# ETHANOL FERMENTATION ABILITY ON VARIOUS SUGARS AND GLUCOSE REPRESSION IN THERMOTOLERANT YEAST *KLUYVEROMYCES MARXIANUS*

(耐熱性酵母 *Kluyveromyces marxianus* における多様な糖に対するエタノール発酵能とグルコース抑制)

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**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF APPLIED MOLECULAR BIOSCIENCE**

**GRADUATE SCHOOL OF MEDICINE**

**YAMAGUCHI UNIVERSITY**

**2011**

# ACKNOWLEDGEMENT

I express my deepest gratitude to my supervisors, Prof. Mamoru Yamada, for creative guidance, attentive supervision and encouragement throughout this research. Besides, he also provides good opportunities and good suggestions, which are valuable to my future career. Your vast knowledge and enthusiastic attitude towards science have been encouraging and motivating.

Beside my advisor, I would like to thank Prof. K. Matsushita, Assoc. Prof. T. Yakushi and Assist. Prof. T. Kosaka for their helpful discussion and valuable advice.

I am particularly grateful to Dr. Noppon Lertwattanasakul, members of Josei laboratory and all Thai students, who study in Yamaguchi University for their valuable comments, discussion, suggestions and friendship during my study in Yamaguchi University.

I am extremely thankful to the Japanese government scholarship (Monbukagakusho; MEXT) for financial support and Department of Biological Chemistry, Yamaguchi University for providing laboratory facilities.

Finally, I greatly appreciate to my parents, my family and my friends for their understanding, encouragement and powerful support.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| *ACT1* | Actin gene |
| *ADH* | Alcohol dehydrogenase gene |
| Ara | arabinose |
| bp | base pair |
| °C | degree Celcius |
| Da | dalton |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| 2-DOG | 2-deoxyglucose |
| *et al.* | Et.alli (Latin), and other |
| etc. | et cetera (Latin), and other things, and so on |
| Fig. | Figure |
| Frt | fructose |
| g | gram |
| Gal | galactose |
| *GAL1* | Galactokinase gene |
| GH32 | glycoside hydrolase family 32 |
| Glc | glucose |
| h | hour (s) |
| i.e. | Ed est (Latin), that is |
| Inu | inulin |
| *INU* | Inulinase gene |
| kb | kilobase (1,000 bp) |
| L, l | litre |
| Man | mannose |
| *Max.* $\gamma_s$ | maximum sugar utilization rate |
| *Max.* $\mu_{x/s}$ | maximum growth rate |
| *Max.* $Y_{p/s}$ | maximum ethanol yield |
| Mel | melibiose |

# LIST OF ABBREVIATIONS

## (Continued)

| | |
|---|---|
| mg | miligram |
| mg/ml | milligram per milliliter |
| *MIG1* | a transcription factor gene involved in glucose repression |
| min | minute |
| ml | milliliter |
| NAD+ | Nicotinamide adenine dinucleotide, oxidized form |
| NADH | Nicotinamide adenine dinucleotide, reduced form |
| NADP+ | Nicotinamide adenine dinucleotide phosphate, oxidized form |
| NADPH | Nicotinamide adenine dinucleotide phosphate, reduced form |
| OD | optical density |
| PCR | polymerase chain reaction |
| Raf | raffinose; |
| RNA | ribonucleic acid |
| rpm | round per minute |
| $\gamma_s$ | specific sugar utilization rate |
| SD | standard deviation |
| Suc | sucrose |
| *SUC2* | Invertase gene |
| $T_m$ | Melting temperature |
| µg | microgram |
| µl | microlitre |
| $\mu_{x/s}$ | specific growth rate |
| *XYL1* | Xylose reductase gene |

