A Thesis for the Degree of Master of Science

Production of ethanol from xylose using xylose metabolic pathway of *Kluyveromyces marxianus* in *Saccharomyces cerevisiae*

효모에서 *Kluyveromyces marxianus* 의 자일로스 대사경로를 이용하여 자일로스로부터 에탄올 생산에 관한 연구

By

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Interdisciplinary Program for

Agricultural Biotechnology

Seoul National University

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ABSTRACT

Xylose is a major fermentable sugar present in lignocellulosic biomass, the second most abundant carbohydrate in nature to glucose. Efficient xylose fermenting strains are required to develop economically viable processes to produce biofuels such as ethanol from biomass. Although *Saccharomyces scerevisiae* has been used for industrial ethanol production, native *S. cerevisiae* cannot utilize xylose as a carbon source because the baker's yeast does not contain the metabolic activity to convert xylose to xylulose.

In yeasts, xylose is converted to xylulose by two oxidoreductases, NAD(P)H-linked xylose reductase (XR;EC1.1.1.21) and NAD^+-linked xylitol dehydrogenase (XDH;EC1.1.1.9), and finally, xylulokinase (XK;EC2.7.1.17) phosphorylates xylulose into xylulose5-phosphate, which is metabolized further via the pentose phosphate pathway.

This study was carried out to clone the xylose metabolic genes from *Kluyveromyces marxianus* and to compare the xylose metabolic activity to ethanol between *K. marxianus* and *Scheffersomyces stipitis*. *K. marxianus* is thermotolerant and could produce ethanol from xylose naturally. The full genome sequences of *K. marxianus* were completed recently.

The three key genes involved in the xylose metabolism were cloned from *K. marxianus* such as *XYL1* for XR, *XYL2* for XDH and *XYL3* for XK. These genes were introduced to *S. cerevisiae* D452-2 used as host.

The engineered *S. cerevisiae* strain (DX123/KM1) containing the *K. marxianus* XR gene along with the *Sch. stipitis* XDH and XK genes was

able to produce 10.5 g/L ethanol from xylose along with specific activity of XR of 0.48 U/mg. Similarly, the genes for XDH and XK from *K. marxianus* were functionally expressed in *S. cerevisiae* and the engineered *S. cerevisiae* strains can convert xylose to ethanol successfully.

All three xylose metabolic genes from *K. marxianus* were introduced to *S. cerevisiae* and the xylose metabolic activity was evaluated in terms of ethanol yield and ethanol productivity. Specific activities of XR, XDH and XK from *Sch. stipitis* were all higher than the corresponding enzymes from *K. marxianus*. The recombinant *S. cerevisiae* strain containing the *K. marxianus* xylose metabolic genes (KM123) produced 10.1 g/L ethanol, 0.33 g ethanol/ g xylose ethanol yield and 0.14 g/L.h ethanol productivity. While the *S. cerevisiae* strain transformed with the *Sch. stipitis* (SS123) produced 10.2 g/L ethanol, 0.35 g ethanol/ g xylose ethanol yield and 0.14 g/L.h ethanol productivity.

In order to improve an ethanol fermentation performance by reducing acetate formation, the *ALD6* gene which can convert acetaldehyde to acetate was disrupted to construct the *S. cereivisae* strain (KM123::Δ*ALD6*). The strain produced 0.2 g/L acetate compared with 2.8 g/L in the control strain. Interestingly, the consumption of xylose and production of ethanol were not affected by disruption of ALD6.

This thesis showed production of ethanol from xylose by the recombinant *S. cerevisiae* containing the three genes (XR, XDH and XK) from *K. marxianus*. The ethanol performance of the *S.cerevisiae* KM123 was comparable to the *S. cerevisiae* strain transformed with the *Sch. stipitis* XR and XDH and *S. cerevisiae* XK.

Keywords : bioethanol, xylose, KmXR, KmXDH, KmXK, *ScALD6*, cofactor imbalance, acetate, metabolic engineering

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CONTENTS

ABSTRACT ····· i

CONTENTS ····· iv

LIST OF FIGURES ····· viii

LIST OF TABLES ····· ix

I. INTRODUCTION ····· 1

1. Bioethanol ····· 1

2. Ethanol production in recombinant *Saccharomyces cerevisiae* ····· 2

3. Xylose metabolism in yeast ····· 3

4. *Kluyveromyces marxianus* and xylose metabolisms ····· 4

5. Xylose reductase (XR) ····· 6

6. Xylitol dehydrogenease (XDH) ····· 7

7. Xylulokinase (XK) ····· 8

8. Acetaldehyde dehydrogenase family (ACDH) ····· 8

9. Cofactors ····· 13

10. Research Objectives ····· 14

II. MATERIALS AND METHODS ····· 15

1. Reagents ····· 15

2. Strains and Plasmids ····· 16
3. DNA Manipulation and Transformation ····· 22
3.1. Enzymes ····· 22
3.2. Transformation of *E. coli* ····· 22
3.3. Preparation of plasmid DNA and yeast genomic DNA ····· 22
3.4. Isolation of DNA fragments and DNA sequencing ····· 23
3.5. Polymerase chain reaction (PCR) ····· 23
3.6. Yeast transformation ····· 24
4. Homologous recombination ····· 24
5. Media and culture conditions ····· 29
5.1 Media ····· 29
5.2 Inoculum ····· 29
5.3 Cultivations in bioreactor ····· 30
6. Analysis ····· 30
6.1 Dry cell mass ····· 30
6.2 Sugar concentrations ····· 31
6.3 Measurement of enzyme activities ····· 31

III. RESULTS AND DISCUSSIONS ····· 36
1. Construction of recombinant *S. cerevisiae* ····· 36
2. Selection of host strain for xylose metabolism ····· 41

3. Confirmation of heterologous expression of *XYL1* ····· 41

3.1 Specific activitiy assay of KmXR ····· 42

3.2. Microaerobic bath fermentation ····· 43

4. Confirmation of heterologous expression of *XYL2* ····· 46

4.1 Specific activitiy assay of KmXDH ····· 46

4.2. Microaerobic bath fermentation ····· 47

5. Conformation of heterologous expression of *XYL3* ····· 51

5.1 Specific activitiy assay of KmXK ····· 51

5.2 Microaerobic bath fermentation ····· 52

6. Comparison of KM123 with *Sch. stipitis* ····· 56

6.1 Specific activitiy assay of KmXK ····· 56

6.2 Microaerobic bath fermentation ····· 60

7. Effects of *ALD6* gene disruption on xylose fermentation in recombinant *S. cerevisiae* ····· 64

7.1 Confirmation of *ALD6* gene disruption ····· 64

7.2 Microaerobic bath fermentation ····· 65

8. Microaerobic batch fermentation in Bioreactor ····· 68

IV. CONCLUSIONS ····· 72

V. REFERENCES ····· 73

국문초록 ··· 83

감사의 글 ·· 88

LIST OF TABLES

Table 1. A list of recombinant *S. cerevisiae* strains used in this study·· 20

Table 2. List of the primers used in this study ····· 21

Table 3. Summary of Microaerobic batch fermentation of *S. cerevisiae* DX23/KM1 ····· 45

Table 4. Summary of aerobic batch fermentation of *S. cerevisiae* ISXK/KM12····· 50

Table 5. Summary of aerobic batch fermentation of *S. cerevisiae* KM12,3 ····· 55

Table 6. Summary of microaerobic batch fermentation of SS12,3 and KM12,3 ····· 63

Table 7. Summary of microaerobic batch fermentation of Km12,3 and Km12,3::△*ald6*····· 67

Table 8. Summary of microaerobic batch fermentation of SS12,3 and KM12,3 in bioreactor ····· 71

LIST OF FIGURES

Figure 1. Metabolic pathway of xylose metabolism in recombinant *S. cerevisiae* harboring *XYL1*, *XYL2* and *XYL3* from *K .marxianus* ····· 11

Figure 2 Strategy to improve the xylose fermentation in recombinant *S. cerevisiae* ····· 12

Figure 3. Genetic maps of plasmids pRS403KmXR, pRS406KmXRXDH and pRS403KmXK ····· 24

Figure 4. Genetic maps of plasmids pRS405KmXDH, pRS406KmXK 27

Figure 5. Schematic representation of *ALD6* gene disruption by homologous recombination. ····· 28

Figure 6. Nucleotide sequence of the *XYL1* gene from *K. marxianus* and its deduced amino acid sequ ····· 37

Figure 7. Nucleotide sequence of the *XYL2* gene from *K. marxianus* and its deduced amino acid sequence ····· 38

Figure 8. Nucleotide sequence of the *XYL3* gene from *K. marxianus* and its deduced amino acid sequence ····· 40

Figure 9. Comparison of the specific activities of xylose reductase (XR) between *S. cerevisiae* D/KmXR , *S. cerevisiae* DX23/KM1 · 42

Figure 10. Microaerobic batch fermentation profiles of *S. cerevisiae*

DX23/pRS403 ····· 44

Figure 11. Comparison of the specific activities of xylitol dehydrogenase (XDH) between *S. cerevisiae* D/KmXDH, *S. cerevisiae* ISXK/KM12 ····· 47

Figure 12. Microaerobic flask batch fermentation profile of *S. cerevisiae* ISXK/pRS406 ····· 49

Figure 13. Comparison of the specific activities of xylulokinase (XK) between *S. cerevisiae* D/KmXK and *S. cerevisiae* KM12,3 ·· 54

Figure 14. Microaerobic flask batch fermentation profiles of and *S. cerevisiae* D/pRS403406 and KM12,3 ····· 54

Figure 15. Comparison of the specific activities of single XR , XDH and XK ····· 58

Figure 16. Comparison of the specific activities of XR, XDH and XK between *S. cerevisiae* SS12,3 and *S. cerevisiae* KM12,3 ····· 60

Figure 17. Microaerobic flask batch fermentation profile of SS12,3 and KN12,3 ····· 62

Figure 18. Electrophoresis of diagnostic PCR product ····· 64

Figure 19. Microaerobic flask batch fermentation profiles of KM12,3 and KM12,3:: Δ*ald6* ····· 66

Figure 20. Microaerobic batch fermentation profiles of SS12,3 and KM12,3 ····· 70

I. INTRODUCTION

1. Bioethanol

Rising concerns over the cost of petroleum and the prospect of global warming are driving the development of technologies for the production of alternative fuels such as ethanol[Ragauskas AJ et al., 2006]. The long-term source of ethanol is from plant matter(biomass) through the fermentation of biomass carbohydrates to ethanol. The conversion of biomass to useable energy is not economical, however, unless hemicellulose is used in addition to the cellulose[Wyman CE, 2003]. Xylose is the second most abundant carbohydrate in nature and its commercial fermentation to ethanol could provide an alternative fuel source for the future.

Renewable lignocellulosic biomass is an attractive feedstock for fuel ethanol production since it is readily available, e.g., as a waste from the pulp and paper or agricultural industries, and also due to the fact that it is renewable with cycles many orders of magnitude shorter compared with those of fossil fuels. Lignocellulose, which is composed of cellulose, hemicellulose, and lignin[Aristidou and Penttila 2000l Saha 2003], is often hydrolyzed by acid treatment; the hydrolysate obtained is then used for ethanol fermentation by microornaisms such as yeast and

bacteria. Becacuse such lignocellulose hydrolysate contains not only glucose, bust also various monosaccharides, such as xylose, mannose, galactose, and arabinose and oligosaccharides, microorganisms should be required to efficiently ferment these sugars for the successful industrial production of ethanol.

2. Ethanol production in recombinant *Saccharomyces cerevisiae*

One of the most effective ethanol-producing yeasts, *Saccharomyces cerevisiae*, has several advantages owing to its high ethanol production from hexoses and high tolerance to ethanol and other inhibitory compounds in the acid hydrolysates of lignocellulosic biomass(Olsson and Hahn-Hagerdal 1993; Hahn-Hagerdal *et al.*, 2001) However, because wild-type strains of this yeast cannot utilize pentoses, such as xylose and arabinose, and celloligosaccharides, ethanol production from a lignocellulose hydrolysate is inadequate. Accordingly, many researchers have engineered yeast capable of xylose fermentation [Ho *et al.*, 1998; Eliasson *et al*. 2000; Hahn-hagerdal *et al*. 2001, Jeffries and Jin 2004]. Natural xylose-fermenting yeasts, such as *Pichia stipitis* [verduyn *et al*., 1985], *Candida shehatae*[Ho *et al*., 1990], and *Candida parapsilosis*[Lee *et al*., 2003], can metabolize xylose via the action of

xylose reductase(XR) to convert xylose to xylitol, and of xylitol dehydrogenase(XDH) to convert xylitol to xylulose. Therefore, ethanol fermentation from xylose can be successfully performed by recombinant *S. cerevisiae* carring heterologous XR and XDH from *P. stipitis*, and xylulokinase(XK) from *S. cerevisiae*[Ho *et al*., 1999; Eliasson *et al*., 2000; Toivari *et al*., 2001]

3. Xylose metabolism in yeast

Xylose can be utilized as a carbon source by various microorganisms such as bacteria, yeast and filamentous fungi [Ostergaard *et al*., 2000]. In bacteria, xylose can be isomerized to xylulose by xylose isomerase. Xylulose is subsequently phosphorylated by xylulokinase into xylulose-5-phosphate of the pentose phosphate pathway (the PP pathway). In general, yeasts convert xylose to xylulose through a two-step reduction and oxidation. Firstly, xylose is reduced to xylitol by NAD(P)H dependent xylose reductase. Then xylitol is either secreted from the cell or oxidized to xylulose by NAD^+ dependent xylitol dehydrogenase. The phosphorylation of xylulose to xylulose-5-phosphate is catalyzed by xylulokinase of the PP pathway [Hähn-Hägerdal *et al*., 2001]. The PP pathway consists of an oxidative stage that converts hexose phosphates to pentose phosphates, providing NADPH needed in biosynthetic

pathways and a non-oxidative stage in which the pentose phosphates are converted into hexose and triose phosphates.

S. cerevisiae is a very attractive organism to work with since it is nonpathogenic. In addition, due to its long history of application in the production of consumable products such as ethanol and baker's yeast, it has been classified as a GRAS (generally recognized as safe) organism. Its large-scale fermentation technology is well developed [Romanos *et al.*, 1992]. Also, it can be easily manipulated at a gene level by recombinant DNA technology because its genome has been completely sequenced [Goffeau *et al.,* 1996]. But *S. cerevisiae* cannot metabolize xylose as a sole carbon source since this yeast does not possess intrinsic enzymes necessary for xylose metabolism [figure 1].

4. *Kluyveromyces marxianus* and it's xylose metabolism

Ethanol production at elevated temperatures has received much attention because of the potential cost savings, which could be obtained by continuous evaporation of ethanol from the broth under reduced pressure (Hacking et al. 1984; Gough et al. 1996, 1997, 1998; Banat et al. 1998). This topic was recently reviewed for yeasts in general, including *K. marxianus*. The advantages described, besides the energy savings due to reduced cooling costs, were higher saccharification and fermentation

rates, continuous ethanol removal, and reduced contamination (Banat et al. 1998). However, the temperature increase has a negative effect on ethanol yield and also reduces the cell viability (Anderson et al. 1986; Ballesteros et al. 1991). *K. marxianus* was reported to produce alcohol at temperatures above 40°C and to have a maximum growth temperature of 47°C (Anderson et al. 1986), 49°C (Hughes et al. 1984), or even 52°C (Banat et al. 1992). Lower ethanol tolerance was observed when *K. marxianus* was compared to S. cerevisiae, and this was correlated with the activity of the plasma membrane ATPase (Rosa and Sa-Correia 1992; Fernanda and Sa- Correia 1992). Hacking et al. (1984) screened yeast strains for their ability to ferment glucose to ethanol at high temperatures. The tolerance of all species seemed to decrease with temperature, but in general, *Kluyveromyces* strains were more thermotolerant than *Saccharomyces*, which in turn can produce higher ethanol yields. Anderson et al. (1986) compared *K. marxianus* strains isolated from sugar mills and CBS strains for ethanol production at high temperatures. The CBS strains produced the same ethanol amounts as the new isolates but with lower cell viability and higher cultivation time. Sakanaka et al. (1996) reported the fusion of a thermotolerant strain of *K. marxianus* with a high ethanol producing strain of *S. cerevisiae*; however, their fermentative capacity was severely impaired and the fusants'

thermostability was lower than for either of the parental cells. Recently XR, XDH, XK of *K. marxianus* were reported. [Biao Zhang *et al*., 2011, Li Lulu *et al.,* 2013, Rongliang Wang et al., 2013]

Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) was produced with *K. marxianus*.[M Ballasteros et al., 2004] Also, by glcose and xylose co-fermentation, ethanol could be produced in *K. marxianus*. [JM laplace 1996]

5. Xylose reductase (XR)

Xylose reductase (XR) is classified as a member of the aldo-keto reductase (AKR) superfamily which is made up of 14 different families and approximately 120 members [Hyndman et al., 2003; Kavanagh et al., 2003]. XR has been purified from many yeasts such as *P. stipitis* [Rizzi *et al.,*1988; Webb *et al*., 1992], *C. shehatae* [Ho *et al*., 1990]. XR from *P. stipitis* is active with various aldose substrates such as D-xylose, D-glucose and D-ribose [Verduyn, C *et al*., 1985].

XR from *P. stipitis*, *C. shehatae*, *C. parapsilosis* can utilize both NADPH and NADH as a cofactor, whereas XR from *C. tropicalis*, *C. utilis*, *C. guilliermondii*, *D. hansenii* exclusively uses NADPH only [Kim *et al*., 2002; Lee *et al*., 2003; Parajo *et al*., 1998; Winkelhausen

and Kuzmanova, 1998; Yokoyama *et al*., 1995].

