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Cloning and expression of xylitol dehydrogenase gene from *Candida shehatae* in *Saccharomyces cerevisiae*

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Abstract

The inability of *Saccharomyces cerevisiae* to utilize xylose is attributed to its inability to convert xylose to xylulose. Low xylose reductase and xylitol dehydrogenase activities in *S. cerevisiae* are regarded as the reason of blocking the pathway from xylose to xylulose. In previous study, we cloned and expressed the xylose reductase gene from *Candida shehatae* in *S. cerevisiae* which enables it to grow in xylose-containing medium. In this study, we investigated the activity of xylitol dehydrogenase gene of *C. shehatae* in *S. cerevisiae*. The xylitol dehydrogenase gene (*XYL2*) from *C. shehatae* was amplified by polymerase chain reaction (PCR). It was placed into plasmid pSFAU to produce the recombinant expression vector pSFAU-XDH. Subsequently, the pSFAU-XDH vector was transformed into *S. cerevisiae* TISTR5339 to produce a recombinant *S. cerevisiae* TISTR5339-XDH. The recombinant *S. cerevisiae* showed higher growth rate than the untransformed strain in media containing xylitol as a carbon source. The specific enzyme activity of xylitol dehydrogenase in the recombinant *S. cerevisiae* was determined. The highest specific activity was 0.805 mU mg^{-1} protein or 0.013 nkat mg^{-1}. This work demonstrates the functionality of the gene xylitol dehydrogenase from *C. shehatae* in *S. cerevisiae*.

Keywords: *Candida shehatae, Saccharomyces cerevisiae, xylitol dehydrogenase, xylose metabolism*

1. Introduction

Ethanol production from lignocellulosic material represents environment-friendly, sustainable alternative to fossil-derived gasoline (1). Lignocellulose is a complex material consisting of cellulose, lignin, and hemicellulose. It can be hydrolyzed by acids or enzymes into pentoses (xylose and arabinose) and hexoses (glucose, galactose and mannose). Hemicellulose consists mainly of xylose and is, next to cellulose, the most abundant renewable carbon source. Efficient bioconversion of xylose to ethanol could make hemicellulose a valuable raw material (2). The hexose sugars (glucose, mannose, and galactose) are relatively easily fermented to ethanol, whereas the pentose sugars (xylose and arabinose) are not (3). Xylose is abundant in the hydrolysates of hemicellulose, for about 10-40% of the total carbohydrates (4). Hence, fuel ethanol

production from pentose-rich sources, such as hardwood or agricultural residues will only be economically viable if xylose is fermented to ethanol (5, 6).

There has been intensive research in the fermentation of xylose to ethanol during the last two decades (7). Xylose can be fermented to ethanol by bacteria, yeasts, and fungi. At present, yeasts give the highest product yields and the highest productivities (3). *Pachysolen tannophilus* (8), *Candida shehatae,* and *Pichia stipitis* (9) are the most thoroughly studied xylose-fermenting yeasts. Although *Pa. tannophilus* is the first known xylose-utilizing yeast that ferments xylose under anaerobic conditions (10), subsequent results show that the yeasts *P. stipitis* and *C. shehatae* ferment xylose more efficiently (11). The well-known bakers' yeast, *Saccharomyces cerevisiae,* generally used in industrial ethanol production, does not ferment xylose, but its isomerization product xylulose (12, 13, 14, 15). This finding implies that *S. cerevisiae* possesses an effective xylulose-metabolising pathway.

In most bacteria, xylulose is formed directly from xylose through the activity of xylose isomerase. However, this conversion in yeasts is performed by the sequential action of two oxidoreductases, xylose reductase (XR, gene *XYL1*) and xylitol dehydrogenase (XDH, gene *XYL2*). Thus, an efficient xylose fermentation may be feasible by providing a xylose-utilizing pathway from a xylose-assimilating organism into fermenting yeasts, such as *S. cerevisiae* or *Schizosaccharomyces pombe* (2). The genes necessary for xylose metabolism have been cloned and expressed in *S. cerevisiae* (16, 17, 18, 19).