# LIST OF ABBREVIATIONS

## (Continued)

| | |
|---|---|
| YP | yeast extract and peptone |
| YPAra | Yeast-peptone-arabinose medium |
| YPD | Yeast-peptone-glucose medium |
| YPDAra | Yeast-peptone-glucose+arabinose medium |
| YPDGal | Yeast-peptone-glucose+galactose medium |
| YPDMan | Yeast-peptone-glucose+mannose medium |
| YPDXyl | Yeast-peptone-glucose+xylose medium |
| YPGal | Yeast-peptone-galactose medium |
| YPXyl | Yeast-peptone-xylose medium |
| YPMan | Yeast-peptone-mannose medium |
| % | percent |
| %w/v | percent weight by volume |

# CHAPTER 1

# General Introduction and Literature Review

## 1.1 General introduction

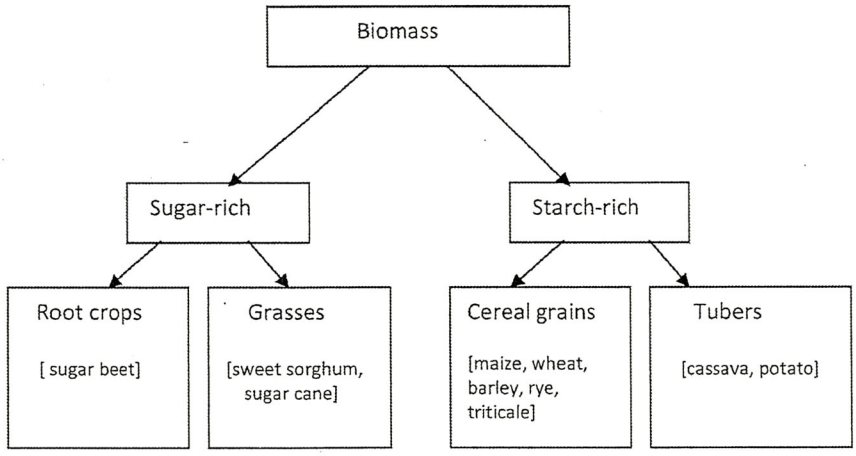
Bioethanol is fermentation alcohol. It refers to ethyl alcohol produced by microbial fermentation processes, as opposed to synthetically produced ethanol from petrochemical sources. It can be utilized as a liquid fuel in internal combustion engines, either in pure form or as a blend in petrol in different proportions (Walker 2011). The main advantages of bioethanol are that the fuel is renewable and that is not a net contributor to greenhouse gas emission (unlike fossil fuels) (Farrell et al. 2006). The ethanol industry of today utilizes raw materials rich in saccharides, such as sugar cane or sugar beets, and raw materials rich in starch, such as corn and wheat (Gong et al. 1999; Gray et al. 2006). The concern about supply of liquid transportation fuels together with the concern about global warming, have turned the interest towards large-scale ethanol production from lignocellulosic materials, such as agriculture and forestry residues (Hahn-Hägerdal et al. 2006). Emerging fuel alcohol processes the exploitation of lignocellulosic feedstocks, scientific and technological constraints involved in depolymerising these materials, and efficiently fermenting the hydrolysate sugars by efficient microorganisms are being overcome (Zaldivar et al. 2001: Hahn-Hägerdal et al. 2006).