XR from *K. marxianus* can utilize NADPH only as a cofactor. The *Km* of the recombinant KmXR for NADPH is 65.67 μM and KmXR activity is 1.295 U/mg, which is lower than those of most reported yeast XRs, and the enzyme has no activity with coenzyme NADH. This result demonstrates that the XR from *K. marxianus* is highly coenzyme specific.[Biao Zhang *et al*., 2011]

6. Xylitol dehydrogenase (XDH)

Xylitol dehydrogenases (EC 1.1.1.9) are classified as members of the medium-chain alcohol dehydrogenease family, and share structural and functional properties with medium-chain sorbitol dehydrogenases [Lunzer *et al.,* 1998]. XDHs have been purified and characterized from various xylose-fermenting yeasts, including *K.marxianus P. stipitis* [Rizzi *et al*., 1989b], *C. shehate* [Yang and Jeffries, 1990]

S. cerevisiae xylitol dehydrogenease(ScXDH), the gene product of the YLR070c ORF, is specific for NAD^+ as a cofactor(Richard *et al*., 1999). The apparent kinetic constants of the ScXDH were: $K_{m,xylitol}$ = 25 mM, K_{m,NAD^+} = 240 μM, $K_{m,xylulose}$ = 1.1 mM , and $K_{m,NADH}$ = 55 μM. These kinetic values suggest that the enzyme actually favors the reverse direction whereby xylulose is reduced to xylitol and that this enzyme

reaction may be incapable of supporting a high flux of xylose through central metabolism which is needed for fermentation of xylose to ethanol.

The highest activity of KmXDH could be observed at pH 9.5 during 55°C. The values of k_{cat}/K_m indicate that KmXDH prefers NAD^+ to $NADP^+$ ($k_{cat}/K_{m\ NAD^+}$ 3681/min mM and $k_{cat}/K_{m\ NADP^+}$ 1361/min mM).[Li Lulu *et al.,* 2013]

7. Xylulokinase(XK)

Xylulokinase(EC 2.7.1.17) that acts to phosphorylate xylulose for entry into the pentose phosphate pathway has been studied in *Lactobacillus pentosus*, *Klebsiella aerogenase*, and *Klebsiella pneumonia* [Lampen, 1953; Stumpf and Horecker, 1956; Feldmann *et al.,* 1992].

The gene encoding xylulokinase (*XKS1*) from *S. cerevisiae* is found on the right arm of chromosome VII and encodes the native XK (ScXK) [Rodriguez-Pena *et al.,* 1998]. Deletion of the *XKS1* gene results in mutants unable to grow on xylulose as the sole carbon source [Richard *et al.,* 2000]. *K.marxianus* had its own XK gene. *KmXYL3* was expressed in *Escherichia coli* BL21 (DE3) cells, and the specific activity of the resulting recombinant purified xylulokinase was 23.5 mU/mg. [Rongliang Wang et al., 2013]

8. Acetaldehyde dehydrogenase family (ACDHs)

Two acetaldehyde dehydrogenase(ACDHs) were originally identified in *S. cerevisiae*;an Mg^{2+}-activated, $NADP^{+}$-dependent cytosolic enzyme [Seegmiller, 1953] and a glucose-repressed, $NAD(P)^{+}$-dependent, mitochondrial enzyme activated by K^{+} and thiols [Jacobson & Bernofsky, 1974]. Five ACDH genes have now been identified in the genome sequence of *S. cerevisiae* S288C. According to the nomenclature of Navarro—Avino *et al*.(1999), the cytosolic ACDHs are encoded by *ALD6*, *ALD2* and *ALD3*, whereas the mitochondrial isoforms are encoded by *ALD4* and *ALD5*. Ald6p and Ald4p, which are Mg^{2+} -and K^{+}-dependent, respectively, are the major isoforms and have been shown to be involved in growth on glucose and on ethanol [Meaden *et al.,* 1998].

In *S. cerevisiae*, acetate is produced as an intermediated of the pyruvate dehydrogenase (PDH) bypass, which converts pyruvate into acetyl-CoA in a series of reactions catalysed by pyruvate decarboxylase(PDC), acetaldehyde dehydrogenase(ACDH) and acetyl-CoA synthetase. This pathway is the sole source of cytosolic acetyl-CoA, which is required for anabolic processes such as lipid biosynthesis [Flikweert *et al.,* 1996]. The reaction catalyze by ACDH also generates reducing equivalents, which are required for a variety of synthetic pathways and redox reactions, in the form of NAD(P)H. ACDH produces acetate by

oxidizing the acetaldehyde produced from pyruvate during the fermentation of sugars and that formed during ethanol oxidation.

One reason for improvement of specific rate of xylose consumption may be that *ALD6* gene disruption led to escape the inhibition of cellular metabolism caused by acetate accumulation in the medium [M. Teresa Ramos *et al.*, 1990].

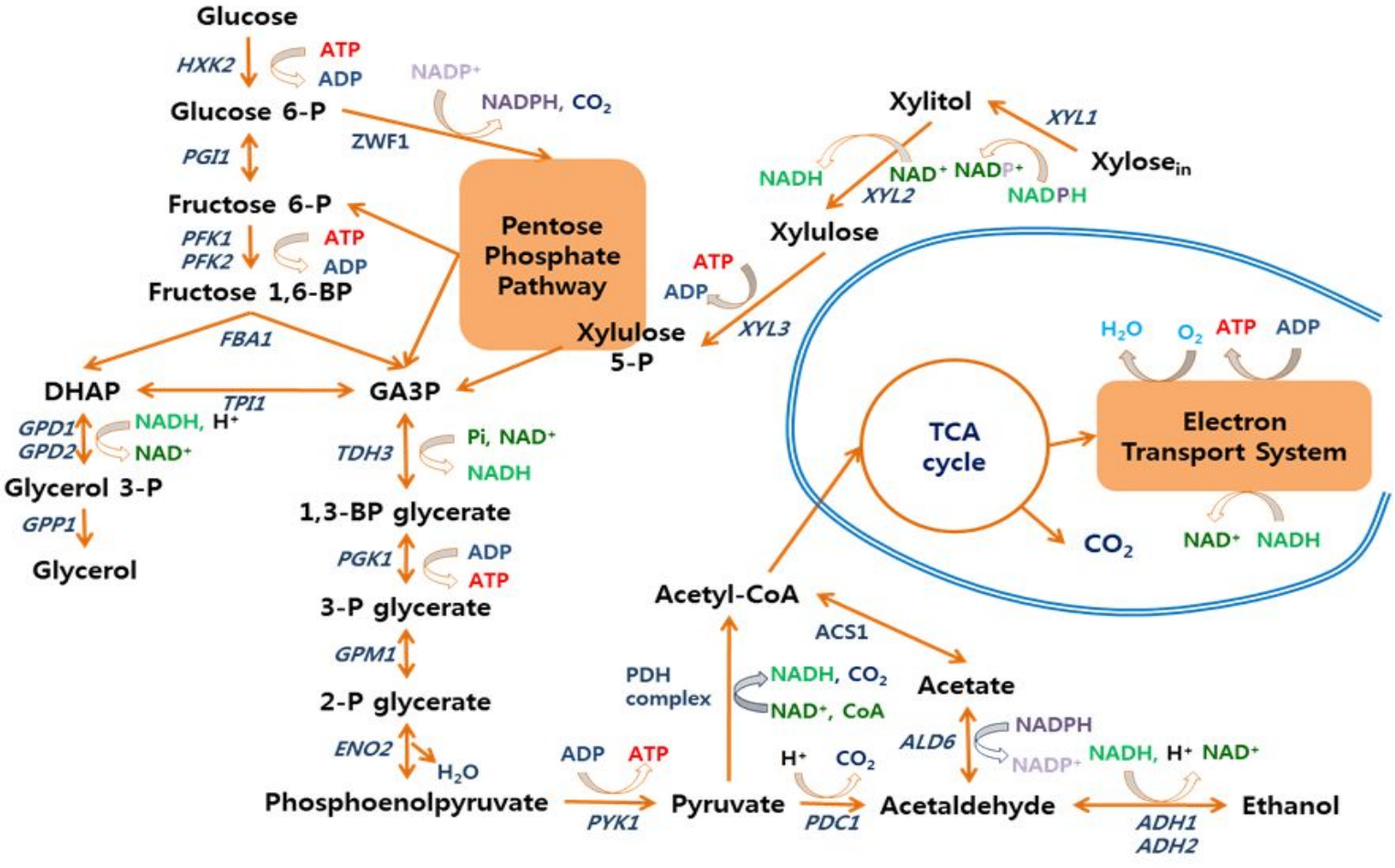

Figure 1. Metabolic pathway of xylose metabolism in recombinant *S. cerevisiae* harboring *XYL1*, *XYL2* and *XYL3* from *K .marxianus*

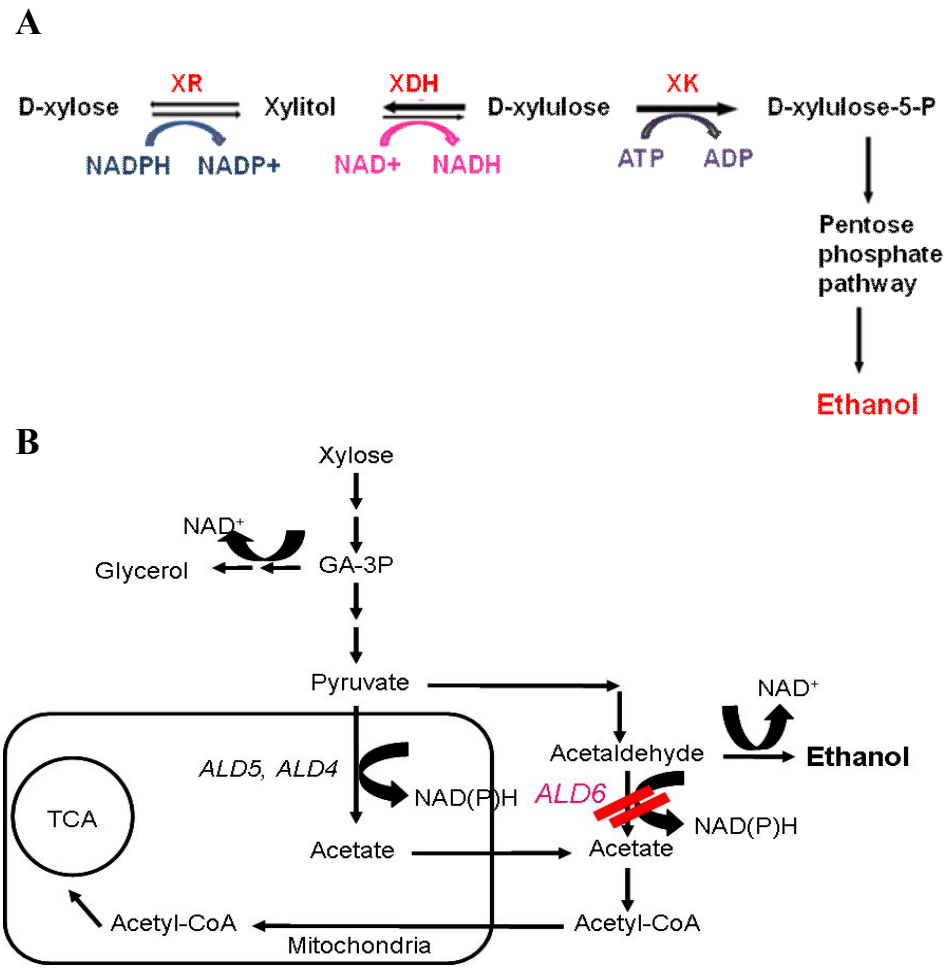

Figure 2. Strategy to improve the xylose fermentation in recombinant *S. cerevisiae*

(A) Process of converting xylose to ethanol and key enzymes

(B) To minimize the acetate formation by *ALD6* gene disruption

9. Cofactors

Heterologous expressions of xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*), either case of which being accompanied by overexpression of xylulokinase (*XKS1 or XYL3*), are known as the prevalent strategies for metabolic engineering of S. cerevisiae to ferment xylose. [K€otter & Ciriacy, 1993; Tantirungkij *et al.,* 1993; Walfridsson *et al.,* 1997; Ho *et al.,* 1998, 1999; Meinander *et al.,* 1999; Eliasson *et al.,* 2000, 2001; Jin *et al.,* 2000.]

But alternative strategy employs overexpression of *GRE3* coding for endogenous aldose reductase instead of *XYL1* to construct efficient xylose-fermenting *S. cerevisiae*. Replacement of *XYL1* with *GRE3* has been regarded as an undesirable approach because NADPH-specific aldose reductase (*GRE3*) would aggravate redox balance with xylitol dehydrogenase (*XYL2*) using NAD+ exclusively. Optimized expression levels of *GRE3, XYL2,* and *XYL3* could overcome redox imbalance during xylose fermentation by engineered S. cerevisiae under oxygen-limited conditions.[S R Kim *et al.,* 2013]

As XR from *K. marxianus* also utilize only NADPH, cofactor imbalance between NADPH dependent XR and NAD+ dependent XDH would be occured. But It is possible to produce ethanol in spite of redox imbalance by combination of XR, XDH and XK.

10. Research Objectives

Key genes like *XYL1*, *XYL2*, *XYL3* involved in the xylose metabolic pathway were introduced to produce ethanol from xylose in recombinant *S. cerevisiae*. *ADL6* gene was disrupted to increase ethanol and decrease byproduct that inhibited ethanol fermentation should be considered.

This study was focused on characterization of genetic factors and process conditions for the production of ethanol from xylose in metabolically engineered *S. cerevisiae*

The specific objectives of this study are as follows.

(1) To clone genes of xylose metabolism *XYL1* encoding XR, *XYL2* encoding XDH and *XYL3* encoding XK from *K. marxianus*.

(2) To compare recombinant strain harboring *K. marxianus* genes with *Sch. stipitis* genes generally used in xylose fermentation.

(3) To express *XYL1, XYL2* and *XYL3* for producing ethanol and minimize the acetic acid formation as inhibitory compound by *ALD6* gene disruption

II. MATERIALS AND METHODS

1. Reagents

All chemicals used were of reagent grade. Glucose, xylitol, agarose, ampicillin, ethidium bromide, yeast synthetic drop-out supplement, yeast nitrogen base (YNB, w/o amino acid), protease inhibitor cocktail, bovine serum albumin, β-nicotinamide adenine dinucleotide phosphate (β-NADPH, β-$NADP^+$), β-nicotinamide adenine dinucleotide(β-NADH, β-NAD^+), EDTA, trizma base, and antifoam were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Protease inhibitor cocktail was purchased from Roche Company. Bacto-peptone, bacto-trypton, yeast extract and bacto-agar were obtained from Difco (Detroit, MI., USA); xylose and glycine from Junsei (Tokyo, Japan); ethanol from Merck (Darmstadt, Germany); HPLC-grade water from J.T. Baker (Phillipsburg, NJ, USA); 2N NaOH and 5N HCl, NaCl, H_2SO_4, and potassium phosphate from Duksan (Ansan, Korea).

2. Strains and plasmids

2.1. Strains

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for the propagation and preparation of plasmid DNA. *S. cerevisiae* D452-2 [*Mata, leu2 his3 ura3 can1*] were used as host strains for the expression of xylose metabolic pathway genes. *S. cerevisiae* D452-2 strain was kindly donated by Prof. Makino at Kyoto university. *S. cerevisiae* ISXK [D452-2 , *Ty1*-delta::P_{GPD}-*XKS1*-T_{GPD}-neo^r] which had been engineered for overepxression of endogenous XK [Lee 2008] was also used as an host for additional engineering.

***XYL1* expression system**

The *XYL1* gene from *K. marxianus* was introduced and expressed in *S. cerevisiae*. The linearized plasmids p403KmXR was transformed into *S. cerevisiae* DX23 strain. *S. cerevisiae* DX23 [D452-2, *ura3::URA3-*PGK_P*-XYL2-*PGK_T*,*PGK_P*-XYL3-*PGK_T] harboring pRS306XDHXK [Jung-Hyun Jo] had XDH, XK from *scheffersomyces stipitis* for production of ethanol from xylose. The plasmid p403KmXR and pRS306XDHXK were linearized by restriction enzyme *Bsm*I.The transformants were named as DX23/KM1.

***XYL1-XYL2* expression system**

The *XYL1* and *XYL2* genes from *K. marxianus* were introduced and

expressed in recombinant *S. cerevisiae*. The linearized plasmids p406KmXRXDH were transformed into recombinant *S. cerevisiae* ISXK strain. The plasmid p406KmXRXDH was linearized by restriction enzyme *Xcm*I. The transformants were named as ISXK/KM12.

***XYL1-XYL2 –XYL3* expression system**

The *XYL1, XYL2* and *XYL3* genes were introduced and expressed in *S. cerevisiae*. The linearized plasmid p406KmXRXDH and p403KmXK was transformed into *S. cerevisiae* D452-2 strain. The plasmid p406KmXRXDH and p403KmXK were linearized by restriction enzyme *Xcm*I. The transformants were named KM12,3.

***XYL1-XYL2 –XYL3* and △*ALD6* expression system**

The linearized plasmid pAUR_d_ALD6 [Lee 2008] was transformed into KM12,3. The plasmid pAUR_d_ALD6 was linearized by restriction enzyme *Sal*I. The transformant was named KM12,3△*ald6*

Strains for confirming *XYL1*, *XYL2* and *XYL3* from *K. marxianus* and *Sch. stipitis*

To confirm activity of each *XYL1*, *XYL2* and *XYL3*, p403Km(Ss)XR, P405Km(Ss)XDH and p406Km(Ss)XK was respectively transformed to *S. cerevisiae* D452-2 strain to construct D/Km(Ss)XR, D/Km(Ss)XDH and D/Km(Ss)XK.

The wild type and recombinant strains were stored on YEPD and YNB

medium in a deep freezer at -80℃ suspended in 15% glycerol.

2.2. Plasmids

pRS306XDHXK plasmids were kindly donated by Jung-Hyun Jo at Seoul National university. *XYL2* and *XYL3* genes coding for NAD+ dependent PsXDH and NADH-dependent PsXK each were cloning to pRS306.

Plasmid pAUR_d_ALD6 was constructed by Sung-Hang Lee. [2008] Truncated *ALD6* gene was isolated from *S. cerevisiae* CEN.PK2-1D genomic DNA and introduced into plasmid pAUR101(Takara, Japan) .

Plasmid pRS403, pRS405, pRS406, pRS403PGK and pRS426GPD were used as mother vectors and cloning vectors for introduction of *XYL1, XYL2, XYL3* genes.

XR was isolated from genomic DNA of *K. marxianus* by the PCR using primers listed in Table 2. This was introduced into plasmid pRS426 to construct plasmid pRS426GPDKmXR. XR was expressed under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. Additionally, for activity assay of single KmXR, XR cassettes from *K. marxianus* and *Sch. Sipitis* was cloned to pRS403 to construct p403GPDKm(Ss)XR [Figure 3].

