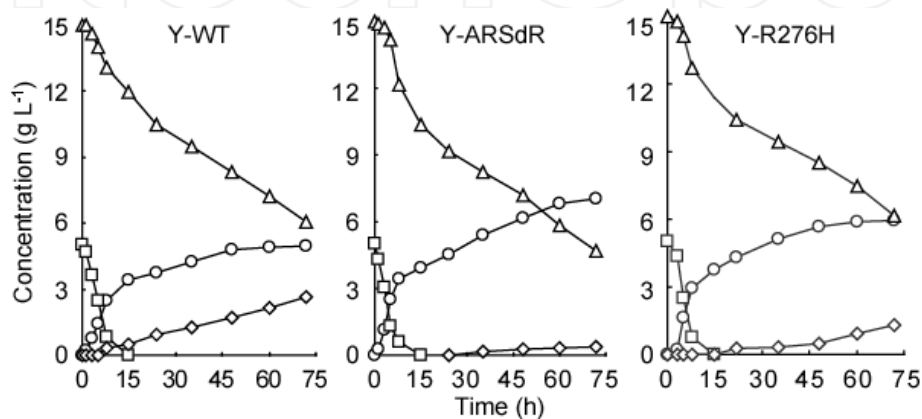


Fig. 8. Xylose fermentation by recombinant *S. cerevisiae* Y-WT, Y-ARSdR and Y-R276H.

Glucose (square); xylose (triangle); xylitol (rhomboid); ethanol (circle).

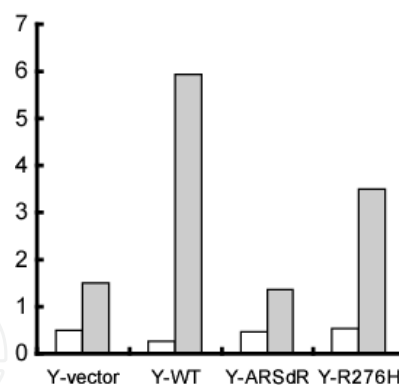
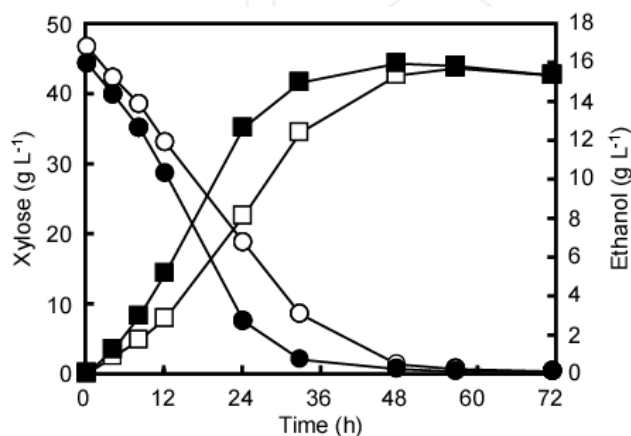


Fig. 9. Ratios of NADH/NAD^+ and $\text{NADPH}/\text{NADP}^+$ in recombinant *S. cerevisiae*.

4.3 Generation of more applicative *S. cerevisiae* strain

It is well known that overexpression of the *XKS1* gene encoding xylulokinase (XK) from *S. cerevisiae* (ScXK) is shown to aid xylose utilization, and that native strain can metabolize (ferment) "xylulose". A flocculent *S. cerevisiae* strain IR-2 was identified as high ability to ferment xylulose; a useful host strain for genetically engineering xylose fermentation (Matsushika et al, 2008ab). Therefore, PsXR, PsXDH (WT or ARSdR mutant) and ScXK genes were integrated on chromosome, and constitutively overexpressed; MA-R4 (vector control) and MA-R5 recombinant strains, respectively (Matsushika et al, 2009).

On a nutrient-rich medium containing 45 g L⁻¹ xylose, MA-R4 and MA-R5 consumed 79% and 95% of the xylose at 33 h, respectively (Fig. 10); MA-R5 produced a maximum of 16.0 g L⁻¹ ethanol at 48 h, while MA-R4 produced no more than 15.3 g L⁻¹. MA-R5 exhibited a 39% higher xylose consumption rate and a 39% higher ethanol production rate than MA-R4. These results suggest that protein-engineering of XDH is applicable to generate industrial recombinant *S. cerevisiae* capable to ferment both hexose and pentose at the same time.



Xylose consumption (circles); ethanol production (squares).

Fig. 10. Xylose fermentation by recombinant *S. cerevisiae*, MA-R4 (open symbols) or MA-R5 (closed symbols).

5. Conclusion

A large body of literature has described the alteration of nicotinamide coenzyme specificity, by which several useful information have already been available. However, the full reversal of coenzyme specificity, in terms of having a mutant enzyme catalytically efficient as the wild type, has been rarely achieved. As illustrated in this chapter, in particular XDH, selection of reference enzyme with close phylogenetic relationship and continuous multiple site-directed mutagenesis including the known “landmark” amino acid residues were necessary for mutant design. Furthermore, it was shown that thermostabilization is dramatically achieved by carefully designed site-directed mutagenesis; introduction of metal ion and subsequently optimization of the binding region. These novel strategies would be very applicable for other dehydrogenase/reductase enzymes with Rossmann-fold type coenzyme-binding mode including a MDR superfamily.

Difference coenzyme specificity between XDH and XR is one of main factors for accumulating unfavourable by-product, xylitol, during xylose-fermentation by recombinant *S. cerevisiae*. In spite of this importance, a few approach based on protein-engineering have been reported for a long time. Recently, several metabolic engineering have been attempted to enhance the pentose-phosphate pathway and/or xylose-uptake; introduction and overexpression of other endogenous genes including xylulokinase (XK), transketolase (TKL1), transaldolase (TAL1) and several hexose-transporter (HXT1-7) genes (Jeffries & Jin, 2004). Combination of these approaches, the protein-engineered enzyme and useful industrial host strain (such as IR-2) should be considered to achieve more effective ethanol production from xylose by recombinant *S. cerevisiae*.

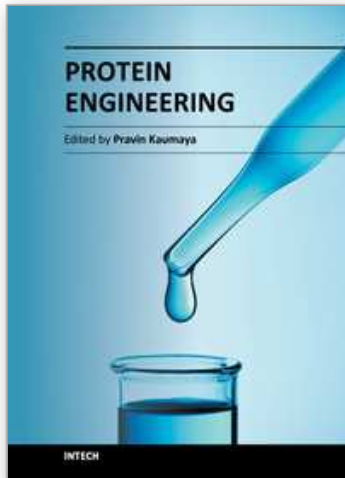
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