## 1.2 Literature Review

### 1.2.1 Feedstocks for bioethanol production

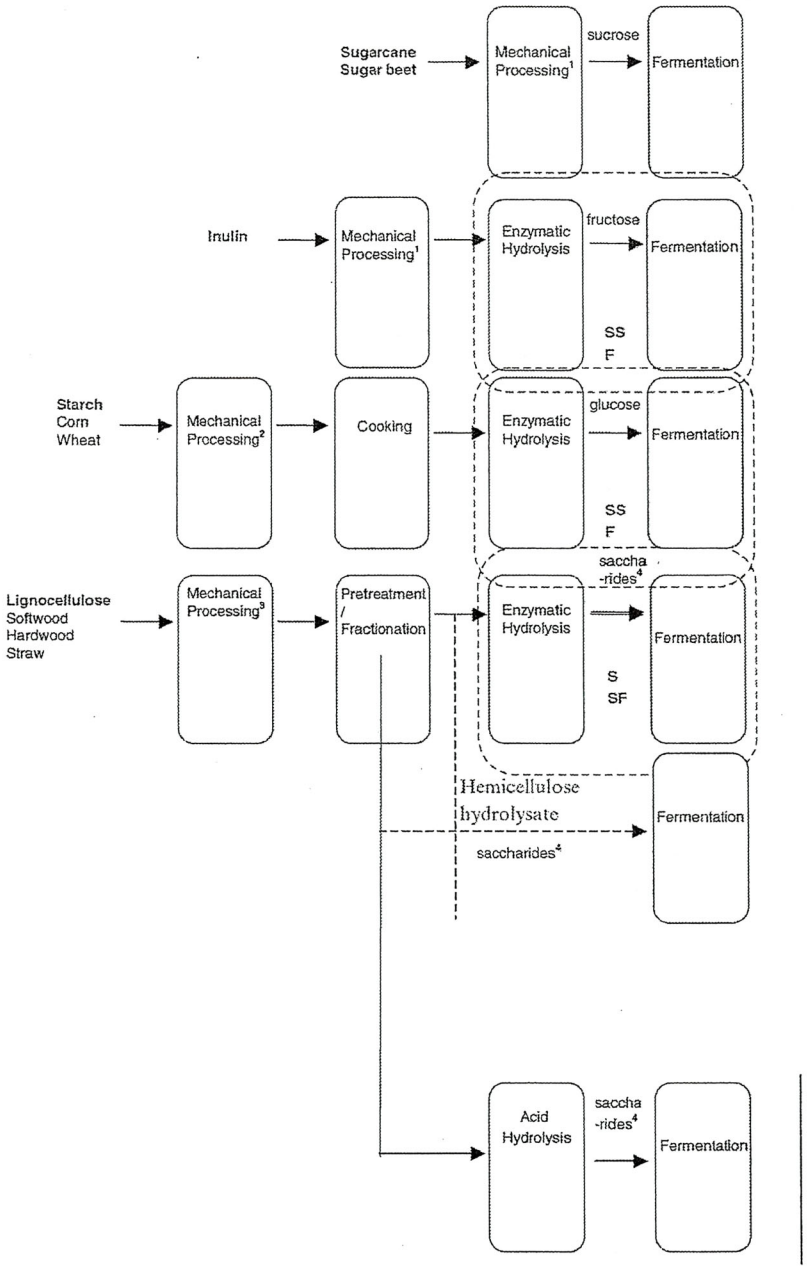
*1.2.1.1 First-generation feedstocks*

In the first-generation technology, bioethanol is produced by converting sugars indirectly through starch from agricultural cereal, which require pre-hydrolysis prior to sugar or directly from crops like sugar cane or sugar beets crops, which contain a readily fermentable sugar source, namely sucrose (Fig. 1.1; Table 1.1) into ethanol via fermentation followed by distillation (Fig. 1.2) (Monceaux 2009; Pasha and Rao 2009). Presently Brazil together with the United States is the world's largest ethanol producer. In Brazil, the raw material for ethanol production is sugar cane (Moreira 2000), whereas in the United States, corn is the major raw material.