XDH also could be isolated from genomic DNA of *K. marxianus* by the PCR using primers listed in Table 2. This was cloned into plasmid pRS403 to construct plasmid pRS403PGKKmXDH . Then, XDH was expressed under the control of the constitutive phosphoglycerate kinase (PGK) promoter. Additionally, for activity assay of single KmXDH, XDH cassettes from *K. marxianus* and *Sch. Sipitis* was cloned to pRS405 to construct p405PGKKmXDH [Figure 4]..

XR and XDH expression cassettes were isolated from pRS426GPDKmXR and pRS403PGKKmXDH plasmids by the PCR using primers listed in Table 2, respectively. They introduced into plasmid pRS406 to construct plasmid pRS406KmXRXDH [Figure 3]. XR and XDH were expressed under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and phosphoglycerate kinase (PGK) promoter respectively.

Finally, XK was isolated from genomic DNA of *K. marxianus* by the PCR using primers listed in Table 2. Plasmid pRS403PGKKmXK was constructed by introducing XK [Figure 3]. XK was expressed under the control of the constitutive phosphoglycerate kinase (PGK) promoter. Additionally, for activity assay of single KmXK, XK cassettes from *K. marxianus* and *Sch. Sipitis* was cloned to pRS406 to construct p406PGKKmXK [Figure 4]..

Table 1. A list of recombinant *S. cerevisiae* strains used in this study

Strains	Genotype	Used plasmid
D452-2	*Matα, leu2 his3 ura3 can1*	
ISXK	D452-2 , *Ty1*-delta:: GPD_P -*XKS1*- GPD_t-neo	pAUR_d_ALD6 [SH LEE 2008],
DX23	D452-2, *ura3::URA3-*PGK_P*-XYL2-*PGK_T*-*PGK_P*-XYL3-*PGK_T	pRS306XDHXK [Jung-Hyun Jo]
DX23/KM1	DX23, *his3::HIS3- *GPD_P *–KmXYL1- *$CYC1_t$	p403GPDKmXR
ISXK/KM12	ISXK, *ura3::URA3- *GPD_P *–KmXYL1- *$CYC1_t$*-*PGK_P*-KmXYL2-*PGK_T	p406KmXRXDH
KM12,3	D452-2, *ura3::URA3-*GPD_P*–KmXYL1-*$CYC1_T$ $_tPGK_P$*-KmXYL2-*PGK_T, *his3::HIS3- *PGK_P *– KmXYL3- *PGK_t	p406KmXRXDH, p403PGKKmXK
Ss12,3	D452-2, *ura3::URA3-*GPD_P*–XYL1-*$CYC1_T$ - $_tPGK_P$ *-XYL2-*PGK_T, *-his3::HIS3- *PGK_P *– XKS1- *PGK_t	p406XRXDH, p403PGKXK [Jung-Hyun Jo]
KM12,3Δ*ald6*	KM12,3, *Ty1*-delta:: GPD_P -*XKS1*- GPD_t-neo	pAUR_d_ALD6 [SH LEE 2008],

Table 2. A list of primers used in this study

Name	**Sequence**	**Relevant work** (Tm)
F_KmXR_ NheI	CTAGCTAGCATGACATACCTCGCACCAACA	Cloning KmXR (50)
R_ KmXR _BglII	GAAGATCTTTATAAGATAAAGGTTGGGAATTCGTTG	Cloning KmXR (50)
F_ KmXDH_ NheI	CTAGCTAGCATGACCAACACTCAAAAAGCCG	Cloning KmXDH (50)
R_KmXDH _ BglII	GAAGATCTTCATTCTGGACCATCAATGATAGTCC	Cloning KmXDH (50)
F_ KmXK_ SpeI	GACTAGTATGTCTACCCCATACTATTTAGG	Cloning KmXDK (50)
R_ KmXK _ BamHI	CGGGATCCTCAACTGGTCCTGCTGCTC	Cloning KmXDK (50)
R_GPDp_SacI	GAGCTCTCATTATCAATACTCGCCAATT	Coning GPDp-CYC1t cassette (50)
F_ CYCt_SacI	GAGCTCGGCCGCAAATTAAAGCCTTCG	Coning GPDp-CYC1t cassette (50)
F_PGKp_SpeI	GACTAGTGTGAGTAAGGAAAGAGTGAGGA	Coning PGKp-PGKt cassette (50)
R_ PGKt_SacII	TCACGCGGTAACGAACGCAGAATTTTCGAGTTA	Coning PGKp-PGKt cassette (50)
F_check_ald6-d	ATGACTAAGCTACACTTTGACACTGC	Checking disruption of *ALD6*
R_check_pAUR_d	AAATACCGCATCAGGCGCCATTC	Checking disruption of *ALD6*

3. DNA Manipulation and Transformation

3.1. Enzymes

Restriction enzymes, calf intestinal alkaline phosphatase (CIP) and Klenow fragment were purchased from New England Biolabs (Beverly, MA, USA). T4 DNA ligase was obtained from Takara(Tokyo,Japan).

3.2. Transformation of *E. coli*

Transformation of *E. coli* was carried out as described by Sambrook et al. [1989]. *E. coli* Top10 was cultured in 5 mℓ LB medium for 12hr. Half a milliliter of the culture was transferred to fresh 50 mℓ LB medium and cultured until OD_{600} reached 0.5. Cells harvested by centrifugation at 6000 rpm for 5 min at 4℃ were resuspended in 5 mℓ of ice-cold 100 mM $CaCl_2$ solution containing 15% (v/v) glycerol. Resuspended cells were aliquoted to 100 μℓ, mixed with DNA, and kept on ice for 30 min. They were subjected to heat-shock at 42℃ for 45 sec, and one mℓ of LB medium was added to the test tubes and incubated at 37℃ for 1 hour to allow the bacteria to express the antibiotic resistance. An appropriate volume of the transformed cells was spread on LB agar plates with an ampicillin selection marker.

3.3. Preparation of plasmid DNA and yeast genomic DNA

Mini-scale preparation of plasmid DNA was carried out using *AccuPrep* ™ Plasmid Extraction Kit from Bioneer Co. (Daejon, Korea) according to the manufacturer's instruction.

Preparation of yeast genomic DNA to obtain a template for the gene was carried out using Masterpure™ Yeast DNA Purification Kit (EPICENTRE, Madison, Wis., USA) according to the manufacturer's instruction.

3.4. Isolation of DNA fragments and DNA sequencing

DNA was digested with restriction enzymes and separated on a 0.1% (w/v) agarose gel. After full separation of the desired DNA band from the gel, the gel containing the DNA fragment was solubilized and further purified by using Gel Extraction Kit from Takara(Tokyo,Japan). DNA sequencing was performed at SolGent co., Ltd (Daejeon, Korea).

3.5. Polymerase chain reaction (PCR)

PCRs were performed with the GeneAmp PCR System 2400 (Applied Biosystems, Lincoln, CA, USA). PCRs for subcloning of *XYL1, XYL2* and *XYL3* were performed in 50 μL of dyemix solution from Enzynomics Co.(Korea) containing 10 pM each of forward and reverse primers (Table 2), and 1 μL genomic DNA of *K. marxianus*. PCRs for

cloning of *XYL1* and *XYL2* cassettes were performed in in 50 μL of dyemix solution from Enzynomics Co.(Korea) containg 10 pM each of forward and reverse primers (Table 2), and 1 μL pRS426GPDKmXR and pRS403PGKKmXDH respectively. After heating the reaction tubes for 5 min at 94℃, 25 cycles of PCR amplification were performed as follows: 30 sec at 94℃, 30 sec at 50℃, and 1min 30 sec at 72℃(time is dependent on product size, 1 min for 1kb), followed by 7 min at 72℃ during the last cycle.

In case of colony PCR, total volume of dyemix solution was 20 μL containg 10 pM each of forward and reverse primers, and colony was put into dyemix solution. The reaction tubes was heated for 10 min at 94℃.

3.6. Yeast transformation

Yeast transformation was carried out by the Alkali-Cation method and MicroPulser™ Electroporation Apparatus (Bio-Rad, Richmond, USA) with the following modifications to increase the transformation efficiency [Wang *et al*., 1996]. Transformants were cultivated and selected on YNB ura^- ,YNB ura^-his^- or YP-AbA(0.5 mg/L) plates.

4. Homologous recombination

Introduction of *XYL1, XYL2* and *XYL3* and disruption of the *ALD6 gene*

were carried by homologous recombination. Two step gene replacement method with some modification was used in this study [Dan Burke, 2000].

XYL1, XYL2 cassettes and *XYL3* were obtained by PCR. The PCR product was cloned into pRS406 having *URA3* marker and pRS403 having *HIS3* marker to make pRS406KmXRXDH and pRS403KmXK. Plasmid pRS406KmXRXDH and pRS403KmXK were linearized with retriction enzyme *Xcm*I and *Bsm*I before transformation into D452-2 strain.

Truncated *ALD6* gene was amplified was obtained by PCR. The PCR product was cloned into pAUR101 that has AUR1-C marker to make pAUR_d_ALD6. Plasmid pAUR_d_ALD6 was linearized with restriction enzyme *Sal* I as shown in before transformation into KM12,3 strain.

Transformants were selected on YP glc with AbA 0.5 mg/L plate and used for diagnostic colony PCR. PCR primers listed in Table 2 were used.

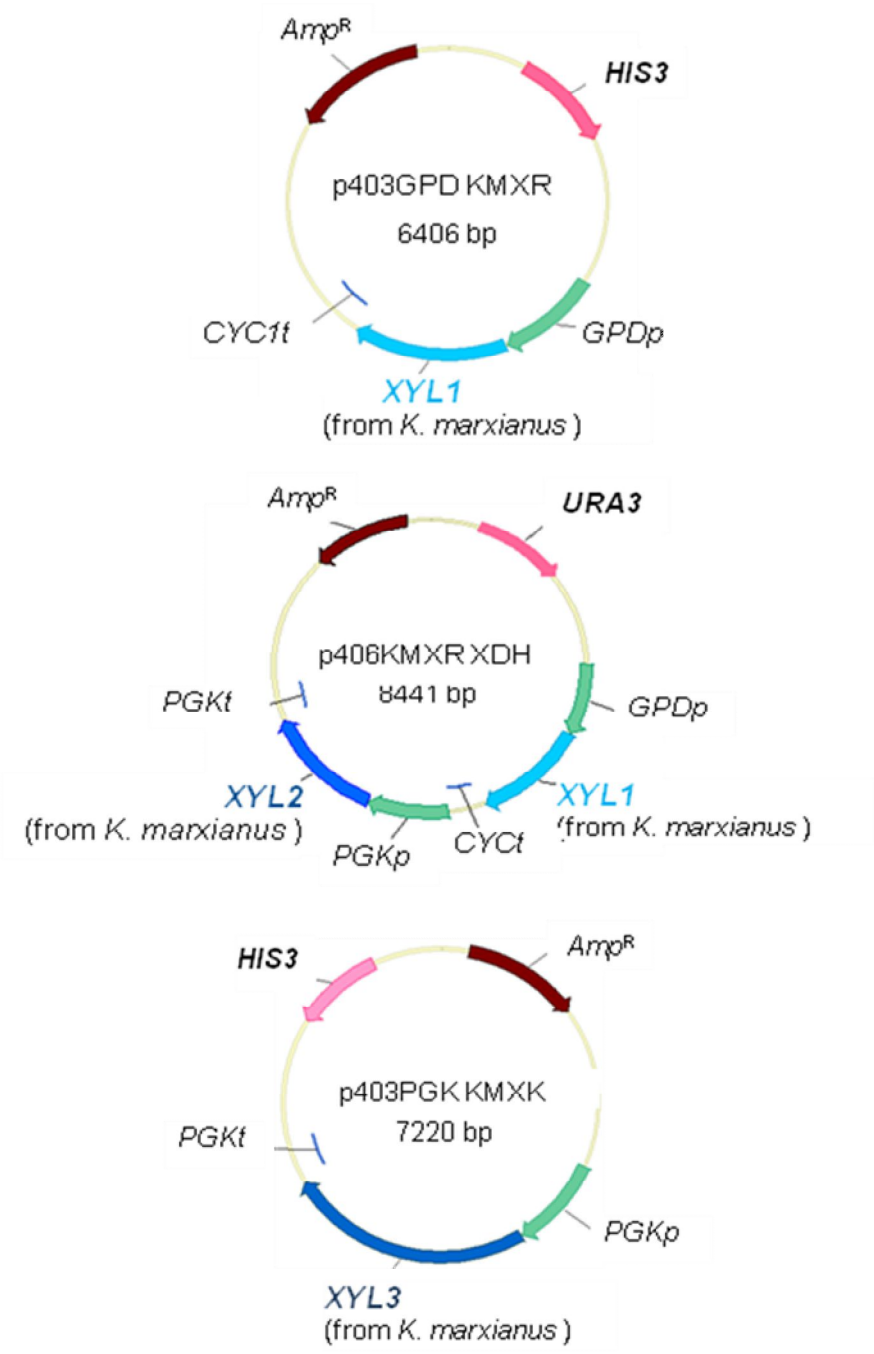

Figure 3. Genetic maps of plasmids pRS403KmXR, pRS406KmXRXDH and pRS403KmXK

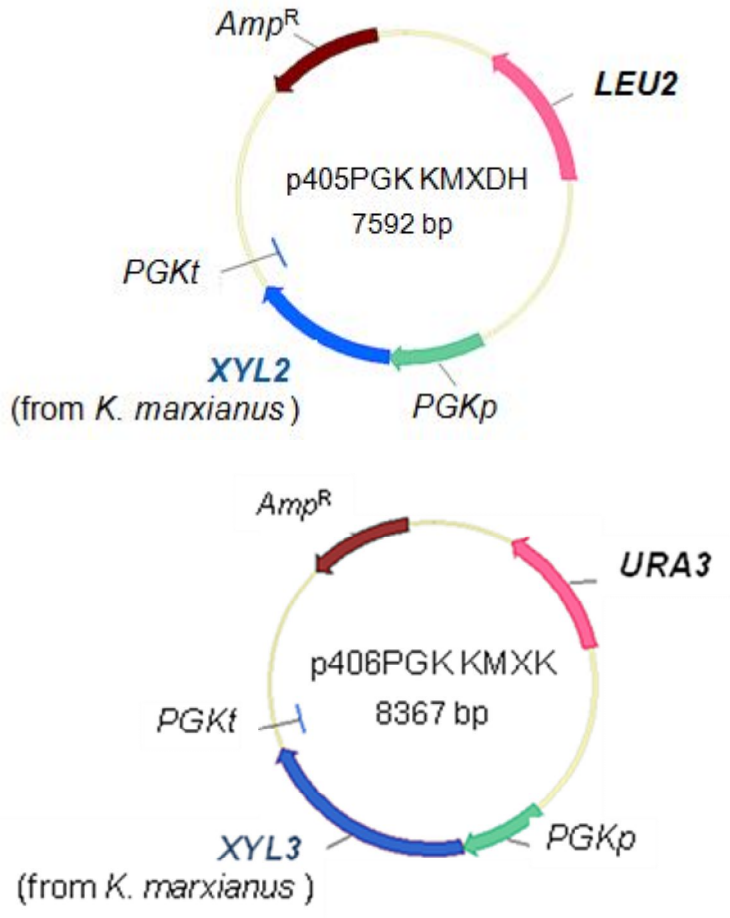

Figure 4. Genetic maps of plasmids pRS405KmXDH, pRS406KmXK

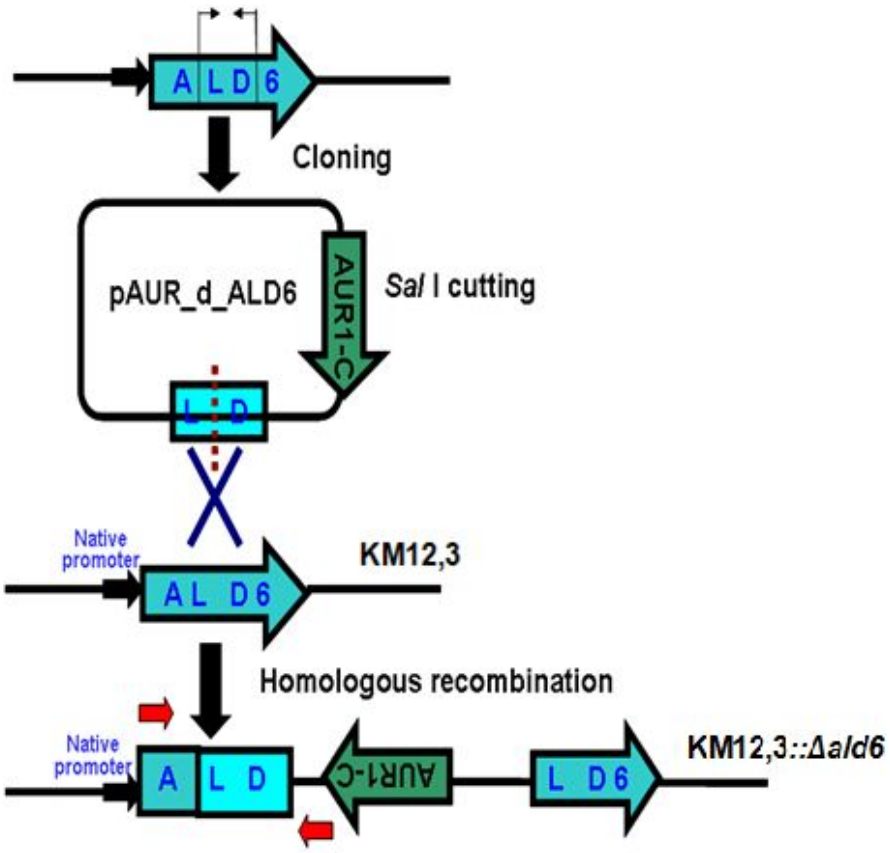

Figure 5. Schematic representation of *ALD6* gene disruption by homologous recombination.

5. Media and culture conditions

5.1 Media

LB medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) was used for *E. coli* cultivation. YEPD-ABA medium (1% yeast extract, 2% bacto-peptone, 2% glucose, Aureobasidin A 0.5 mg/L) and YNB medium (0.6 g/L) which lacked appropriate amino acid were used for selection of yeast strains. YEPX (1% yeast extract, 2% bacto-peptone, 4% xylose) were used for cultivation of yeast strains.