The expression of *XYL1* and *XYL2* is the basis for the further modification and is crucial for the utilization of xylose. The two genes expressed in various recombinant *S. cerevisiae* strains are originally from *P. stipitis*, which enable the recombinant *S. cerevisiae* to ferment xylose to ethanol under anaerobic conditions (7). Together with *P. stipitis, C. shehatae* is also known as one of the best native ethanol producers from xylose. Therefore, *C. shehatae* could also be another source for XR and XDH gene. The gene *XYL1* encoding XR of *C. shehatae* was expressed in *S. cerevisiae* (20). Later on, it was reported that the specific XR activity of the recombinants harbouring the *C.shehatae* gene controlled by the *PGK1* promotor had twice the activity of that harbouring the *P. stipitis* gene controlled by the same promoter (21). However, cloning of the *XYL2* gene of *C. shehatae* and its expression in *S. cerevisiae* has not been reported previously. In this study, cloning and expression of the *C. shehatae XYL2* gene in *S. cerevisiae*, as well as its specific activity are investigated.

2. Materials and Methods

2.1 Strains and plasmids

Escherichia coli TOP10[F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ*80lacZΔ M15 ΔlacX74recA1 araΔ139Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 nupG*] (Invitrogen) was used for the subcloning. *S. cerevisiae* strain TISTR 5339 was used as a host for DNA transformation. *C. shehatae* TISTR 5843 and *S. cerevisiae* strains were obtained from MIRCEN, Thailand Institute of Science and Technology Research. The yeast expression vector pSFAU, which contains an *SFA* gene (Hyperresistance to formaldehyde) as a selection was constructed from previous work (22).

2.2 Media and culture condition

Escherichia coli TOP10 and *E. coli* transformants were cultured in Luria-Bertani medium (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) with or without antibiotics at 37°C. The yeast strains were grown on yeast peptone dextrose (YEPD) medium (Bacto peptone 20 g/L, yeast extracts 10 g/L, glucose 10 g/L or

varied amount of sugar tested). For the selection of the recombinants, formaldehyde was added to the medium with a final concentration of 5.5 mM (22).

2.3 Enzymes and reagents

The restriction enzymes and the T4 DNA Ligase were purchased from New England Biolabs (Ipswich, MA, USA) and Fermentas (Glen Burnie, MD, USA). The plasmid DNA extraction kit was obtained from Bio-Rad (Hercules, CA, USA). The AccuPrime™ Pfx DNA polymerase was purchased from Invitrogen (Carlsbad, CA, USA). The DNA gel purification kit was obtained from Qiagen (Hilden, Germany). The PCR purification kit was purchased from Roche (Indianapolis, IN, USA).

2.4 Nucleic acid manipulations and transformations of bacteria and yeast

Genomic DNA was isolated from *C. shehatae* and *S. cerevisiae* using a standard protocol (23). Standard techniques were used for nucleic acid manipulations (24). Bacterial transformation was performed by the method of Dagert and Ehrlich (1979) (25). Yeast transformations were performed by electroporation (26).

2.5 Amplification of *XYL2* gene

The *C. shehatae XYL2* gene was amplified from the genomic DNA by PCR amplification. Oligonucleotide primers (forward: 5'GGGGAAT-TCATGACTGCTAACCCTTCG3' and reverse: 5' GGATCCCTATTCAGGGCCATCAAT 3') were derived from the published sequence of the *C. shehatae XLY2* gene (Genbank accession no. FJ040172.1). The *Eco*RI restriction site was added to the 5' end of the gene and the *Bam*HI restriction site was added to the 3' end of the gene. The DNA was amplified in 50-ml reaction mixtures. Each reaction contained 100 ng of template DNA, 0.3 mM of each primer, 1 × AccuPrime™ Pfx Reaction mix (including dNTP) supplemented with 0-5 mM $MgSO_4$ and 0-1 M KCl, and 2.5 U AccuPrime™ Pfx DNA polymerase. Thirty cycles of denaturation, annealing and polymerization were carried out for 15 sec at 95 °C, 43 °C and 68 °C, respectively. The PCR products were excised from the gel and purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instruction.