From: Walker, 2011

**Fig. 1.1**—Bioethanol from first generation feedstocks




From: Rudolf et al., 2009

**Fig. 1.2**—Raw materials and their processing for ethanol production

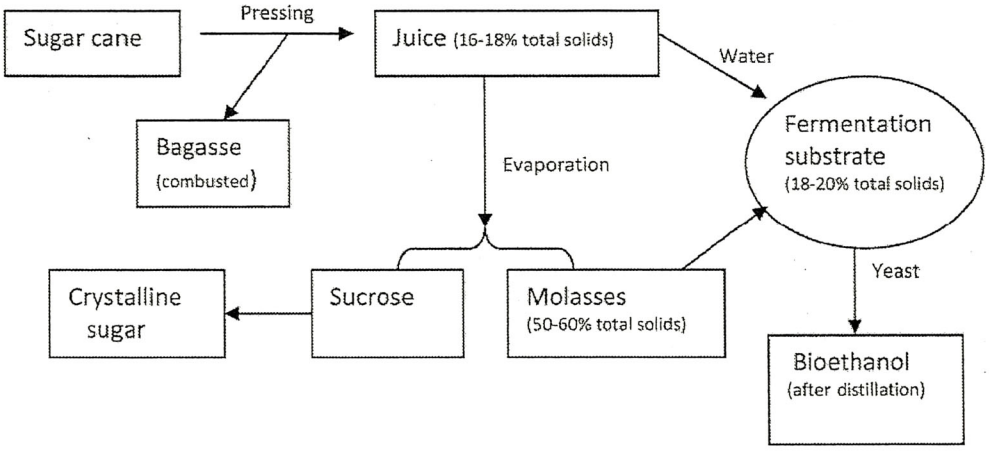
[1] Slicing and extraction
[2] Milling (Dry-mill process), steeping (Wet-mill process)
[3] Chipping or milling
[4] Glucose, mannose, xylose, galactose, arabinose, cellobiose and oligosaccharides

**Table 1.1** Composition of sugar-based feedstock for bioethanol production

| Composition | Sugar cane juice (g/L) | Sugar cane molasses (g/Kg) | Sugar beet molasses (g/Kg) |
|---|---|---|---|
| Total solids | 140–190 | 735–875 | 759–854 |
| Total sugars | 105–175 | 447–587 | 477–530 |
| Sucrose | 98–167 | 157–469 | 443–530 |
| Reducing sugars | 6–11 | 97–399 | 1.2–10 |
| Raffinose | - | - | 4.7–21 |
| Nitrogen | 0.08–0.3 | 0.25–1.5 | 1.3–2.3 |
| Phosphorus | 0.02–0.1 | 0.3–0.7 | 0.15–0.52 |
| Potassium | 0.7–1.5 | 19–54 | 15–52 |
| Calcium | 0.1–0.5 | 6–12 | 0.75–3.8 |
| Magnesium | 0.1–0.5 | 4–11 | 0.1–2.7 |

From: Walker, 2011

Yeasts such as *Saccharomyces cerevisiae* can directly ferment sugar cane juice (~15% sucrose), or the residual molasses (~50% sucrose) from sugar refining processes. The juice can be processed either into crystalline sugar or directly fermented to ethanol, as per many Brazilian industrial plants (Fig. 1.3) (Walker 2011).




From: Walker, 2011

**Fig. 1.3**—Sugar cane processing for sugar and bioethanol production

Starch is a storage compound consisting of glucose linked via α-1,4 and α-1,6 glycosidic linkages (amylose and amylopectin) (Gray et al. 2006). Regarding

starchy crops for bioethanol production, maize-to-bioethanol processes are differentiated into 2 main types: dry and wet milling (Fig. 1.2) (Abbas 2007; O'Brien et al. 2009; Rudolf et al. 2009). Rudolf et al. (2009) explained the difference between wet and dry mill processes as in the following sentences. In a wet mill process corn is initially steeped with water at 49–53°C, which softens the hulls and causes the grains to swell. Subsequently, the wet grain is milled and germs, fibers and gluten are separated, after which the starch is dried. In a dry mill plant, the raw material is initially ground to increase the surface area without separating the different grain component. For bioethanol production, more complete starch hydrolysis is required. This is accomplished using exogenous amylolytic enzymes, including: α- and β-amylases (for liquefaction); amyloglucosidase (or glucoamylase) required to debranch amylopectin fractions (comprising 75–90% of starch, depending on cereal source) and glucanases (for viscosity reduction) (Walker 2011). The bioconversion of starch to ethanol required industrial enzymes produced by bacteria such as *Bacillus spp.* and fungi such as *Aspergillus* spp. (Nair and Ramachandran 2008).