5.2 Inoculation

Recombinant *S. cerevisiae* stock was transferred to a test-tube containing 5 ml of appropriate medium and incubated overnight at 30°C in a shaking incubator (Vision, Korea). Pre-culture was carried out in a 500 ml baffled flask with 100 ml or 200 ml working volume at 30°C for 12 h in YPD (2% glucose) media at fully aerobic condition (250 rpm). The inocula were prepared by growing cells overnight to an OD_{600} of over 10. The cells were then harvested in 50 mL autoclaved falcon, resuspended in 50 mL DDW and then transferred to the bioreactor, giving an initial OD_{600} of approximately 5.

In case of flask culture, recombinant cell stock was transferred to test-tube containing 5ml YPD (2%glucose) and incubated overnight at 30°C,

fully aerobic condition (250 rpm). This cells were harvested in 2 ml eppen tube, and then transferred to 250ml glass flask containing 50ml YPX (4% xylose), giving an initial) OD_{600} of approximately 5.

5.3 Cultivations

Batch flask cultivations took place in a 250 ml glass flask with 50 ml working volume at 30 °C, oxygen-limited condition (80 rpm). YEP medium (1 % (w/v) yeast extract, 2 % (w/v) peptone) supplemented with 4 % (w/v) xylose was used for batch flask cultivation.

Bioreactor cultivations took place in a 1L B.Braun multi-fermenter (BIOSTAT-Q,Germany) with 0.5 L working volume. Medium acidity was automatically controlled at pH 5.5 by addition of 2 N NaOH from Duksan (Korea) and temperature of 30℃ was maintained throughout the cultivation. Foam was removed by the addition of Antifoam 204 (Sigma Chemical Co., USA). YEP medium (1 % (w/v) yeast extract, 2 % (w/v) peptone) supplemented with 4 % (w/v) xylose was also used for batch fermentations. The fermentation experiments were performed under oxygen limited condition at 0.3 vvm and 200 rpm.

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6. Analysis

6.1 Dry cell mass

Dry cell mass concentration was estimated by measuring absorbance at 600 nm by a spectrometer (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Optical density was converted into dry cell mass by using the following conversion equation.

$$\text{Dry cell mass (g/L)} = 0.30 \times OD_{600}$$

6.2 Sugar and ethanol concentrations

Concentrations of glucose, xylose, xylitol, glycerol, acetic acid and ethanol were measured by a high performance liquid chromatography (Agilent 1260infinity, U.S.A) equipped with the Carbohydrate Analysis column (Phenomenex, USA). The carbohydrate analysis ion exclusion column heated at 60°C was applied to analyze the 20 ul of diluted culture broth. Detection was made with a reflective index detector at 35°C. HPLC operation conditions were set according to the instruction manual of the column supplier. H_2SO_4 (5 mM) solution was used as mobile phase at a flow rate of 0.6 ml/min.

6.3 Measurement of enzyme activities

Sample preparation

50 ㎕ of the recombinant *S. cerevisiae* stock was transferred to a test-tube containing 5 ml of YPD media and incubated overnight at 30 °C in

a shaking incubator (Vision, Korea) at 250 rpm.

Samples of steady-state cultures were harvested by 13200 rpm centrifugation, giving them same OD_{600} at 20. After washing cells twice, centrifuge them and threw supernatant away. Then, cells were resuspended with 500 μl Y-PER (THERMO #78991) and appropriated buffers containing protease inhibitor cocktail and agitated for 20 minutes at room temperature. After centrifugation of the samples for 10 minutes at 4℃, 13200 rpm, the supernatant was used for crude extract.

Xylose reductase (XR) activity

Preparation of crude cell extract and measurement of xylose reductase activity were one using the method described by Jeun [Jeun *et al.*, 2003] with some modification.

The standard assay volume of 200 μL contained 50 mM Potassium phosphate buffer (pH 6.0), 0.4 mM NADPH or NADH and 2M xylose. Xylose reductase activity was measured microplate reader reader (Molecular Devices Co., Sunnyvale, CA, USA) by monitoring the oxidation of NADPH or NADH at 30℃ and 340 nm.

One unit of XR activity was defined as the amount of enzyme that can oxidize one micromole of NADPH or NADH per minute. Specific

enzyme activity (U/mg protein) was estimated by dividing enzyme activity by the cellular protein concentration.

Xylitol dehydrogenase (XDH) activity

Preparation of crude cell extract and measurement of xylitol dehydrogenase activity were one using the method described by Jin [Jin *et al*., 1998] with some modification.

The standard assay volume of 200 μL contained 50 mM Tris-HCl buffer (pH 8.5), 50 mM $MgCl_2$, 4 mM NAD^+ and 300 mM xylitol. Xylitol dehydrogenase activity was measured microplate reader reader (Molecular Devices Co., Sunnyvale, CA, USA) by monitoring the reduction of NAD^+ at 30℃ and 340 nm.

One unit of XDH activity was defined as the amount of enzyme that can reduce one micromole of NAD^+ per minute. Specific enzyme activity (U/mg protein) was estimated by dividing enzyme activity by the cellular protein concentration.

Xylulokinase (XK) activity

Preparation of crude cell extract and measurement of xylulokinase activity were one using the method described by Lee [Lee *et al*., 2000] with some modification.

The xylulokinase reaction is done by coupling the reduction of pyruvate to lactase by lactate dehydrogenase. Xylulokinse produces a small amount of ADP to allow pyruvate kinase to convert phosphoenolpyruvate into lactate. Xylulokinase activity was determined by measuring the rate of NAD^+ production at 340 nm.

Xylulose+ATP -----XK------> Xylulose 5-phosphate + ADP

ADP + Phosphoenolpyruvate -----PK------> Pyruvate + ATP

Pyruvate + NADH ----LDH-------> Lactate + NAD^+

The standard assay volume of 200 μL contained 20 mM glycylglycin buffer(pH 7.4), 0.2 mM NADH, 1.1 mM ATP, 2.3 mM phosphoenolpyruvate, 5 mM $MgSO_4$, 2 U pyruvate kinase, 2 U lactate dehydrogenase and 8.5 mM xylulose. Xylulokinase activity was measured microplate reader reader (Molecular Devices Co., Sunnyvale, CA, USA) by monitoring the oxidation of NADH at 30℃ and 340 nm.

One unit of XK activity was defined as the amount of enzyme that can oxidize one micromole of NADH per minute. Specific enzyme activity (U/mg protein) was estimated by dividing enzyme activity by the cellular protein concentration.

Protein concentration

Protein concentration was determined by the protein assay kit (Bio-Rad, Richmond, CA, USA) based on the method of Bradford using bovine serum albumin (BSA) as standard.

III. RESULTS AND DISCUSSIONS

1 . Cloning and sequence analysis of *XYL2, XYL2, XYL3* genes from *K. marxianus*

K. marxianus genome is currently being sequenced but the projects are listed as incomplete [http://genomesonline.org/cgibin/GOLD/bin/Search.cgi]. Seqeuncing of *XYL1, XYL2, XYL3* from *K. marxianus* was done [http://ergo.integratedgenomics.com/ERGO/login.cgi,], thus primer for cloning genes was able to be constructed. ORF of each genes were successfully obtained by PCRs with following primers

A 990 bp ORF of KmXR encoding a 329 amino acid protein [fig. 6], a 1,065 bp ORF of KmXDH encoding a 354 amino acid protein [fig. 7], a 1,812 bp ORF of KmXK encoding a 603 amino acid protein could be found. [fig. 8]

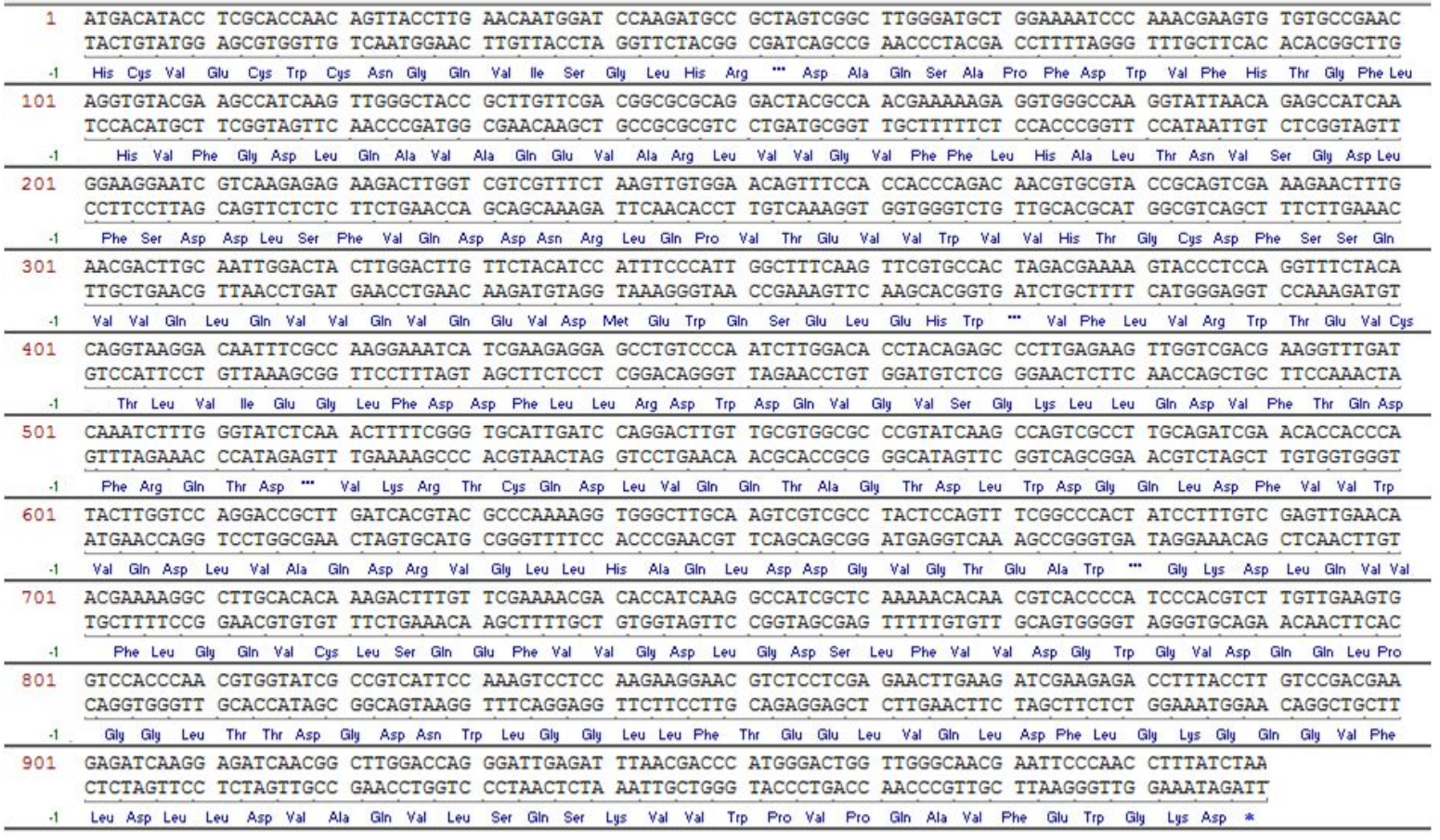


Figure 6. Nucleotide sequence of the *XYL1* gene from *K. marxianus* and its deduced amino acid sequence.

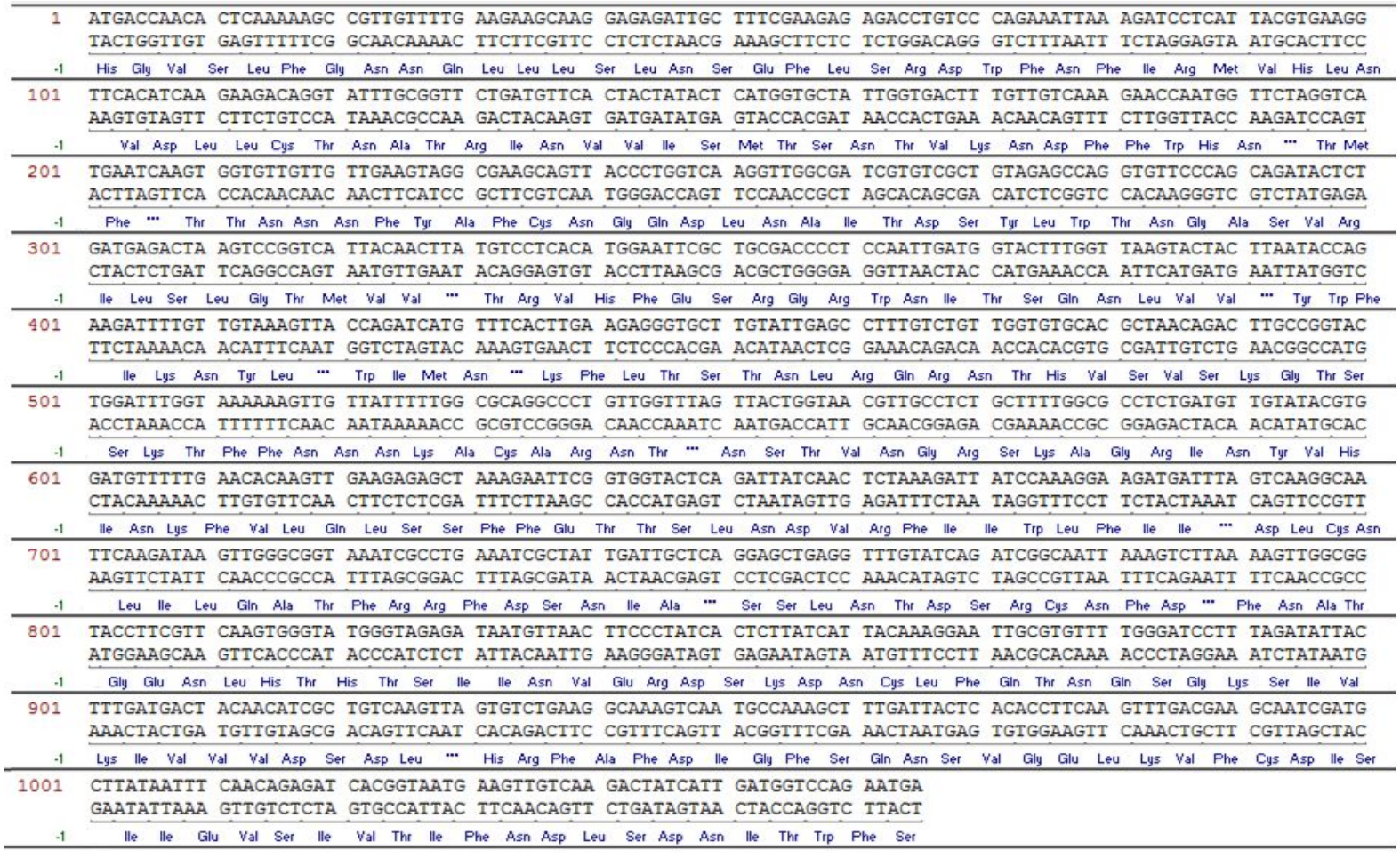
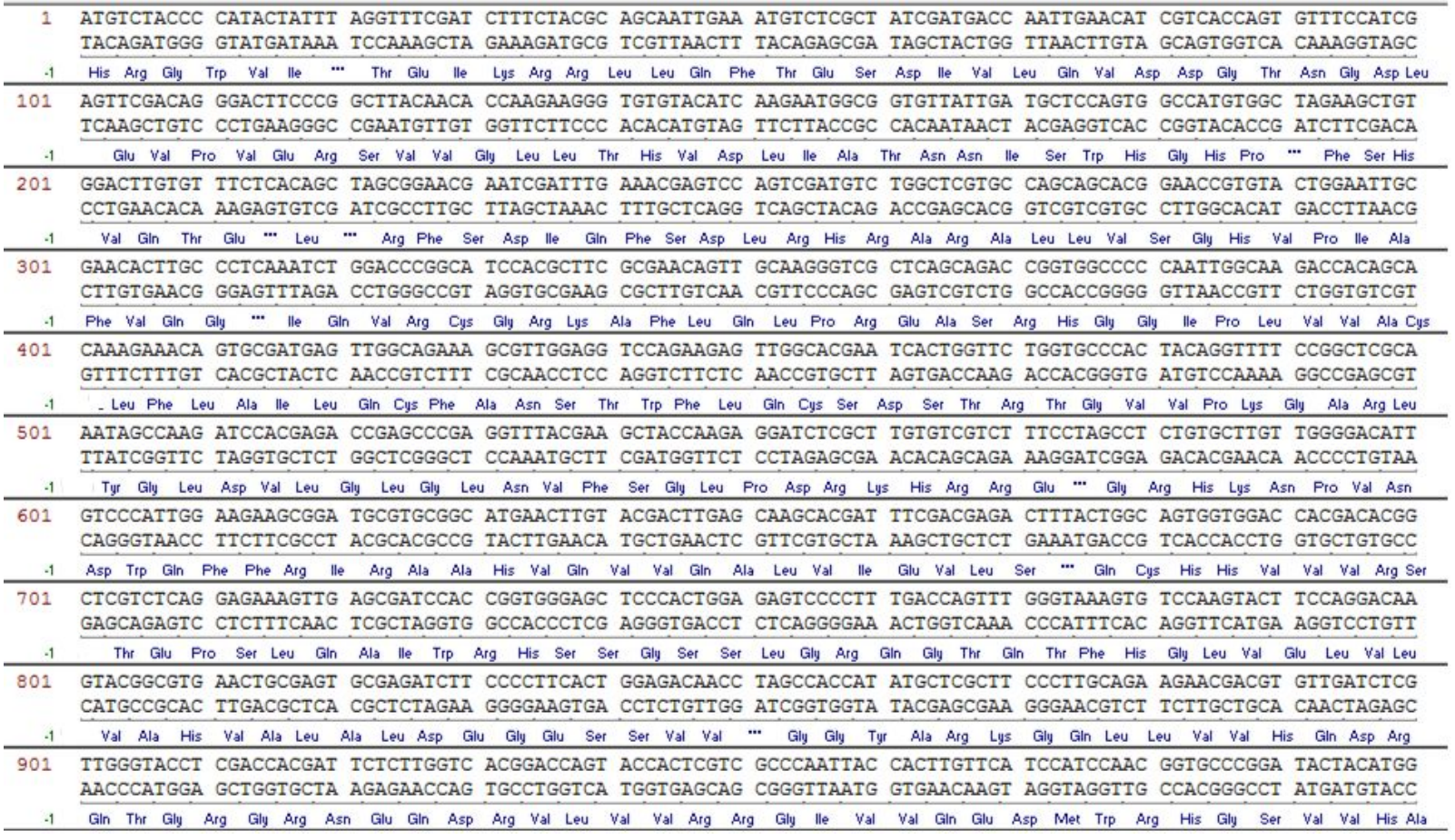