2.6 Construction of the expression vector

The yeast expression vector pSFAU was opened at the unique *Eco*RI/ *Bam*HI site downstream of the *SFA* gene, which positions downstream of *ADH2* promoter. Then the *XYL2* gene of *C. shehatae* was inserted as an *Eco*RI/ *Bam*HI fragment to generate the plasmid pSFAU-XDH (Figure 2). The recombinant plasmid was sent to sequencing at 1st Base Laboratory (Malaysia) in order to confirm the correction of the inserted gene. The primers Seq_SFA_1102-1121 (5'GAAGATTTGCATAACGGTG3') and Seq_p GBDU_EcoRI50_RV (5¢GAACTTGCGGGGTTTT-TC3') were used for the sequencing reaction.

2.7 Preparation of crude cell extracts

Yeast cells were grown in the YEPD medium, then harvested by centrifugation, washed in sterile distilled water and resuspended in a buffer (0.1 M sodium phosphate, 0.5 mM EDTA, pH 7.0). One milliliter of the resuspension buffer per gram of wet cells and glass beads were added to the cells, which were subsequently disrupted by vortexing. The ruptured cell mixture was centrifuged at 13,000 rpm for 5 min and the supernatant was stored at -70°C prior to the analysis of the protein concentration and the enzyme activities. The protein concentration was determined by the method of Lowry (27), with bovine serum albumin as a standard.

2.8 Enzyme assays

The activity of xylitol dehydrogenase was assayed according to the method from Smiley and Bolen (28). The specific activity of the enzyme was expressed as nanomoles of the converted NAD^+ per milligram protein per minute (U mg^{-1}).

3. Result and discussion

3.1 Cloning of the gene *XYL2* from *C. shehatae*

The *XYL2* gene, encoding xylitol dehydrogenase (Genbank accession number FJ040172.1, NCBI Genbank) was amplified by polymerase chain reaction from the genomic DNA of *C. shehatae* TISTR 5843. The amplicon was visualized and the band with the length of about 1.1 kb was gel-purified (Figure 1).

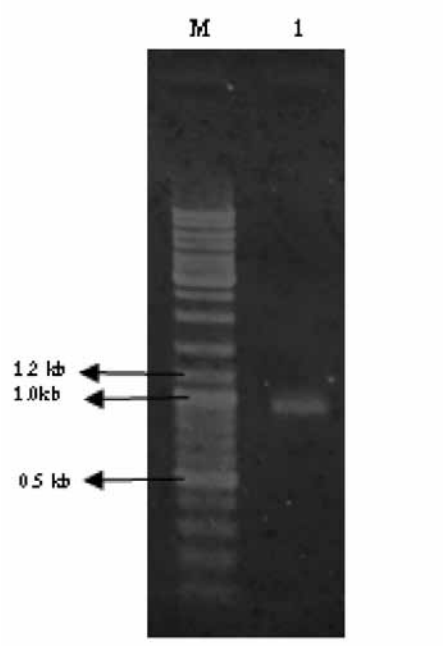


Figure 1. PCR amplification of *XYL2* (M-marker; 1-gel-purified *XYL2*)

The purified target fragment was inserted into the *Eco*RI/ *Bam*HI site of pSFAU, generating a pSFAU-XDH vector (Figure 2). The gene *XYL2* was placed downstream of the existing *SFA* gene, and thus, sharing the same promoter (*ADH1*) with the *SFA* gene. The ligation product was used to transform the competent *E. coli* Top 10 cells. To verify the results, plasmids from the transformants were analyzed with the restriction endonucleases *Eco*RI/ *Bam*HI. The length of the insert fragments derived from restriction digestion was about 1.1 kb. The correction of the inserted gene was confirmed by sequencing. The pSFAU-XDH vector was transformed into *S. cerevisiae* TISTR 5339, by which the transformants were selected by their abilities to grow on media containing 5.5 mM formaldehyde.