*1.2.1.2 Second-generation feedstocks*

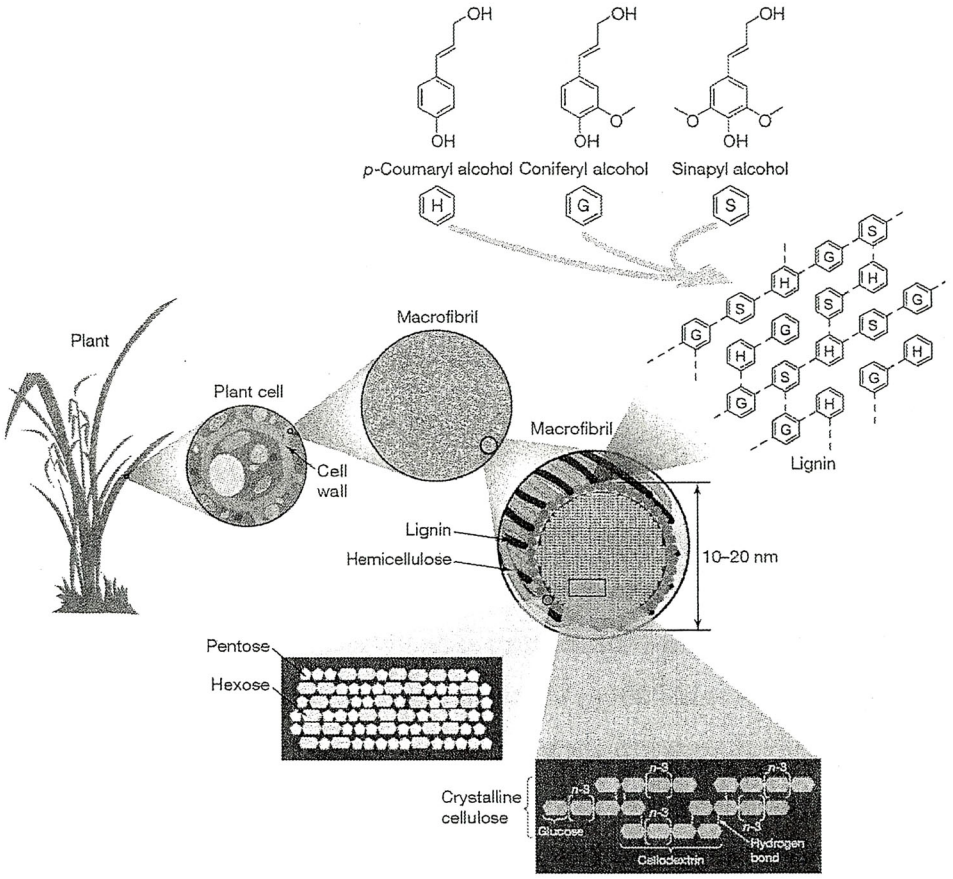
Utilization of first-generation feedstocks for future biofuel production is ultimately unsustainable due to compete for food and land-use. To satisfy the increasing demand for fuel ethanol and to respond to the demand for reducing green house gas emission (Farrell et al. 2006), ethanol for transportation has to be produced from other raw materials than saccharides and starch. In the second-generation technology, ethanol is produced through cellulose from non-food biomass sources, such as lignocellulose, the most abundant alternative raw material (Claassen et al 1999). Besides, inulin is considered as a raw material for fuel ethanol production (Claassen et al 1999).

1.2.1.2.1 Lignocellulose

Lignocellulose is composed of cellulose, hemicellulose and lignin, which are associated to each other in a complex plant cell wall matrix (Fig. 1.4). Cellulose is a polymer of β-1,4-linked glucose units, where the repeating unit is the disaccharide cellobiose. Cellulose chains associate very strongly to each other by

hydrogen bonds and give cellulose a highly crystalline structure (Delmer and Amor 1995). Hemicellulose, in turn, is a complex heteropolymer consisting of different saccharides, both hexose and pentose sugars. The composition of hemicellulose varies widely between different plant species. Hemicellulose from herbaceous plants and hardwood contains large fractions of xylan and arabinan, whereas softwood hemicellulose has a low content of xylan but is rich in mannan. Lignocellulose also contains lignin, a complex hydrophobic polymer of substituted aromatic rings, that is very resistant to chemical or biological degradation (Lee 1997). Table 1.2 summarizes the carbohydrate composition of potential lignocellulosic raw materials for ethanol production.