Figure 7. Nucleotide sequence of the *XYL2* gene from *K. marxianus* and its deduced amino acid sequence


1 ATGTCTACCC CATACTATTT AGGTTTCGAT CTTTCTACGC AGCAATTGAA ATGTCTCGCT ATCGATGACC AATTGAACAT CGTCACCAGT GTTTCCATCG
TACAGATGGG GTATGATAAA TCCAAAGCTA GAAAGATGCG TCGTTAACTT TACAGAGCGA TAGCTACTGG TTAACTTGTA GCAGTGGTCA CAAAGGTAGC
-1 His Arg Gly Trp Val Ile *** Thr Glu Ile Lys Arg Arg Leu Leu Gln Phe Thr Glu Ser Asp Ile Val Leu Gln Val Asp Asp Gly Thr Asn Gly Asp Leu
101 AGTTCGACAG GGACTTCCCG GCTTACAACA CCAAGAAGGG TGTGTACATC AAGAATGGCG GTGTTATTGA TGCTCCAGTG GCCATGTGGC TAGAAGCTGT
TCAAGCTGTC CCTGAAGGGC CGAATGTTGT GGTTCTTCCC ACACATGTAG TTCTTACCGC CACAATAACT ACGAGGTCAC CGGTACACCG ATCTTCGACA
-1 Glu Val Pro Val Glu Arg Ser Val Val Gly Leu Leu Thr His Val Asp Leu Ile Ala Thr Asn Asn Ile Ser Trp His Gly His Pro *** Phe Ser His
201 GGACTTGTGT TTCTCACAGC TAGCGGAACG AATCGATTTG AAACGAGTCC AGTCGATGTC TGGCTCGTGC CAGCAGCACG GAACCGTGTA CTGGAATTGC
CCTGAACACA AAGAGTGTCG ATCGCCTTGC TTAGCTAAAC TTTGCTCAGG TCAGCTACAG ACCGAGCACG GTCGTCGTGC CTTGGCACAT GACCTTAACG
-1 Val Gln Thr Glu *** Leu *** Arg Phe Ser Asp Ile Gln Phe Ser Asp Leu Arg His Arg Ala Arg Ala Leu Leu Val Ser Gly His Val Pro Ile Ala
301 GAACACTTGC CCTCAAATCT GGACCCGGCA TCCACGCTTC GCGAACAGTT GCAAGGGTCG CTCAGCAGAC CGGTGGCCCC CAATTGGCAA GACCACAGCA
CTTGTGAACG GGAGTTTAGA CCTGGGCCGT AGGTGCGAAG CGCTTGTCAA CGTTCCCAGC GAGTCGTCTG GCCACCGGGG GTTAACCGTT CTGGTGTCGT
-1 Phe Val Gln Gly *** Ile Gln Val Arg Cys Gly Arg Lys Ala Phe Leu Gln Leu Pro Arg Glu Ala Ser Arg His Gly Gly Ile Pro Leu Val Val Ala Cys
401 CAAAGAAACA GTGCGATGAG TTGGCAGAAA GCGTTGGAGG TCCAGAAGAG TTGGCACGAA TCACTGGTTC TGGTGCCCAC TACAGGTTTT CCGGCTCGCA
GTTTCTTTGT CACGCTACTC AACCGTCTTT CGCAACCTCC AGGTCTTCTC AACCGTGCTT AGTGACCAAG ACCACGGGTG ATGTCCAAAA GGCCGAGCGT
-1 _ Leu Phe Leu Ala Ile Leu Gln Cys Phe Ala Asn Ser Thr Trp Phe Leu Gln Cys Ser Asp Ser Thr Arg Thr Gly Val Val Pro Lys Gly Ala Arg Leu
501 AATAGCCAAG ATCCACGAGA CCGAGCCCGA GGTTTACGAA GCTACCAAGA GGATCTCGCT TGTGTCGTCT TTCCTAGCCT CTGTGCTTGT TGGGGACATT
TTATCGGTTC TAGGTGCTCT GGCTCGGGCT CCAAATGCTT CGATGGTTCT CCTAGAGCGA ACACAGCAGA AAGGATCGGA GACACGAACA ACCCCTGTAA
-1 Tyr Gly Leu Asp Val Leu Gly Leu Gly Leu Asn Val Phe Ser Gly Leu Pro Asp Arg Lys His Arg Arg Glu *** Gly Arg His Lys Asn Pro Val Asn
601 GTCCCATTGG AAGAAGCGGA TGCGTGCGGC ATGAACTTGT ACGACTTGAG CAAGCACGAT TTCGACGAGA CTTTACTGGC AGTGGTGGAC CACGACACGG
CAGGGTAACC TTCTTCGCCT ACGCACGCCG TACTTGAACA TGCTGAACTC GTTCGTGCTA AAGCTGCTCT GAAATGACCG TCACCACCTG GTGCTGTGCC
-1 Asp Trp Gln Phe Phe Arg Ile Arg Ala Ala His Val Gln Val Val Gln Ala Leu Val Ile Glu Val Leu Ser *** Gln Cys His His Val Val Val Arg Ser
701 CTCGTCTCAG GAGAAAGTTG AGCGATCCAC CGGTGGGAGC TCCCACTGGA GAGTCCCCTT TGACCAGTTT GGGTAAAGTG TCCAAGTACT TCCAGGACAA
GAGCAGAGTC CTCTTTCAAC TCGCTAGGTG GCCACCCTCG AGGGTGACCT CTCAGGGGAA ACTGGTCAAA CCCATTTCAC AGGTTCATGA AGGTCCTGTT
-1 Thr Glu Pro Ser Leu Gln Ala Ile Trp Arg His Ser Ser Gly Ser Ser Leu Gly Arg Gln Gly Thr Gln Thr Phe His Gly Leu Val Glu Leu Val Leu
801 GTACGGCGTG AACTGCGAGT GCGAGATCTT CCCCTTCACT GGAGACAACC TAGCCACCAT ATGCTCGCTT CCCTTGCAGA AGAACGACGT GTTGATCTCG
CATGCCGCAC TTGACGCTCA CGCTCTAGAA GGGGAAGTGA CCTCTGTTGG ATCGGTGGTA TACGAGCGAA GGGAACGTCT TCTTGCTGCA CAACTAGAGC
-1 Val Ala His Val Ala Leu Ala Leu Asp Glu Gly Glu Ser Ser Val Val *** Gly Gly Tyr Ala Arg Lys Gly Gln Leu Leu Val Val His Gln Asp Arg
901 TTGGGTACCT CGACCACGAT TCTCTTGGTC ACGGACCAGT ACCACTCGTC GCCCAATTAC CACTTGTTCA TCCATCCAAC GGTGCCCGGA TACTACATGG
AACCCATGGA GCTGGTGCTA AGAGAACCAG TGCCTGGTCA TGGTGAGCAG CGGGTTAATG GTGAACAAGT AGGTAGGTTG CCACGGGCCT ATGATGTACC
-1 Gln Thr Gly Arg Gly Arg Asn Glu Gln Asp Arg Val Leu Val Val Arg Arg Gly Ile Val Val Gln Glu Asp Met Trp Arg His Gly Ser Val Val His Ala

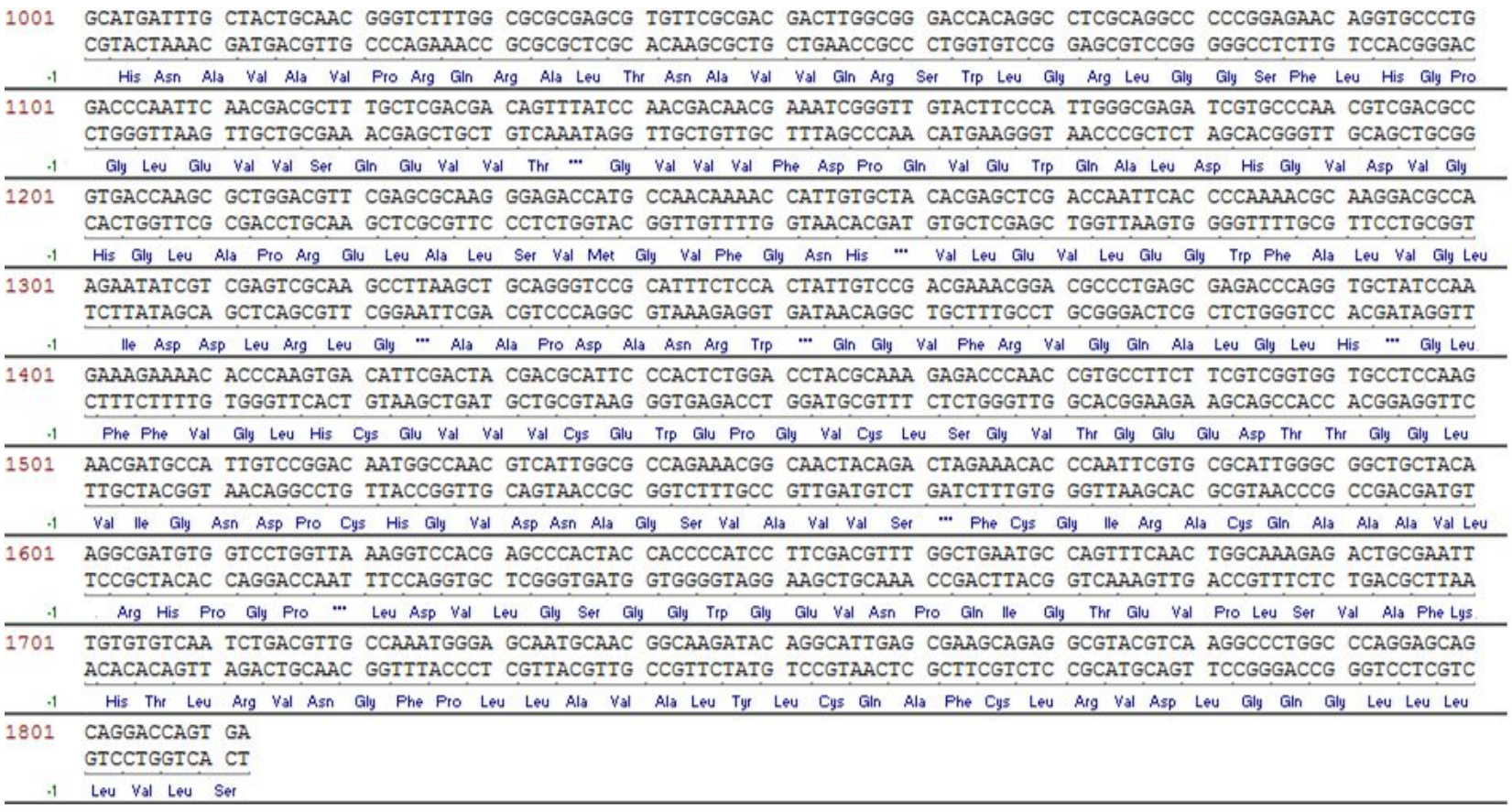


Figure 8. Nucleotide sequence of the *XYL3* gene from *K. marxianus* and its deduced amino acid sequence

2 . Construction of recombinant *S. cerevisiae*

The *K. marxianus* gene *XYL1* encoding KmXR was integrated into the *HIS3* locus of *S. cerevisiae* DX23 and cells selected on YNB *his*- plate were checked by colony PCR whether vectors for transferring *XYL1* gene were inserted to exact location. Several colonies showing appropriate size of PCR product were picked and fermented with 40 g/L xylose. One colony having best fermenting ability was chosen and stored as cell stocks. Introducing *XYL1, XYL2* to *URA3* locus of *S. cerevisiae* ISXK and *XYL1, XYL2* to *URA3* as well as *XYL3* to *HIS3* of *S. cerevisiae* D452-2 were also done and confirmed same way.

To confirm activity of XYL1, XYL2, XYL3 from *K. marxianus* and compare with each genes of *Sch. Stipitis*, each genes was integrated to *S. cerevisiae* D452-2. D/Km(Ss)XR, D/Km(Ss)XDH, D/Km(Ss)XK were constructed..

The linearized plasmid pAUR_d_ALD6 was integrated into the *ALD6* locus of KM12,3 in order to disruption of *ALD6* gene using YP-AbA plate. All strains were confimed whether having or deleting right genes or not.

3. Confirmation of heterologous expression of *XYL1*

To confirm expression of exogenous *XYL1* gene in *S. cerevisiae*, it was transformed to DX23 host strain that show expression of *XYL2* and *XYL3*

surely. If *XYL1* was expressed well, XR would be produced and *S. cerevisiae* could produce ethanol from xylose.

3.1 Specific activitiy assay of KmXR

Cofactor specificity of xylose reductase (XR) was checked by measuring enzyme activity. Specific activities of a single KmXR in D/KmXR and the recombinant *S. cerevisiae* DX23/KM1 in the presence of NADPH and NADH were 0.58, 0.06 and 0.48, 0.03 U/mg-protein, respectively [Figure 9]. It also seems that KmXR in recombinant strain was almost dependent to NADPH. It is similar with single KmXR in D/KmXR in an aspect to both cofactor affinity and specific activities.

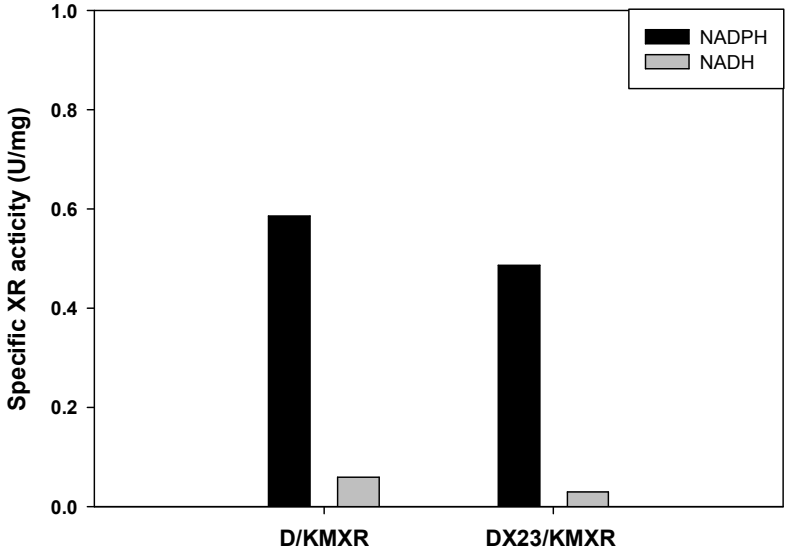


Figure 9. Comparison of the specific activities of xylose reductase (XR) between *S. cerevisiae* D/KmXR , *S. cerevisiae* DX23/KM1

3.2 Microaerobic bath fermentation

Microaerobic batch flask fermentations were performed with DX23/KM1 and DX23/pRS403 control strain. YEPD medium containing 4 %(w/v) xylose was used. Figures 10 and Table 3 showed Microaerobic batch fermentation profiles and fermentation results.

As control strain didn't have entire xylose metabolic pathway, it couldn't produce ethanol, but recombinant strain DX23/KM1 surely produce ethanol. Therefore, heterologous expression of *XYL1* could be confirmed in *S. cerevisiae*.

S. cerevisiae DX23/KM1 consumed 34.1 g/L xylose then, produced 6.67 g/L of final dry cell weight and 10.5 g/L of ethanol for 96 h at initial O.D. 5. Ethanol yield of *S. cerevisiae* DX23/KM1 was 0.31 g ethanol/g xylose and its productivity was 0.11 g/L·h.