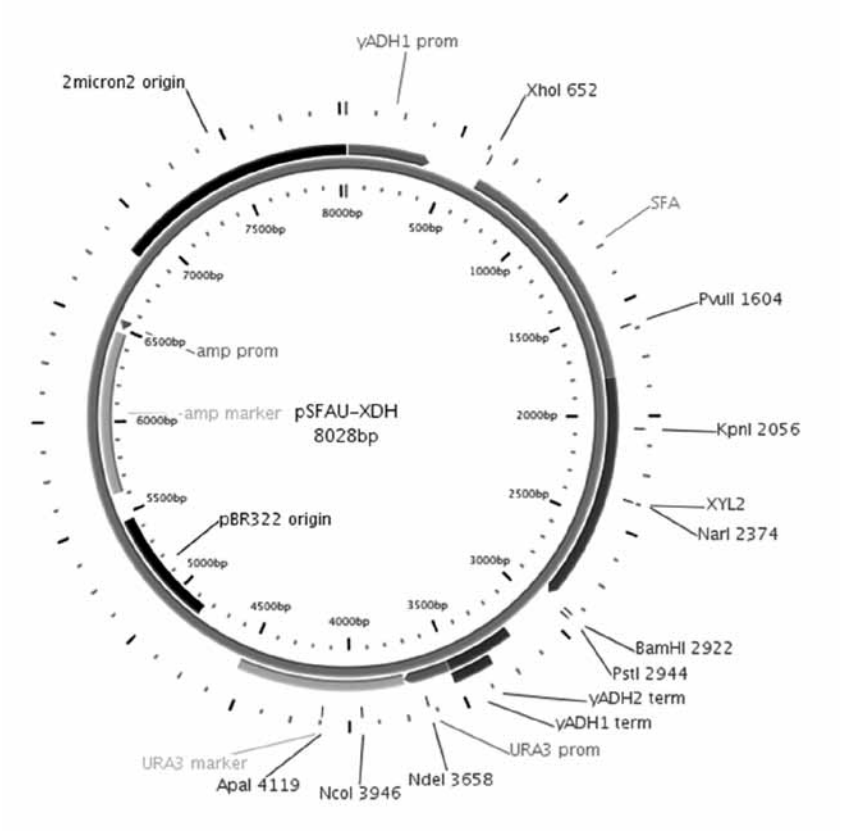
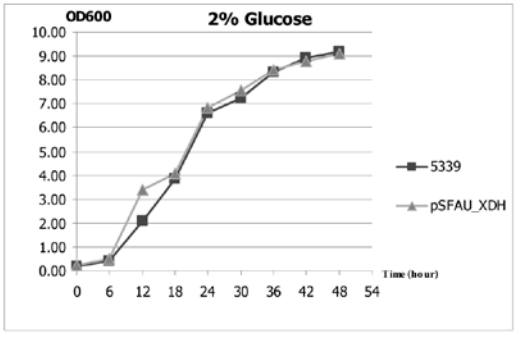


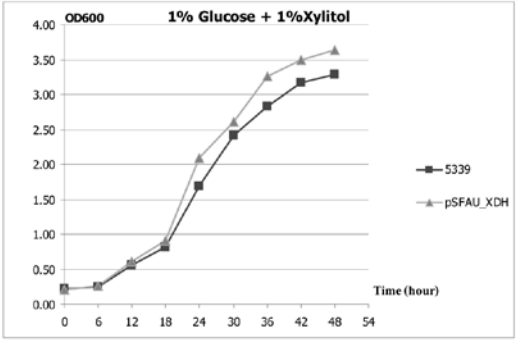
Figure 2. Recombinant expression vector pSFAU-XDH

3.2 Growth of the recombinant *S. cerevisiae* TISTR 5339-XDH

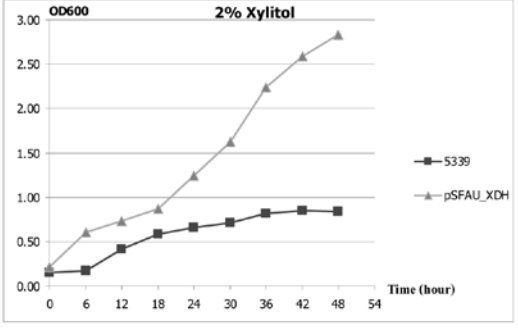
The recombinant expression vector pSFAU-XDH was transformed into *S. cerevisiae* TISTR 5339 cells by electroporation. Positive transformants were selected on YEPD plates containing 5.5 mM formaldehyde. After an incubation of the identified positive recombinant, the strain was inoculated, then a growth experiment was performed and the XDH activity was determined. The recombinant *S. cerevisiae* TISTR 5339-XDH was inoculated into YEP medium containing 2% xylitol, 2% glucose, and 1% glucose plus 1% xylitol. The growth study of both strains is shown in figure 3.