Cellulose and hemicellulose must be hydrolyzed to monosaccharides by thermochemical and enzymatic methods to enable ethanolic fermentation by yeast (Fig. 1.2). A first hydrolysis step in which hemicellulose is converted to oligo-, di- and mono-saccharides may also be referred to as a pre-treatment and fractionation step.




From: Rubin, 2008

**Fig. 1.4—Structure of lignocellulose**. The main component of lignocellulose is cellulose, a β (1–4)-linked chain of glucose molecules. Hydrogen bonds between different layers of the polysaccharides contribute to the resistance of crystalline cellulose to degradation. Hemicellulose, the second most abundant component of lignocellulose, is composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose and xylose. Lignin is composed of three major phenolic components, namely p-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). Lignin is synthesized by polymerization of these components and their ratio within the polymer varies between different plants, wood tissues and cell wall layers. Cellulose, hemicellulose and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall.

**Table 1.2** Carbohydrate composition of typical lignocellulosic feedstocks

| | Salix*(hardwood) Sassner et al. (2006) | Spruce* (softwood) Söderström et al. (2003) | Corn Stover* Öhgren et al. (2006, 2007) | Bagasse* Rudolf (2007) |
|---|---|---|---|---|
| Glucan | 43 | 50 | 42 | 43 |
| Galactan | 2.0 | 2.3 | 1.1 | 0.4 |
| Mannan | 3.2 | 12 | - | - |
| Xylan | 15 | 5.3 | 20 | 26 |
| Arabinan | 1.2 | 1.7 | 2.9 | 1.5 |

*Values shown as percentages based on dry raw material. The remainder constitutes mainly lignin and ash.

From: Rudolf et al., 2009

A major obstacle to the effective utilization of lignocellulose is the chemically unreactive nature of cellulose. The lignin-hemicellulose matrix, cellulose crystallinity and its low surface area make the lignocellulose very resistant to enzymes (Berlin et al. 2006). Making cellulose accessible to the enzyme is an essential factor in order to increase the rate of hydrolysis (Philippidis and Smith 1995). Therefore, pretreatment is necessary before enzymatic hydrolysis. Enzymatic hydrolysis has been recognized as an attractive method for hydrolysis of cellulose and hemicelluloses contained in pretreated biomass suspension (Himmel et al. 1996). The methods of lignocellulose pretreatment can be classified in three groups: physical, chemical and biological pretreatments.

**Table 1.3** Pre-treatment technologies for lignocelluloses

| Pre-treatment methods | Examples |
|---|---|
| Physical | Milling (mechanical comminution), microwave irradiation, ultrasound, thermal processes (pyrolysis at >300°C, steam explosion using 160–260°C, 0.69–4.83 MPa pressure, followed by rapid decompression), thermochemical processes (weak acid, high temperature), extrusion. |
| Chemical | Alkali-pretreatment, ammonia fibre expansion (AFEX) technologies, organosolv (ACOS), liming (calcium hydroxide), sulphur dioxide, liquid hot water (LHW) and wet oxidation (hot water plus oxygen at 200°C), $CO_2$ explosion, $SO_2$ explosion, ozonolysis, $H_2O_2$ delignification, supercritical fluid and ionic liquid pre-treatments (e.g. n-butyl-methy-lilidazolium chloride ~300°C). |
| Biological | Microbial (e.g., white-rot fungi such as *Phanerochaete chrysosporium, Trametes versicolor*) and enzymatic (e.g., peroxidase and laccase) pretreatments (delignification). |

From: Walker, 2011

1.2.1.2.2 Inulin

In addition to the far more abundant lignocellulosic feedstocks, inulin-rich plants, such as Jerusalem artichoke have been considered for fuel ethanol production (Ohta et al. 1993; Szambelan et al. 2004).