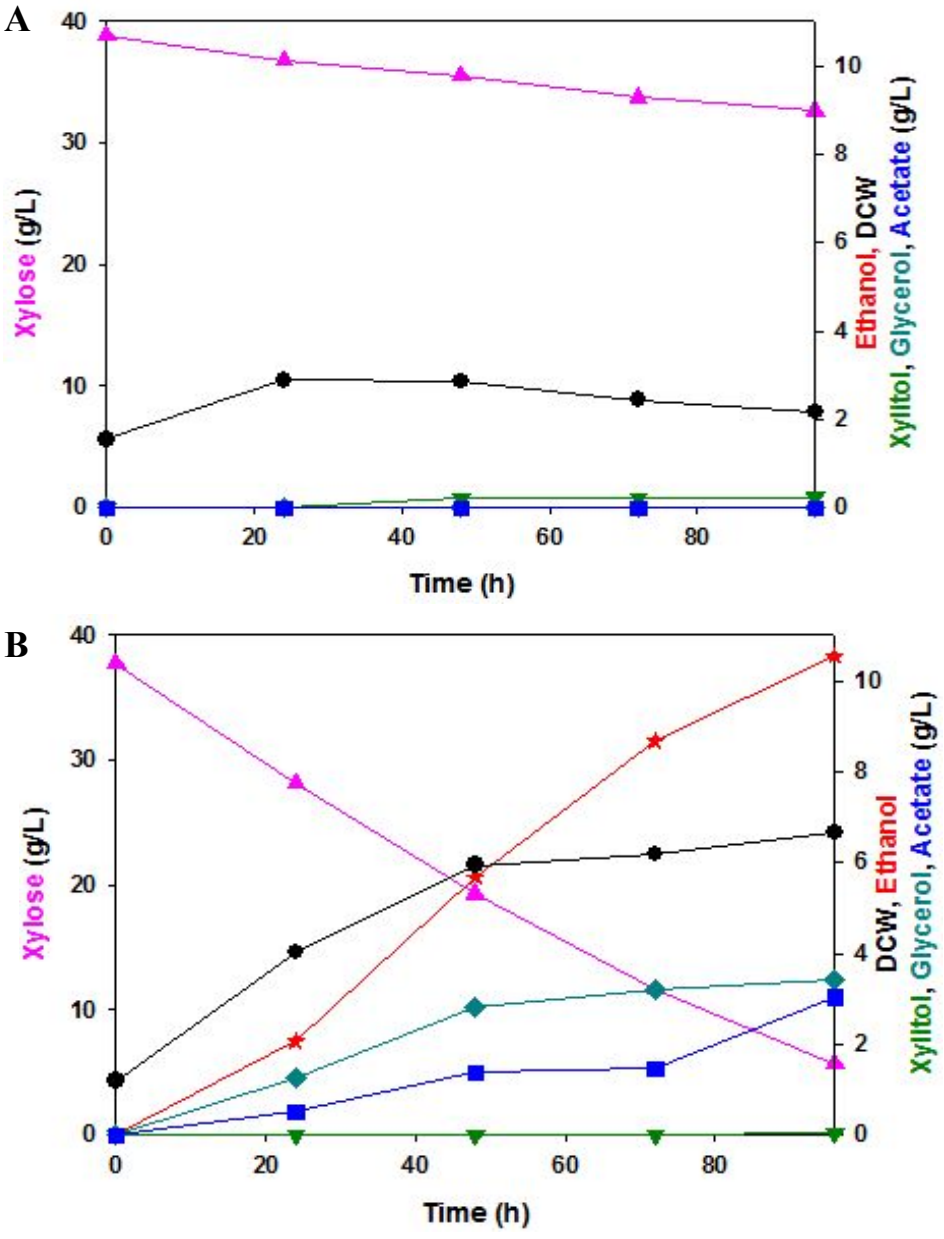


Figure 10. Microaerobic batch fermentation profiles of *S. cerevisiae* DX23/pRS403 (A) and *S. cerevisiae* DX23/KM1(B) in YEPD containing 4 %(w/v) xylose at 30℃, 80 rpm, initial OD : 5

(●: Dry cell weight, ▲: Xylose, ▼: Xylitol, ◆: Glycerol, ■: Acetate, ★: Ethanol)

Table 3. Summary of Microaerobic batch fermentation of *S. cerevisiae* DX23/KM1

Strains	Dry cell weight (g/L)	Final ethanol concentration (g/L)	Ethanol yield (g ethanol/ g xylose)	Ethanol productivity (g/L-hr)	xylose consumption rate (g/L-hr)
DX23/KM1	6.67	10.5	0.31	0.11	0.28

4. Confirmation of heterologous expression of *XYL2*

To confirm expression of exogenous *XYL2* gene in *S. cerevisiae*, *XYL1* which was verified to express well in *S. cerevisiae* and *XYL2* was transformed to ISXK host strain that show expression of *XYL3*surely. If *XYL2* was expressed well, XDH would be produced and *S. cerevisiae* could produce ethanol from xylose.

4.1 Specific activitiy assay of KmXDH

Expression of XDH was detected by measuring enzyme activity. Specific activities of single KmXDH in D/KmXDH and the recombinant *S. cerevisiae* ISXK/KM12 in the presence of NAD+ were 0.57 and 0.21 U/mg-protein respectively. It also seems that KmXDH in recombinant strain was almost dependent to NAD+. It is similar with single KmXDH in D/KmXDH in an aspect to both cofactor affinity but specific activities is somewhat lower that of KmXDH [Figure 11].

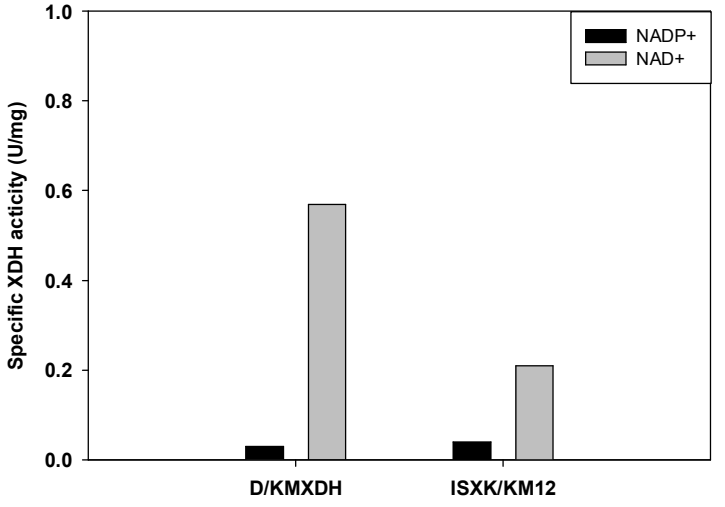


Figure 11. Comparison of the specific activities of xylitol dehydrogenase (XDH) between *S. cerevisiae* D/KmXDH, *S. cerevisiae* ISXK/KM12.

4.2 Microaerobic bath fermentation

Microaerobic batch flask fermentations were performed with ISXK/KM12 and ISXK/pRS406 control strain. YEPD medium containing 4 %(w/v) xylose was used. Figures 12 and Table 4 showed Microaerobic batch fermentation profiles and fermentation results.

As control strain depleted XR, XDH enzyme among entire xylose metabolic pathway, it couldn't produce ethanol, while recombinant strain ISXK/KM12 surely produce ethanol. Therefore, heterologous expression of *XYL2* in *S. cerevisiae* could be confirmed.

S. cerevisiae ISXK/KM12 consumed 27.5 g/L xylose then, produced 4.89 g/L of final dry cell weight and 9.26 g/L of ethanol for 96 h at initial O.D. 5. Ethanol yield of *S. cerevisiae* DX23/KM1 was 0.34 g ethanol/g xylose and its productivity was 0.10 g/L·h.

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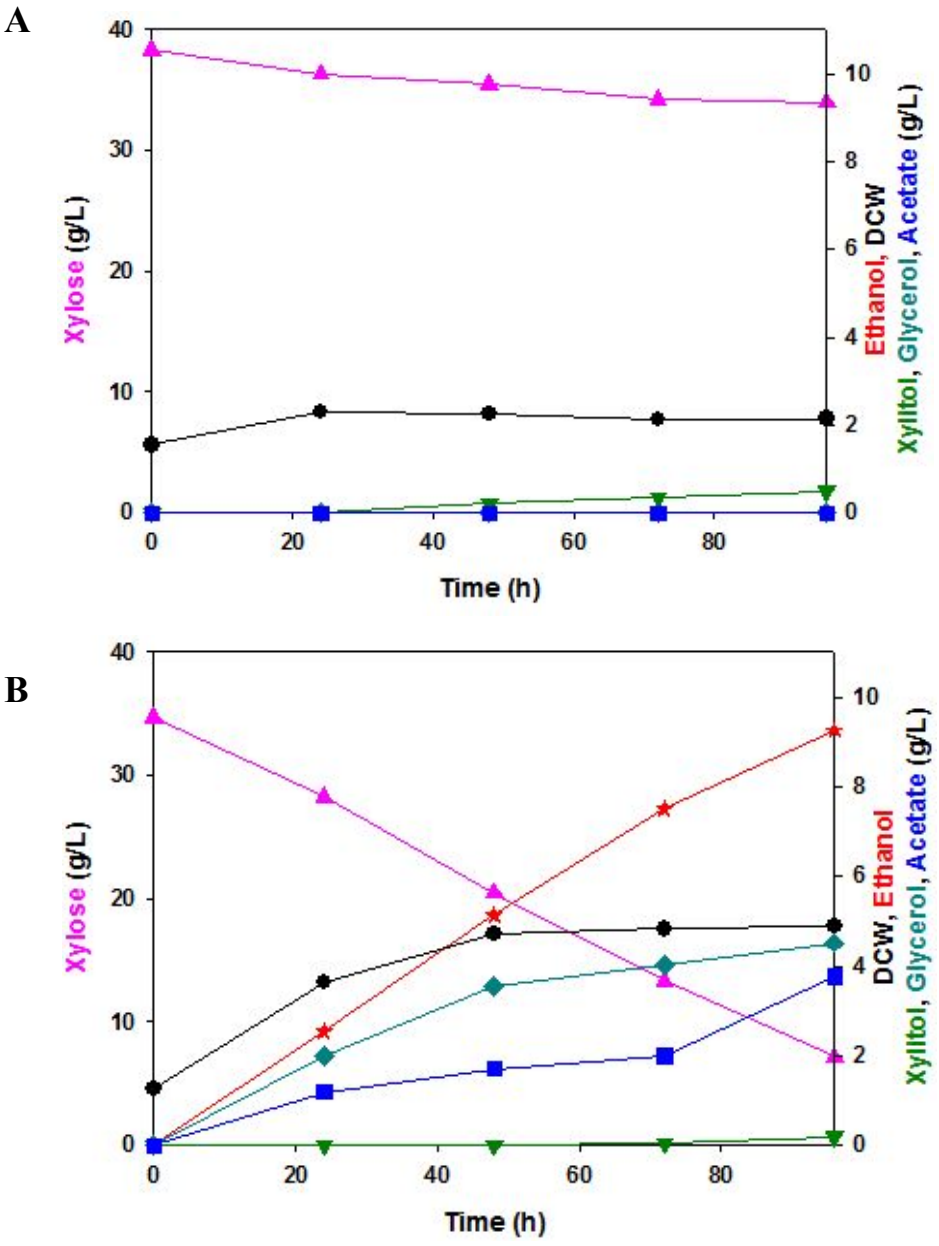


Figure 12. Microaerobic flask batch fermentation profile of *S. cerevisiae* ISXK/pRS406 (A) and *S. cerevisiae* ISXK/KM12 (B) in YEPD containing 4 %(w/v) xylose at 30℃, 80 rpm, initial OD : 5

(●: Dry cell weight, ▲: Xylose, ▼: Xylitol, ◆: Glycerol, ■: Acetate, ★: Ethanol)

Table 4. Summary of aerobic batch fermentation of *S. cerevisiae* ISXK/KM12

Strains	Dry cell weight (g/L)	Final ethanol concentration (g/L)	Ethanol yield (g ethanol/ g xylose)	Ethanol productivity (g/L-hr)	xylose consumption rate (g/L-hr)
ISXK/KM12	4.89	9.26	0.34	0.10	0.29

5. Confirmation of heterologous expression of *XYL3*

To confirm expression of exogenous *XYL3* gene in *S. cerevisiae*, *XYL1* and *XYL2* which were proved to express in *S. cerevisiae* and *XYL3* were transformed to *S. cerevisiae* D452-2 strain that could not metabolize xylose. If *XYL3* was expressed well, XK would be produced and *S. cerevisiae* could produce ethanol from xylose through entire combination of XR, XDH and XK.

5.1 Specific activitiy assay of KmXK

Expression of xylulokinase (XK) was detected by measuring enzyme activity. Specific activity of KmXR, XDH in recombinant *S. cerevisiae* KM12,3 was 4.40, 4.60 U/mg-protein respectively [Figure 13]. Secific activities of single KmXK and KmXK of KM12,3 are similar each other.

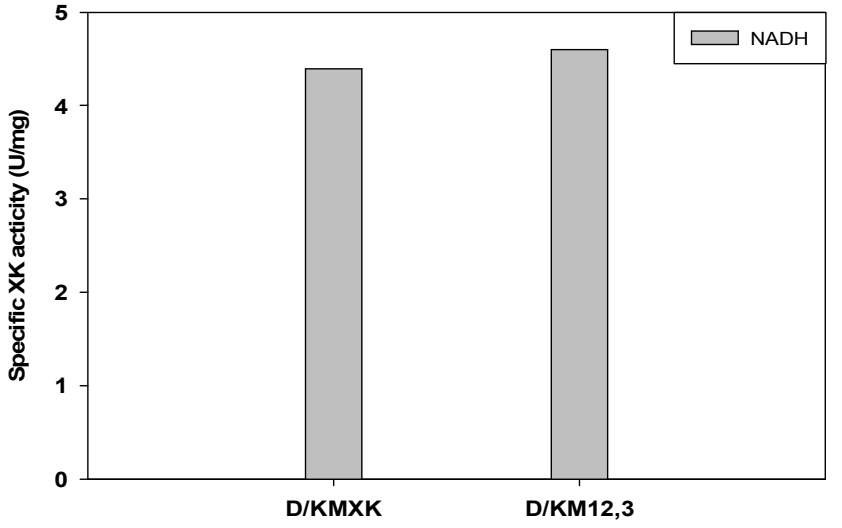


Figure 13. Comparison of the specific activities of xylulokinase (XK) between *S. cerevisiae* D/KmXK and *S. cerevisiae* KM12,3

5.2 Microaerobic bath fermentation

Microaerobic batch flask fermentations were performed with KM12,3 and D/pRS403,406 control strain. YEPD medium containing 4 %(w/v) xylose was used. Figures 14 and Table 5 showed Microaerobic flask batch fermentation profiles and fermentation results.

As control strain depleted entire xylose metabolic pathway, it couldn't produce ethanol, while recombinant strain KM12,3 surely produce ethanol. Therefore, heterologous expression of *XYL3* in *S. cerevisiae* could be confirmed as well as expression of *XYL1* and *XYL2*.

S. cerevisiae KM12,3 consumed 32.3 g/L xylose then, produced 6.69

g/L of final dry cell weight and 11.1g/L of ethanol for 96 h at initial O.D. 5. Ethanol yield of *S. cerevisiae* KM12,3 was 0.31 g ethanol/g xylose and its productivity was 0.12 g/L·h.

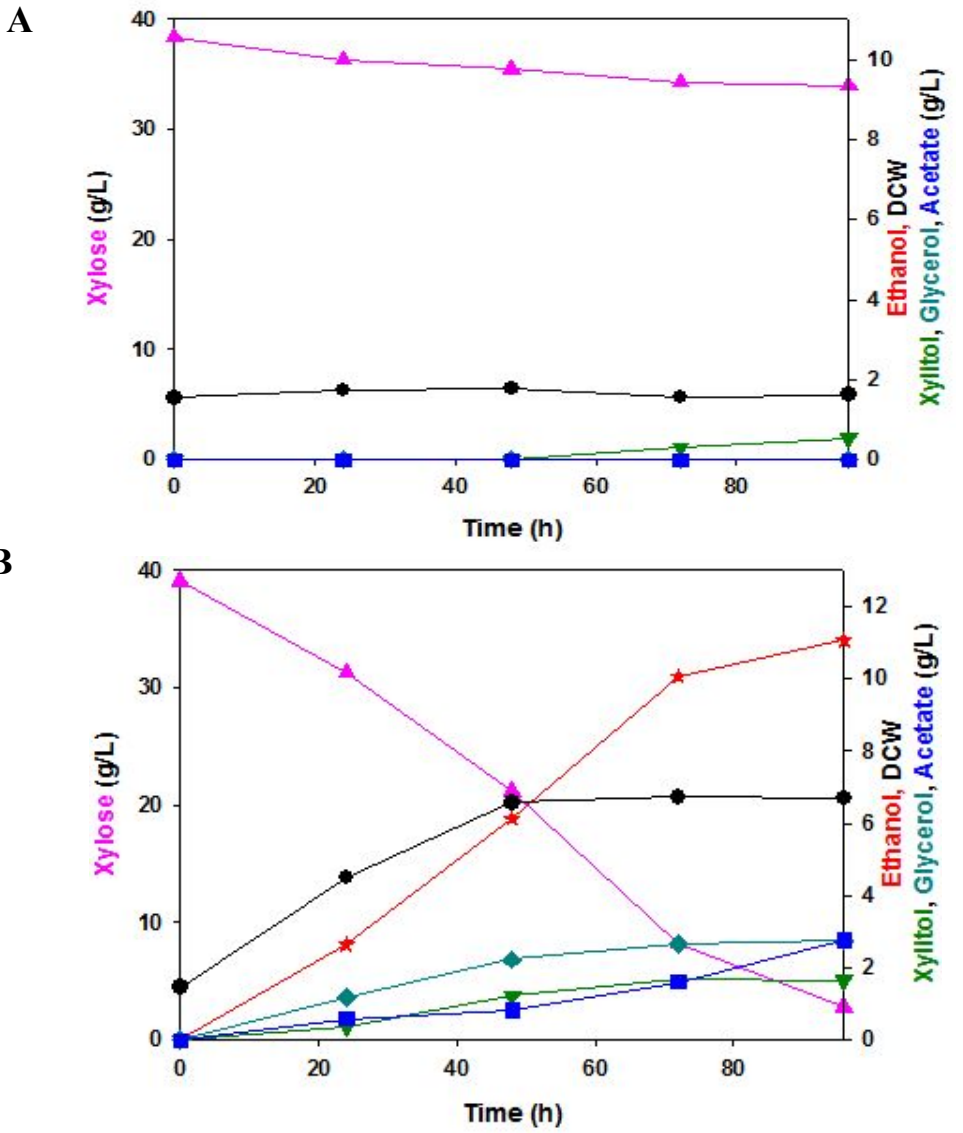


Figure 14. Microaerobic flask batch fermentation profiles of and *S. cerevisiae* D/pRS403406 (A) and *S. cerevisiae* KM12,3 (B) in YEPD containing 4 %(w/v) xylose at 30℃, 80 rpm, itintial OD : 5 .