(A)



(B)



(C)

Figure 3. The growth of *S. cerevisiae* TISTR 5339 (■) and the recombinant strain containing pSFAU-XDH (▲) on (A) 2% glucose, (B) 1% glucose + 1% xylitol, and (C) 2% xylitol.

The result indicated that both strains, TISTR 5339 and TISTR 5339-XDH, could grow at almost the same rate in medium containing 2% glucose (Figure 3A). The recombinant strain showed slightly higher growth rate in medium containing 1% glucose plus 1% xylitol (Figure 3B). A significant difference in the growth was found when *S. cerevisiae* TISTR 5339 and the recombinant strain were cultivated in media containing 2% xylitol as a carbon source (Figure 3C). The recombinant *S. cerevisiae* TISTR 5339-XDH could grow and reach OD_{600} about 3, after 48 hours incubation, whereas the TISTR 5339 strain could reach OD_{600} around 0.8. However, the growth of the recombinant *S. cerevisiae* in the xylitol-containing medium was slower than that in the glucose-containing medium. The same tendency was found when the final cell density was observed. A rich medium was used for the growth analyses, and quite possibly some of the growth on xylitol could be due to other carbon sources found in the YEP. Nevertheless, the difference in the growth rate between both strains indicated that the recombinant *S. cerevisiae* TISTR 5339-XDH could utilize xylitol for its growth and thus, the xylitol dehydrogenase gene from *C. shehatae* could be expressed in *S. cerevisiae*.

3.3 The XDH activity in recombinant *S. cerevisiae* TISTR 5339-XDH

Since xylitol dehydrogenase is an NAD-linked enzyme, the activity of xylitol dehydrogenase can be indirectly determined by a rapid reduction of its coenzyme, NAD^+, to NADH when xylitol is used as a substrate. The conversion of NAD^+ to NADH can be detected by a change of the absorbance at 340 nm, which is the absorbance of NADH, while NAD^+ is absorbed at 260 nm. For this purpose, cell extracts of *S. cerevisiae* TISTR5339 and its recombinant were prepared and used as crude enzymes for the reaction. Figure 4 shows the absorbance of NADH that occurred during the time period of the reaction, by which xylitol was used as a substrate, cell extracts of *S. cerevisiae* as crude enzymes and NAD^+ as a coenzyme.

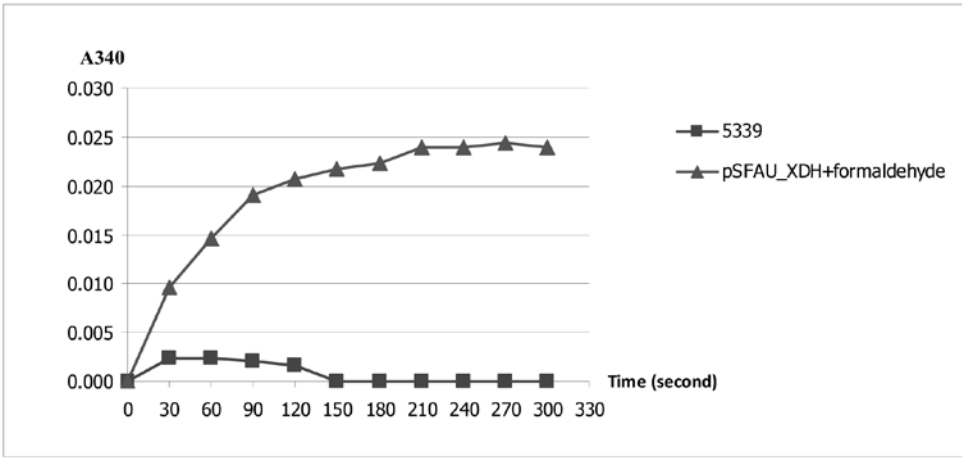


Figure 4. The absorbance of NADH at 340 nm of *S. cerevisiae* TISTR5339 (■) and the recombinant strain containing pSFAU-XDH (▲).