Inulin is a polymer of β-2,1 linked fructose units, terminated by a glucose residue through a sucrose-type linkage at the reducing end (Chi et al. 2009) and its conversion to ethanol resembles that of starch (Fig. 1.2). Some yeast species such as *Kluyveromyces marxianus* and *Saccharomyces rosei* can ferment inulin directly to ethanol (Duvnjak et al. 1981; Margaritis and Merchant 1983). Inulin can also be hydrolysed with inulinases and simultaneously or subsequently fermented to ethanol (Ohta et al. 1993; Vandamme and Derycke 1983). The hydrolysis of inulin can also be catalyzed, albeit very slowly, by invertase (EC 3.2.1.26) (Rouwenhorst et al. 1990b). The separation classification of these two enzymes has been disputed (Arnold 1987). In addition to substrate specificity, considerable structural differences also exist with the invertase of *Saccharomyces* var. *marxianus cerevisiae* (Rouwenhorst et al. 1990a). This inulinase is secreted into the culture fluid and also retained with the cell wall. Both forms of inulinase have the same carbohydrate content of 34% of its mass as carbohydrate but a molecular mass is different. The former one has a molecular mass about 165 kDa and consists of two protein subunits. The later one has an average size of 350 kDa with a tetramer form. In *S. cerevisiae*, invertase is secreted in the culture fluid as a dimer with a molecular mass of 270 kDa, whereas the enzyme, which is mainly retained in the cell wall, is an octamer of about 800 kDa. Both invertase forms contain up to 50% carbohydrate (Esmon et al. 1987). The amino acid sequences of the amino-terminal ends of invertase and inulinase revealed a little homology (Rouwenhorst et al. 1990a). Contrary to invertase, inulinase is able to hydrolyze fructans like inulin and levan (Snyder and Phaff 1960; Snyder and Phaff 1962). These polysaccharides do not enter the cell wall of yeasts (Scherrer et al. 1974) and hydrolysis must occur outside the cell wall.

### 1.2.2 Microorganisms for Ethanol production

Ethanol fermentation is a biological process in which fermentable sugars are converted to ethanol and $CO_2$ by microorganisms, which is the key factor in the conversion of sugars to ethanol. Ideally, the microorganism should give a high ethanol yield, have a high ethanol tolerance, be resistant to inhibitors of hydrolyzates, with no oxygen requirement, and a broad substrate utilization range (Pasha and Rao 2009). It is also desirable to have a strain with a high sugar consumption rate and productivity, minimal nutrient requirement, high salt tolerance, high shear tolerance, thermotolerance, safety for humans and no spore formation (Picataggio and Zhang 1996). Development for obtaining microorganisms, which satisfies all those characteristics, is now in progress, however, no single microorganism has been obtained (Pasha and Rao 2009).

The yeast, *S. cerevisiae*, is the predominant microorganism responsible for ethanolic fermentation and is the major cell factory in industrial bioethanol production processes. *S. cerevisiae* has a number of physiological characteristics advantageous in the industrial context over bacteria, other yeast and filamentous fungi, (Hahn-Hagerdal et al. 2007). *S. cerevisiae* grows both under aerobic and anaerobic conditions, and performs well in industrial fermentation conditions. It tolerates a wide range of pH, with an optimum at acidic pH, which makes *S. cerevisiae* fermentation less susceptible to infection than e.g. bacterial fermentation. *S. cerevisiae* has an optimal temperature around 30–35°C and tolerates temperatures up to around 40°C, with. This yeast is also tolerant to high concentrations of sugar and ethanol, and it tolerates relatively high osmotic pressures (Pasha and Rao 2009).

*S. cerevisiae* can utilize sucrose, glucose, fructose, galactose and mannose (Lindén et al. 1992; Nilsson et al. 2002). However, *S. cerevisiae* lacks the ability to ferment a number of mono-, di-, and trisaccharides derived from starch, cellulose and hemicellulose such as cellobiose, arabinose and xylosbiose (Lynd et al. 2002; Zhang and Lynd 2005). Also, the hexose sugar galactose is only consumed upon glucose depletion, which prolongs the fermentation time of galactose-rich substrates (Johnston and Carlson 1992). Moreover, *S. cerevisiae* has some

drawbacks related to the metabolism that is accumulation of glycerol, which is produced for reducing co-factor NADH under anaerobic conditions (Oura 1977).