(●: Dry cell weight, ▲: Xylose, ▼: Xylitol, ◆: Glycerol, ■: Acetate, ★: Ethanol)

Table 5. Summary of aerobic batch fermentation of *S. cerevisiae* KM12,3(96 h – Point of maximum ethanol production)

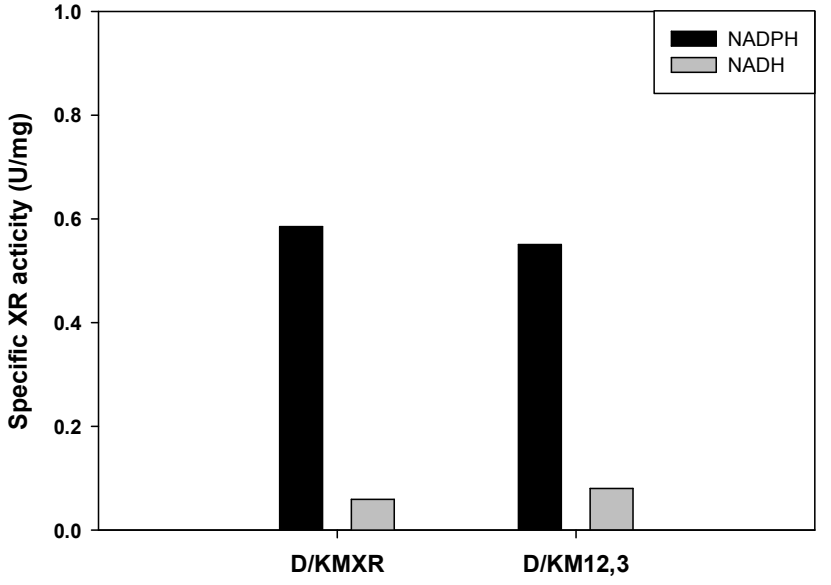
Strains	Dry cell weight (g/L)	Final ethanol concentration (g/L)	Ethanol yield (g ethanol/ g xylose)	Ethanol productivity (g/L-hr)	xylose consumption rate (g/L-hr)
KM12,3	6.69	11.1	0.31	0.12	0.38

6. Comparison of with *Sch. stipitis*

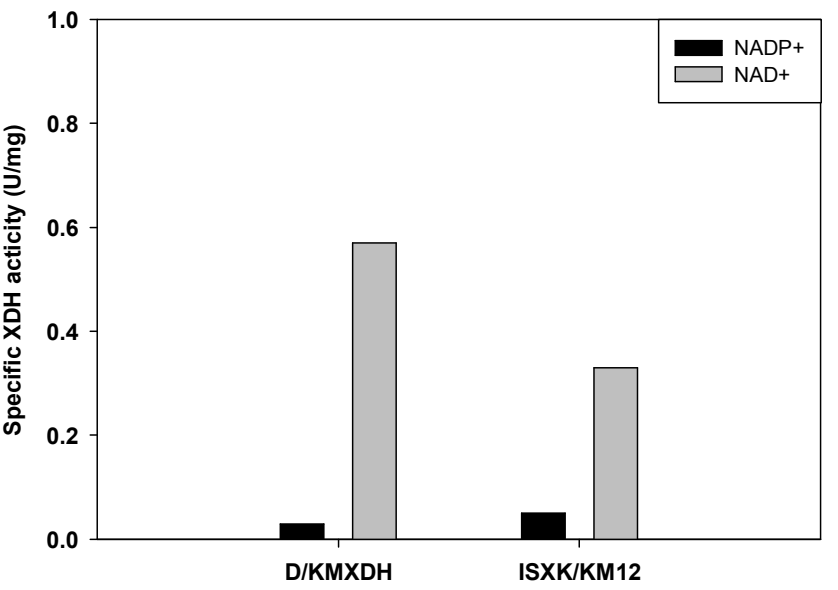
6.1 Specific activity

To compare genes from *Sch. stipitis*, At first, activity of single KmXR in D/KmXR was compared with single SsXR in D/SsXR. XR from *K. marxianus* prefered NADPH as the coenzyme and specific activity was very low with NADH in contrast with XR from *Sch. Stipitis* dependent both NADPH and NADH. Considered NADPH only, specific activity of KmXR was 0.58 U/mg-protein and 0.8 times lower than that of SsXR. Activity of single KmXDH in D/KmXDH was compared with single SsXDH in D/SsXDH. XDH from *K. marxianus* prefered NAD+ as the coenzyme and specific activity was very low with NADP+. These properties are similar with XDH from *Sch. Stipitis*. Considered NAD+ only, specific activity of KmXDH was 0.56 U/mg-protein and 0.35 times lower than that of singleSsXDH. Activity of single KmXK in D/KmXK was compared with single SsXK in D/SsXK. XK from *K. marxianus*. Specific KmXK activity was 4.4 U/mg-protein and 6 times higher compared with that of single SsXK. Specific KmXK activity of 4.3 U/mg-protein in was obtained for the recombinant *S. cerevisiae* KM12,3, which was similar with specific D/KmXK activity [Figure 15].

A


1.0
0.8
0.6
0.4
0.2
0.0
Specific XR acticity (U/mg)
NADPH
NADH
D/KMXR
D/KM12,3

B


1.0
0.8
0.6
0.4
0.2
0.0
Specific XDH acticity (U/mg)
NADP+
NAD+
D/KMXDH
ISXK/KM12

C

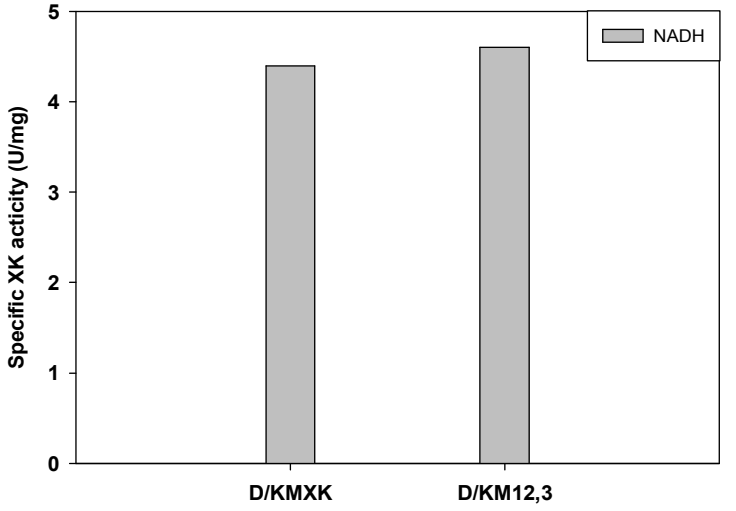
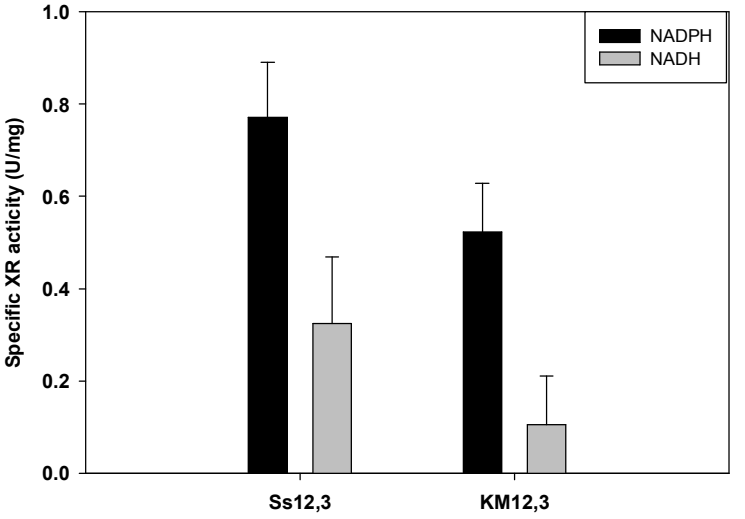


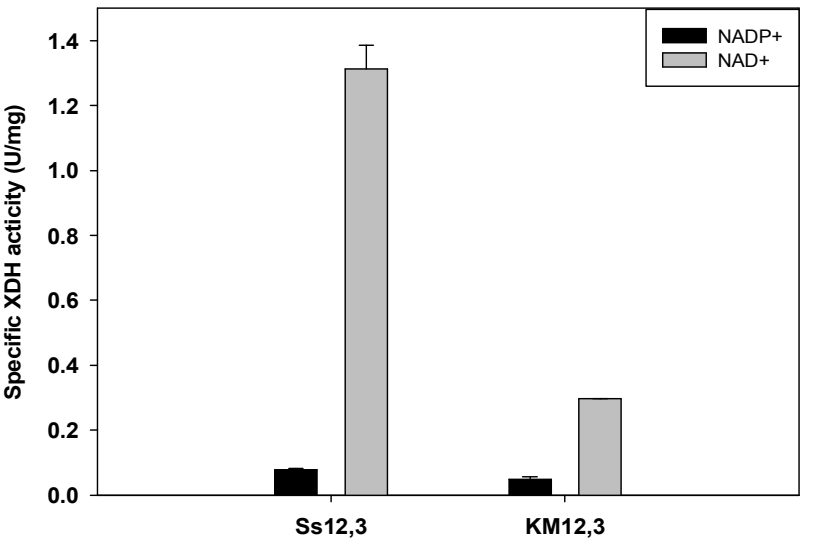
Figure 15. Comparison of the specific activities of single XR (A), XDH (B) and XK (C)

Specific activities of KM12,3 and SS12,3 were shown in figure 16. KM12,3 activities were all lower than those of Ss12,3 .

A


NADPH
NADH
Specific XR acticity (U/mg)
1.0
0.8
0.6
0.4
0.2
0.0
Ss12,3
KM12,3

B


NADP+
NAD+
Specific XDH acticity (U/mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ss12,3
KM12,3

C

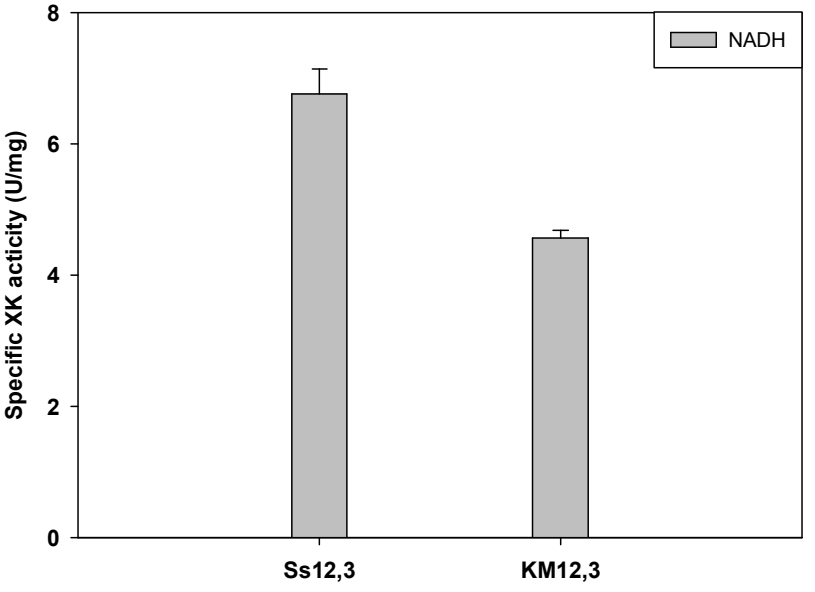


Figure 16. Comparison of the specific activities of XR (A), XDH (B) and XK (C)between *S. cerevisiae* SS12,3 and *S. cerevisiae* KM12,3

6.2 Microeaerobic fernmtnetion

Microaerobic batch flask fermentations were performed with Both KM12,3 and Ss12,3. YEPD medium containing 4 %(w/v) xylose was used. Figures 17 and Table 6 showed Microaerobic flask batch fermentation profiles and fermentation results.

At 72 h, KM2,3 strain produce 10.1 g/L of ethanol at initial OD : 5, similar with Ss12,3 strain. Also, KM12,3 accumulates more byproduct such as xylitol, glycerol, acetate. These phenomenones might be occured

by difference of specific activities and cofactor imbalance between NADPH dependent KmXR and NAD+ dependent KmXDH. As specific activity of KmXDH is much lower than that of SsXDH, xylitol would be more accumulated than in case of Ss12,3.

KmXR was totally dependent in NADPH, supply of NAD+ was not occured within KmXR – KmXDH process. NADH was converted to NAD+ though the pathway of producing Glycerol and this NAD+ could fulfill depletion of cofactor in expressing KmXDH.[J W Kim 2012]. Acetate was accumulated in cells as fermentation lasted for a long time. Cell growth and xylose metabolism were inhibited over 2 g/L of acetate [S H Lee 2011]. But when Acetaldehyde was converted to acetate, NADPH was produced and it might help working of KmXR, xylose uptake. So, It is possible for Km12,3 to produce more acetate for supplying NADPH.

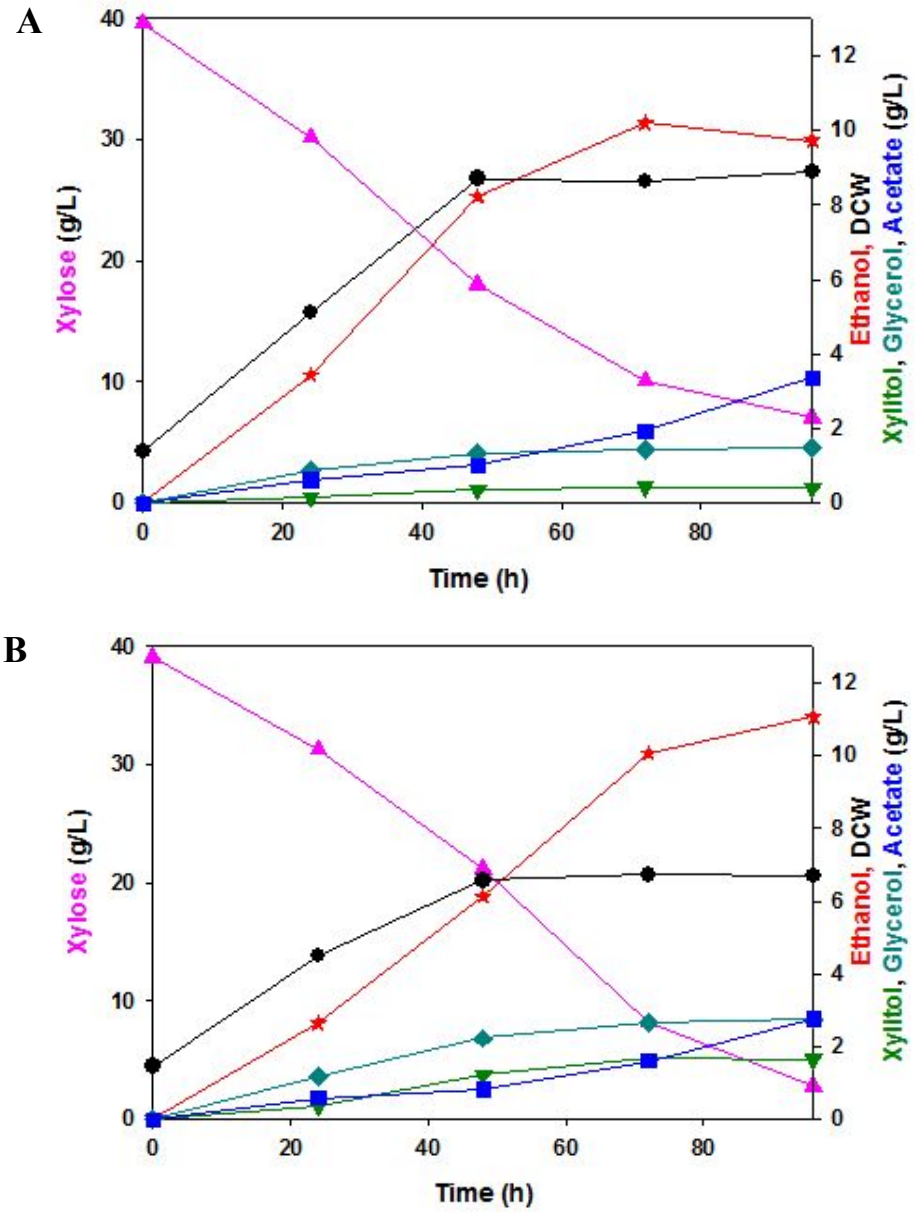


Figure 17. Microaerobic flask batch fermentation profile of SS12,3 (A) and KM12,3 (B) YEPD containing 5 %(w/v) xylose at 30℃, pH 5.5.

(●: Dry cell weight, ▲: Xylose, ▼: Xylitol,

◆ : Glycerol, ■: Acetate, ★: Ethanol)

Table 6. Summary of microaerobic batch fermentation of SS12,3 and KM12,3 (72 h)

Strains	Xylose consumption rate (g/L-hr)	ethanol concentration (g/L)	Ethanol productivity (g/L-hr)	Ethanol yield (g ethanol /g xylose)	Dry cell mass (g/L)	Xylitol	Glycerol	Acetate
SS12,3	0.41	10.2	0.14	0.35	8.64	0.43	1.45	1.94
KM12,3	0.43	10.1	0.14	0.33	6.72	1.68	2.65	1.60

7. Effect of *ALD6* gene disruption on xylose fermentation in recombinant *S. cerevisiae*

7.1 Confirmation of *ALD6* gene disruption

The *ALD6* gene was disrupted by introducing linearized pAUR_d_ALD6 plasmid by single crossover homologous recombination event. Disruption cassette shown in Figure 15 was transformed into KM12,3 strain.

Disruption of *ALD6* gene was confirmed by diagnostic PCR using appropriate primers (Table 2). Expected-sized PCR product was obtained as shown in Figure 18.

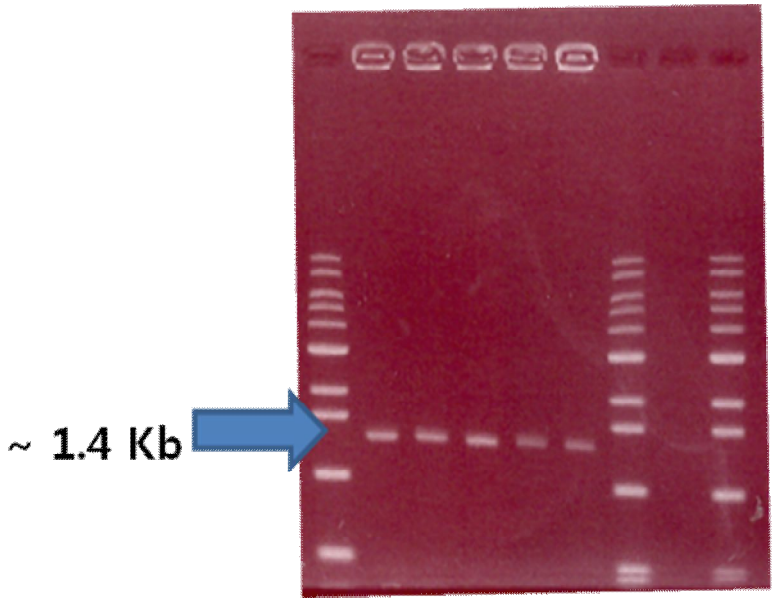


Figure 18. Electrophoresis of diagnostic PCR products.

Lane 1, 2, 3, 4, 5 : KM12,3:: △*ald6*, lane 6 : KM12,3

7.2 Microaerobic batch fermentation

The effects of the *ALD6* gene disruption on cell growth and xylose metabolism in recombinant yeast strains: Km12,3 and Km12,3:: △*ald6* were examined in microaerobic flask batch cultivations. YEPD medium containing 4 % (w/v) xylose was used. Figure 19 showed microaerobic batch fermentation profiles. Table 7 showed comparison of microaerobic batch fermentation data obtained for two strains.