The specific activity of xylitol dehydrogenase from this experiment was 0.805 mU min^{-1} mg^{-1} or 0.013 nkat mg^{-1}. Comparing with the other XDH activities that are reported from previous publications (Table 1), the XDH activity from this work is considered low. This might be caused from many factors such as medium, or promoter that was used in the cloning. Previous studies showed that carbon source in the media play a critical role in the activity of XDH (19, 29, 30, 31,32). In this work YEPD medium was used, which contains glucose as a carbon source. Glucose was reported to give the lowest activity of XDH when it is used as a carbon source in the media. Tamburini et al (29) reported that the highest XDH activity in *C. tropicalis* was detected when xylose plus glycerol were used as carbon sources, while glucose and xylitol gave the lowest activity of XDH. Ko et al (30) presented a significant increase of about 280%, 100% or 40% of the *C. tropicalis* XDH activity when arabinose, xylose, or glycerol was used in the media, respectively. On the other hand, no major change was found when glucose, fructose, sorbose were used as carbon sources (30). Tantirungkij et al (19) also reported an increase in the XDH activity of the genetic engineered *S. cerevisiae* strains when xylose was used in the medium instead of glucose. The effect of carbon source in the XDH activity was also reported on *Pachysolen tannophilus* (31). Bolen and Detroy (31) found that the highest XDH activity of *P. tannophilus* was observed when arabinose was used as a carbon source. The XDH activities of *P. tannophilus* decreased when xylose, galactose, fructose/ glycerol/ mannitol/ mannose/ sorbitol were used as carbon sources (31). Glucose was found to give the lowest XDH activity (31). Alexander (32) also reported a major change of the XDH activity when glucose or xylose was added in the media, namely higher XDH activity was observed from the cell that grew in xylose-containing medium.

Table 1. Summary of XDH activities that have been reported from selected publications

XDH activity	Strain	Construct	Medium/ condition	Ref.
0.805 mU mg^{-1} or 0.013 nkat mg^{-1}	*S. cerevisiae* TISTR 5339	*ADH: SFA: XYL2*	YEPD + 5.5 mM formaldehyde; 150 rpm, 30 °C, 24 h.	This work
0 – 60 nkat mg^{-1}	*C. tropicalis* DSM 7524	-	AM minimal medium with various carbon source mixture (xylose, xylitol, glucose, fructose, galactose) varied from 30 – 50 g L^{-1}	(29)
46.4 U mg^{-1}	*C. tropicalis* ATCC 20913	-	Xylitol fermentation medium; 200 rpm; 30 °C	(30)
0.36 – 15.03 U mg^{-1}	*S. cerevisiae* H158	Various combination of *ADH1* and *PGK* promoter + *XYL1* and *XYL2* in various direction	Synthetic complete medium (0.67% yeast nitrogen base w/o amino acid)	(33)
25 – 452 mU mg^{-1}	*S. cerevisiae* SH1089/ AM12/ or TJ1	Internal promoter: *XYL2;* coexpresses with *XYL1*	YEP + xylose or glucose 20 g/L; 500 rpm; 30 °C, 36 h.	(19)
2 – 79 mU mg^{-1}	*Pichia stipitis*	-	YEP + xylose or glucose 20 g/L; 500 rpm; 30 °C, 36 h.	(19)
76 – 1482 U mg^{-1}	*Pachysolen tannophilus*	-	CCY production medium + various sugar 50 g/ L (arabinose, fructose, galactose, glucose, glycerol, mannose, mannitol, sorbitol, xylose); 150 rpm, 30 °C, 72 h	(31)
< 2 - 228 U mg^{-1}	*Pachysolen tannophilus*	-	YEP + 5% glucose or 5 xylose; 200 rpm; 30 °C or 37 °C; 48 h.	(32)

Another factor that was shown to effect the activity of XDH is the promoter that is used in the cloning, and the coexpression of the *XYL1* gene, encoding for xylose reductase, as thoroughly investigated by Walfridsson et al (33). Higher activities were obtained for *XYL2* that was controlled by the *PGK1* promoter, when *XYL1* and *XYL2* were transcribed in the same direction with *XYL2* downstream of *XYL1* and the ratio of XR:XDH was 0.06 (33). They also reported that the presence of the *ADH1* promoter-terminator fragment enhanced the activity of the structural gene expressed under the control of the *PGK1* promoter up to 20-fold (33). The highest specific activities of XR and XDH were observed when the *ADH1* promoter-gene-terminator was placed upstream of the *PGK1* promoter-gene-terminator (33). In this work, *XYL2* was placed downstream of the selective marker gene *SFA*, by which both genes were controlled by one promoter, the *ADH2* promoter. This construct could cause the low activity of XDH, in addition to the use of glucose-containing medium. Since this is a preliminary result, further optimizations such as changing carbon sources or the cloning strategy by using appropriate promoter are needed to be done in order to enhance the activity of xylitol dehydrogenase from *C. shehatae* in *S. cerevisiae*. Nevertheless, this work demonstrates the functionality of the gene xylitol dehydrogenase from *C. shehatae* in *S. cerevisiae* since the cloning of the *XYL2* gene of *C. shehatae* in *S. cerevisiae* has not been reported previously.

4. Conclusion

A recombinant plasmid containing the xylitol dehydrogenase gene (*XYL2*) from *C. shehatae* was constructed. *S. cerevisiae* that harbours this plasmid showed higher growth rate than the untransformed strain in the media containing xylitol as a carbon source. The specific activity of the xylitol dehydrogenase enzyme in the recombinant *S. cerevisiae* was 0.805 mU mg^{-1} protein or 0.013 nkat mg^{-1}. It will need further investigations to optimize and increase the activity of this enzyme. However, the functionality of the gene xylitol dehydrogenase from *C. shehatae* in *S. cerevisiae* had been demonstrated in this work.

5. Acknowledgment

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6. References

(1) Träff KL, Otero Cordero RR, van Zyl WH, Hahn-Hägerdal B. Deletion of the GRE3 aldose reductase gene and its Influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expressing the *XylA* and *Xks1* genes. Appl Environ Microbiol. 2001;67(12): 5668–74.

(2) Amore R, Otter PK, Kiister C, Ciriacy M, Hoilenberg CP. Cloning and expression in *Saccharomyces cerevisiae* of the NAD(P) H-dependent xylose reductase encoding gene *(XYL1)* from the xylose-assimilating yeast *Pichia stipitis*. Gene. 1991;109: 89-97.

(3) Hahn-Hägerdahl B, Lindén T, Senac T, Skoog K. Ethanolic fermentation of pentoses in lignocellulose hydrolysates. Appl Biochem Biotechnol 1991;131(28/29): 131-44.

(4) Ladisch MR, Lin KW, Voloch M, Tsao GT. Process considerations in the enzymatic hydrolysis of biomass. Enzyme Microb Technol. 1983;5: 82-102.

(5) Hinman ND, Wright JD, Hoagland W, Wyman CE. Xylose fermentation: an economic analysis. Appl Biochem Biotechnol. 1989;20/21: 391–401.

(6) Von Sivers M, Zacchi G. Ethanol from lignocellulosics: a review of the economy. Bioresour Technol. 1996;56: 131–40.

(7) Skoog, K, Hahn-Hägerdal B. Effect of oxygenation on xylose fermentation by *Pichia stipitis*. Appl Environ Microbiol. 1990;56: 3389–94.

(8) Slininger PJ, Bolen PL, Kurtzman CP. *Pachysolen tannophilus*: properties and process considerations for ethanol production from D-xylose. Enzyme Microb Technol. 1987; 9(1): 5-15.

(9) Prior BA, Kilian, SG, du Preez JC. Fermentation of D-xylose by the yeasts *Candida shehatae* and *Pichia stipitis*. Proc Biochem. 1989;24(1): 21-32.

(10) Kurtzman CP. Biology and phys.iology of the xylose-fermenting yeast *Pachysolen tannophilus*. Adv Biochem Eng Biotechnol. 1983;27: 73-83.

(11) du Preez JC, Bosch M, Prior BA. The fermentation of hexose and pentose sugars by *Candida shehatae* and *Pichia stipitis*. Appl Microbiol Biotechnol. 1986;23: 228-33.

(12) Chiang LC, Gong CS, Chen LF, Tsao GT. D-Xylulose fermentation to ethanol by *Saccharomyces cerevisiae*. Appl Environ Microbiol. 1981; 42: 284-9.