As shown in Table 7, two strains show differences in acetate concentration and cell growth. Accumulation of Acetate was reduced in deletion of *ALD6,* so cell growth of Km12,3::△*ald6* would not inhibited by acetate compared with Km12,3. But rate of xylose consumption and production of ethanol was slightly increased, because Km12,3 already uptaked xylose as much as Km12,3::△*ald6* in spite of being acetate. This could be related to supply of NADPH during *ALD6* precess. Reduction of acetated also decreased concentration of NADPH and this affected expression of KmXR negatively, thus, it seems that concentration of glycerol in Km12,3::△*ald6* was 1.3 folds increased than that of KM12,3 for balancing cofactors.

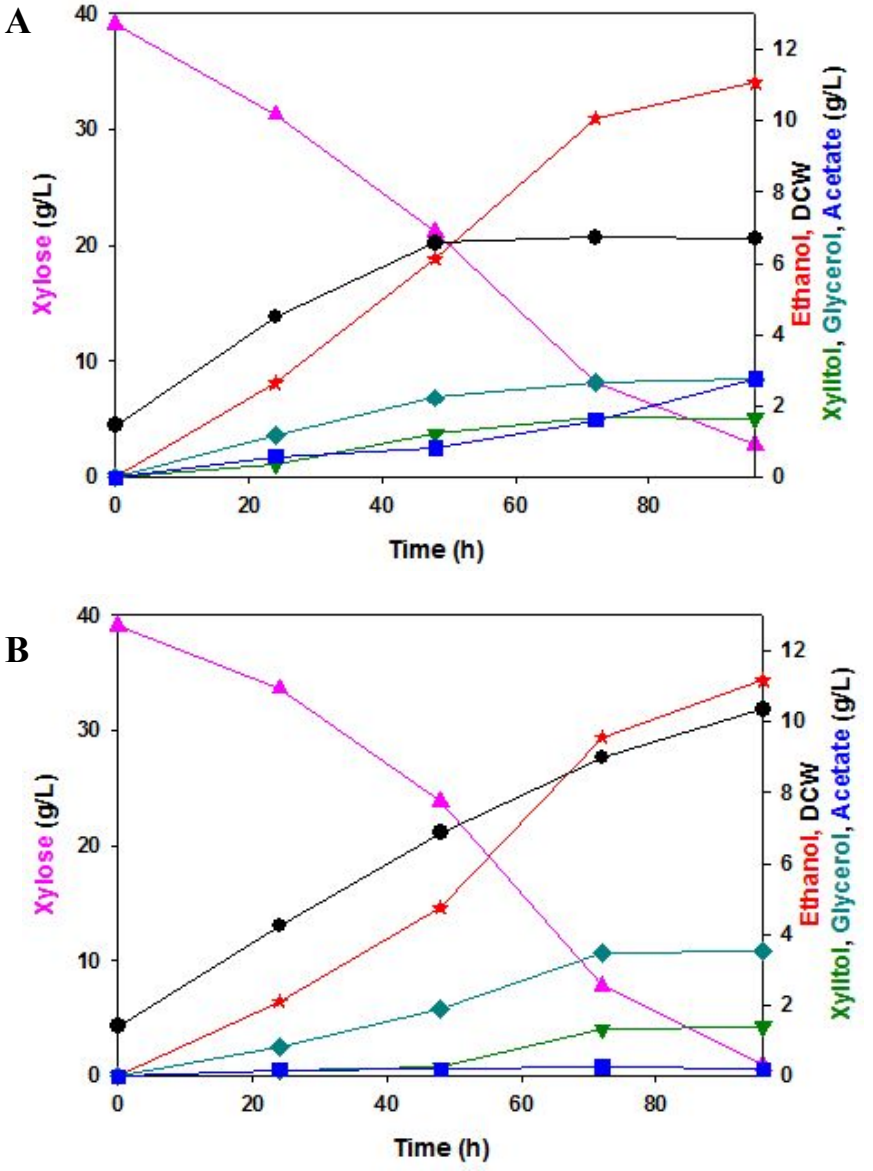


Figure 19. Microaerobic flask batch fermentation profiles of KM12,3 (A) and KM12,3:: Δ*ald6* (B) in YEPD containing 4 %(w/v) xylose at 30℃, 80 rpm, initial OD : 5.

(●: Dry cell weight, ▲: Xylose, ▼: Xylitol,


: Glycerol,
: Acetate,
: Ethanol)

Table 7. Summary of microaerobic batch fermentation of Km12,3 and Km12,3::△*ald6* (96h)

Strains	Xylose consumption rate (g/L-hr)	ethanol concentration (g/L)	Ethanol productivity (g/L-hr)	Ethanol yield (g ethanol /g xylose)	Dry cell mass (g/L)	Xylitol	Glycerol	Acetate
KM12,3	0.38	11.1	0.12	0.31	6.69	1.65	2.74	2.76
Km12,3::△*ald6*	0.40	11.2	0.12	0.30	10.4	1.41	3.55	0.19

8 . Microaerobic batch fermentation in Bioreactor

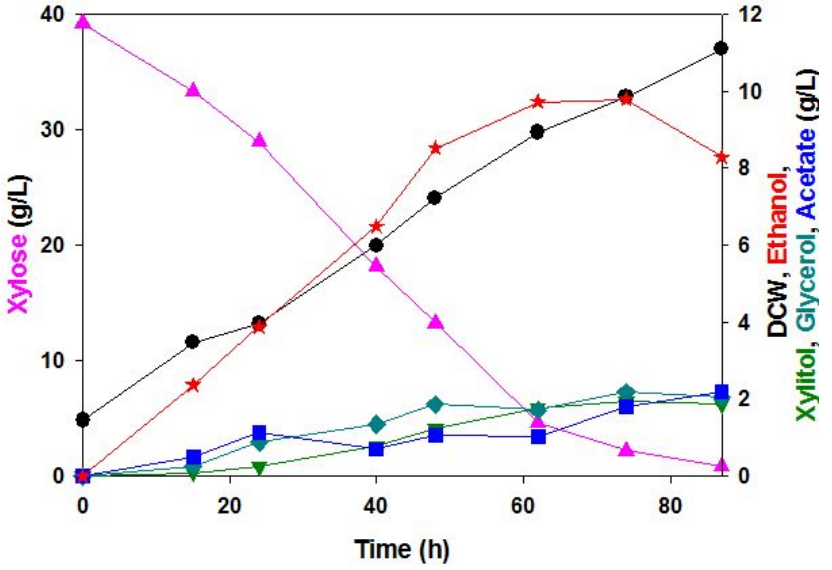
In spite of deletion of *ALD6*, ethanol yield and productivity of Km12,3 was higher than that of Km12,3::⊿*ald6*. Finally, Km12,3 examined in microaerobic batch cultivations compared with Ss12,3. YEPD medium containing 4 % (w/v) xylose was used. Figure 20 showed microaerobic batch fermentation profiles and Table 8 showed comparison of microaerobic batch fermentation data obtained for two strains As xylose is depleted in the medium, ethanol was produced from the xylose metabolism. Ethanol wasn't used as a cosubstrate for generation of ATP and NAD(P)H at microaerobic cultivation condition.

At 76 h, KM2,3 strain produce 7,62 g/L of ethanol at initial OD : 5, = 0.8 folds decrease compared with SS12,3 strain. KM12,3 accumulate more byproduct such as xylitol, glycerol, and acetate. These phenomenones might be occurred by difference of specific activities and cofactor imbalance between NADPH dependent KmXR and NAD+ dependent KmXDH. As specific activity of KmXDH is much lower than that of SsXDH, xylitol would be more accumulated than in case of SS12,3.

KmXR was totally dependent in NADPH, supply of NAD+ was not occurred within KmXR – KmXDH process. NADH was converted to NAD+ though the pathway of producing Glycerol and this NAD+ could

fulfill depletion of cofactor in expressing KmXDH. [J W Kim 2012].

This study produced ethanol from xylose using genes from K. marxianus first. Productivity and yield would be more increased through optimizing process and fermentation conditions



A.

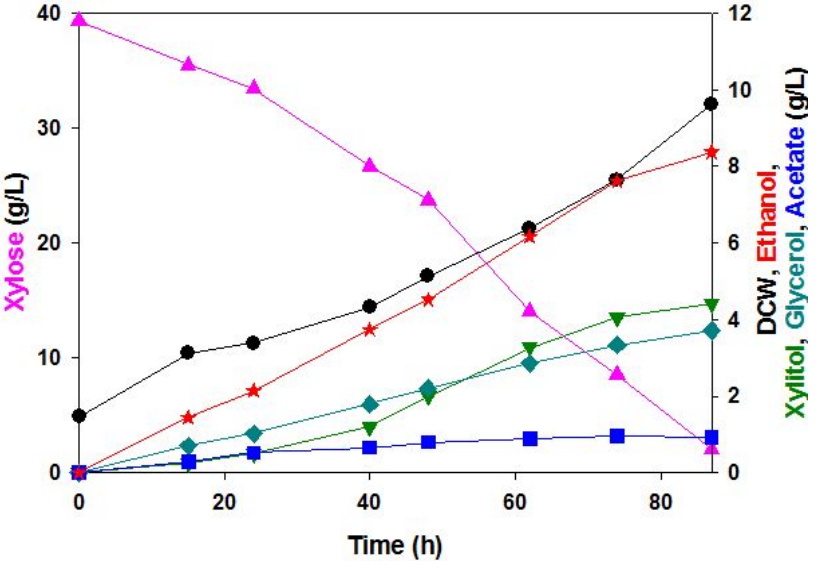


Figure 20. Microaerobic batch fermentation profiles of SS12,3 (A) and KM12,3 (B) in YEPD containing 4 %(w/v) xylose at 30℃, 200 rpm, 0.3 vvm, initial OD : 5.

(●: Dry cell weight, ▲: Xylose, ▼: Xylitol,

◆ : Glycerol, ■: Acetate, ★: Ethanol

Table 8. Summary of microaerobic batch fermentation of SS12,3 and KM12,3 in bioreactor

Strains	Xylose consumption rate (g/L-hr)	ethanol concentration (g/L)	Ethanol productivity (g/L-hr)	Ethanol yield (g ethanol /g xylose)	Dry cell mass (g/L)	Xylitol	Glycerol	Acetate
SS12,3	0.50	9.76	0.13	0.27	9.87	1.94	1.82	1.82
KM12,3	0.42	7.62	0.10	0.25	7.65	4.06	3.34	0.96

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) The genes coding for **XR, XDH** and **XK** from ***K. marxianus*** were functionally expressed in *S. cerevisiae.* and the recombinant *S. cerevisiae* strain (KM123) able to produce 10.1 g/L ethanol from xylose using the xylose metabolic pathway of *K. marxianus* was successfully constructed.

(2) Microaerobic batch fermentation by KM123 resulted in 10.1 g/L final ethanol concentration, 0.33 g ethanol/g xylose ethanol yield and 0.14 g/L·h productivity which are comparable to the values of 10.2 g/L ethanol, 0.35 g ethanol/ g xylose ethanol yield and 0.14 g/L.h ethanol productivity in the strain expressing *Sch. stipitis* XR, XDH and XK (SS123).

(3) The disruption of the ACD6 gene reduced acetate concentration to 0.2 g/L from 2.8 g/L in KM123 but did not affect the rates of xylose consumption and ethanol production.

V. REFERENCES

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국 문 초 록

바이오에탄올은 친환경적이고, 현재 운송 수단에 바로 이용 가능하여 경제성, 효율성이 우수하므로 화석 연료의 대체에너지로서 주목 받고 있다. Xylose는 목질계에서 포도당에 이어서 2번째로 풍부한 발효 가능한 탄소원이다. 바이오매스로부터 에탄올을 경제적으로 생산하기 위해서는 효율적으로 xylose를 발효할 수 있는 재조합 균주 개발이 반드시 필요하다. *Saccharomyces cerevisiae*는 산업적으로 에탄올 생산 균주로 사용되지만, xylose를 탄소원으로 이용하지 못한다. 이는 xylose를 xylulose로 전환하는 대사활성이 없기 때문이다. 효모에서, xylose는 2종류의 산화환원효소인, NAD(P)H의존적 XR과 NAD^+ 의존적인 XDH에 의해서 xylulose로 전환이 된다. 마지막으로 XK에 의해 xylulose는 xylulose-5-phosphate로 전환이 되며 이것은 5탄당 인산회로에 의해서 이후의 대사가 진행된다.

이 연구는 xylose 대사경로를 자체적으로 갖고 있는 균주 중 *Kluyveromyces marxianus*를 선택하여 관련 유전자들을 얻고, 이들을 *S. cerevisiae*에 도입하여 xylose에서 에탄올을 생산하고자 하였다.

필요한 유전자는 XR을 암호화하는 *XYL1*, XDH를

암호화하는 *XYL2,* XK를 암호화하는 *XYL3*이다. 먼저, *S. cerevisiae* 내에서 *XYL1, XYL2, XYL3* 각각의 유전자 발현 여부를 확인하였다. *KmXYL1*이 도입된 DX23/KM1에서 *KmXYL1*의 specific activity는 0.48 U/mg이었고, xylose 발효에서 10.5 g/L ethanol을 생산하면서 *KmXYL1*의 발현을 확인할 수 있었다. *KmXYL2*를, 발현이 검증된 *KmXYL1*과 함께 도입한 ISXK/KM12에서 *KmXYL2*의 specific activity는 0.21 U/mg, 9.26 g/L ethanol을 생산했다. *KmXYL3* 역시 발현이 확인된 *KMXYL1, 2*과 함께 도입하여 KM123을 구축했고 *KmXYL*3의 specific activity는 4.6 U/mg, 11.1 g/L ethanol 생성함으로서, *S. cerevisiae* 내에서 *KmXYL1, 2, 3* 유전자가 모두 발현됨을 확인하였다. 따라서, *K. marxianus*의 xylose 대사 경로를 이용해 xylose로부터 에탄올을 생산하는 균주를 성공적으로 구축했다.

이 균주를 현재 많이 이용되는 *Scheffersomyces stipitis* 유래의 *XYL1,2,3*와 비교해 보았다. *K. marxianus*는 NADPH 의존적 XR과 NAD+ 의존적 XDH 사이에 조효소가 불균형을 이룰 가능성이 높은데, NADPH와 NADH 모두 의존적인 *Sch. stipitis* 유래의 XR과 비교했을 때 xylose 발효에 미치는 영향을 보고자 하였다. specific activity는 XR, XDH, XK 모두 *Sch. stipitis* 유전자들이 더 높았지만, 40 g/L xylose 발효에서

KM123이 약 10.1 g/L의 에탄올을 생성해 Ss123과 유사했고 Ethanol yield는 0.33 ethanol g/xylose g, productivity는 0.14 g/L.h로 역시 유사했다. 조효소의 불균형이 있지만 유전자들 간의 조화가 에탄올 생성에 미치는 영향도 매우 중요하다고 사료된다.

Km12,3에서 에탄올 생산을 증가시키고자 초산을 생성하는 *ALD6* 유전자를 파쇄하였다. (KM12,3::*ΔALD6*) 이를 미세호기조건에서 발효한 결과 KM12,3::*ΔALD6*가 세포 성장이 좋고 acetate 축적이 눈에 띄게 줄어들었지만, xylose의 소비와 ethanol 생성에는 크게 영향을 미치지 않았다. 이는 KM123 균주 자체로 xylose 소비가 좋았고, *ALD6* 유전자를 파쇄로 NADPH의 생성이 저해되어 XR의 기능에 도움을 주지 못했을 것으로 생각된다.

본 연구에서는 *K. marxianus* 유전자를 이용해 xylose에서 에탄올을 생성하였으며, 표면적인 조효소의 불균형이 있더라도 에탄올을 생성할 수 있음을 분명하게 보여주었고 이는 유전자들 간의 조화가 다른 중요한 요소이기 때문으로 사료된다.

주요어 : 바이오에탄올, xylose, KmXR, KmXDH, KmXK, *ScALD6*, 조효소 불균형, 초산, 대사공학

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께예요. 제 석사 생활동안 방장이셨던 영욱오빠, 꼭 여자친구 응원하겠습니다. 사수이신 진우오빠, 이것 저것 물어봐도 다 받아주시고 알려주셔서 너무 고마웠고 감사했습니다. 실험 잘 마무리 되어서 졸업 준비 잘 되었으면 좋겠습니다. SRC에서 동거동락했던 선기오빠, 실험도 방장일도 너무 잘하셔서 역시 선기오빠구나 합니다. 오빠와의 인연도 참 오래되었는데 항상 응원할께요. 동기이지 선배인 정현오빠, 학부 때보다 좀 더 친해진 것 같아 좋았습니다. 오빠한테 실험적으로 많은 도움을 얻었습니다. 감사해요. 제일 달달한 신혼을 보내고 계신 형로오빠, 미국가게 되신 거 다시 한 번 축하드리고, 새로운 생활에 잘 적응하실거라 믿습니다.

SPC의 또 다른 동거동락자 효란아, 항상 웃는 얼굴로 궂은 일 마다 않고 해줘서 너무 고마웠어. 언니로서 더 잘해주지 못해 항상 미안했고 맛난 거 함 먹자. 피부미인 민지야, 8층에 있을 때는 교류가 별로 없었는데 그래도 마지막 학기에 같이 지내서 참 좋았어. 효란이랑 같이 2년 차인데도 막내일 하느라 너무 고생이 많았은데, 민지랑도 맛난 거 먹어야지. 같은 동네 주민 인영이, 실험할 땐 뚝부러진데 다른 데는 약간 허당인 모습이 진짜 매력적이야. 많은 일을 맡아서 힘들었을 텐데 잘 마무리하길 응원할께.

아끼는 후배 현욱아, 현욱이랑도 거의 7년 째 인연을 맺고 있네. 대학원 때 오히려 못 챙겨줘서 항상 미안했어. 둘 다 여유가 좀 생기면 자주 보고 연락하자. 힘든 거 있으면 언제든지 연락하고. 이제 신입생 벗어난 지영이랑 상미, 상미는 어서 쾌차해서 건강한 모습으로 빨리 볼 수 있었으면 좋겠다. 시원하게 웃는 얼굴이 예쁜 지영아, 갑작스런 변화에 맘 고생 했을텐데 밝은 모습 유지해서 기특한 거 같아. 논문도 도와줘서 너무 고맙고.

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