(13) Gong CS, Chen LF, Flickinger MC, Chiang LC, Tsao GT. Production of ethanol from D-Xylose by using D-xylose isomerase and yeasts. Appl Environ Microbiol. 1981;41: 430-6.

(14) Senac T, Hahn-Hägerdal B. Intermediary metabolite concentrations in xylulose- and glucose-fermenting *Saccharomyces cerevisiae* cells. Appl Environ Microbiol. 1990;56: 120-6.

(15) Wang PY, Shopsis C, Schneider H. Fermentation of a pentose by yeasts. Biochem Biophys Res Comm 1980; 94(1): 248-54.

(16) Hallborn J, Walfridsson M, Airaksinen U, Ojamo H, Hahn-Hägerdal B, Penttilä M, Keräsnen S. Xylitol production by recombinant *Saccharomyces cerevisiae*. Nature Biotechnol. 1991;9(11): 1090–5.

(17) Ho NWY, Chang SF. Cloning of yeast xylulokinase gene by complementation of *E. coli* and yeast mutations. Enzyme Microb Technol. 1989;11: 417–21.

(18) Kötter P, Amore R, Hollenberg CP, Ciriacy M. Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, *XYL2*, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. Curr Genet. 1990;18: 493–500.

(19) Tantirungkij M, Nakashima N, Seki T, Yoshida T. Construction of xylose-assimilating *Saccharomyces cerevisiae*. J Ferm Bioeng. 1993; 75: 83–8.

(20) Bruinenberg PM, de Bot PHM, van Dijken JP, Scheffers WA. NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. Appl Microbiol Biotechnol. 1984;19(4): 256–60.

(21) Govindem R, Pillay B, van Zyl WH, Pillay D. Xylitol production by recombinant *Saccharomyces cerevisiae* expressing the *Pichia stipitis* and *Candida shehatae XYL1* genes. Appl Microbiol Biotechnol. 2001;55: 76-80.

(22) Koolkoksong T, Boonmee A. Construction of a vector containing a formaldehyde hyperresistance gene as a selective marker. KKU Res Jour.

2010;15(5): 351-9.

(23) Rose MD, Winston F, Hieter P. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press;1990.

(24) Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. Cold spring Harbor, NY: Cold Spring Harbor Laboratory;1982.

(25) Dagert M, Ehrlich SD. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene. 1979;6: 23-8.

(26) Grey M, Brendel M. A ten minute protocol for transforming *Saccharomyces cerevisiae* by electroporation. Curr Genet. 1992;22: 335-6.

(27) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193: 265.

(28) Smiley KL, Bolen PL. Demonstration of D-xylose reductase and D-xylitol dehydrogenase in *Pachysolen tannophilus*. Biotechnol Lett. 1982;4(9): 607-10.

(29) Tamburini E, Bianchini E, Bruni A, Forlani G. Cosubstrate effect on xylose reductase and xylitol dehydrogenase activity levels, and its consequence on xylitol production by *Candida tropicalis*. Enzyme Microb Technol. 2010; 46(5): 352-9.

(30) Ko BS, Jung HC, Kim JH. Molecular cloning and characterization of NAD(+)-dependent xylitol dehydrogenase from *Candida tropicalis* ATCC 20913. Biotechnol Prog. 2006;22(6): 1708-14.

(31) Bolen PL, Detroy RW. Induction of NADPH-linked D-xylose reductase and NAD-linked xylitol dehedrogenase activities in *Pachysolen tannophilus* by D-xylose, L-arabinose, or D-galactose. Biotechnol Bioeng. 1985;27(3): 302-7.

(32) Alexander NJ. Temperature sensitivity of the induction of xylose reductase in *Pachysolen tannophilus*. Biotechnol Bioeng. 1985;27(12): 1739-44.

(33) Walfridsson M, Anderlund M, Bao X, Hahn-Hägerdal B. Expression of different levels of enzymes from the *Pichia stipitis XYL1* and *XYL2* genes in *Saccharomyces cerevisiae* and its effects on product formation during xylose utilisation. Appl Microbiol Biotechnol. 1997;48(2): 218-24.