Yeasts other than *Saccharomyces sp.* are misleadingly referred to as nonconventional yeast (NCY) (Boekhout and Kurtzman 1996). In fact NCY constitute the majority of yeast species, whereas *Saccharomyces sp.* has very unusual qualities due to its adaptation to industrial use (Rudolf et al. 2009). Among NCY are yeast that consume the pentose sugars xylose (Skoog and Hahn-Hägerdal 1988), arabinose (Dien et al. 1996; Fonseca et al. 2007), starch (Spencer-Martins and van Uden 1977), and starch and lignocellulose derived di- and trisaccharides (Ryabova et al. 2003). Among NCY are also those that endure much lower pH than *Saccharomyces sp.*, such as *Zygosaccharomyces sp.* (Thomas and Davenport 1985) as well as those that perform ethanolic fermentation at temperatures above 40°C, such *Hansenula polymorpha* (Ryabova et al. 2003) and *K. marxianus* strain is able to grow at 52°C and ferment ethanol at 50 ° C (Banat et al. 1992).

### 1.2.3 Uptake of sugars in yeast

#### *1.2.3.1 Common hexose sugars*

Transport of the common hexose sugars, glucose, fructose or mannose in *S. cerevisiae* is only through facilitated diffusion (Boles and Hollenberg 1997) mediated by several transporters, the Hxt proteins, with different kinetic properties and modes of regulation, although the $K_m$ value is higher than that for glucose (Reifenberger et al. 1997). In *Pichia stipitis*, xylose fermenting yeast, exhibits glucose transport by both low-and high- affinity proton symport systems that operate simultaneously (Kilian and van Uden 1988). In *K. lactis*, glucose transport appears to process by facilitated diffusion, Hgt and Rag proteins, with different affinities (Billard et al. 1996; Goffrini et al. 1990). Gasnier (1987) reported that glucose can enter in *K. marxianus* ICG 2587 through two distinct transporters: a high-affinity glucose transporter, which is a $H^+$-sugar symporter and a low-affinity transporter, which is not associated with $H^+$ movement. Moreover, the regulation of sugar transporters in *K. marxianus* CBS 937 was revealed (De Bruijne et al. 1988) that

cells grown in glucose medium during the exponential phase (high glucose concentration) only express a constitutive low-affinity transporter, while cells under the stationary phase (low glucose level) express three proton symporters. These symporters appear to be subject to catabolite inactivation in the presence of glucose, fructose or mannose (De Bruijne et al. 1988). By the function of hexokinase (*HXK2*), glucose and mannose are converted to be glucose-6-phosphate and mannose-6-phosphate, respectively, the latter is then isomerized to fructose-6-phosphate by phosphomannose isomerase (van Maris et al. 2006).

Galactose is taken up in *S. cerevisiae* by a dedicated member of the *HXT* family, the galactose permease Gal2p, (van Maris et al. 2006), while galactose transport in *K. lactis* is through Hgt, a constitutive high-affinity glucose transporter (Baruffini et al. 2006). Both organisms transport galactose via facilitated diffusion transporters. On the other hand, *K. marxianus* transports galactose through a proton symporter (De Bruijne et al. 1988; Van Leeuwen et al. 1991), for which higher energy is expended compared to other hexose transportation. An ATP level is involved in the control of $H^+$-galactoside symport (Van den Broek et al. 1987). After taken up into cells, galactose is subsequently converted into glucose-6-phosphate via the Leloir pathway, which consisting of four catalytic steps (Leloir 1951). The main regulators: a transcriptional activator, Gal4p (or KlLac9p); a repressior, Gal80p; and a ligand senser, ScGal3p (or KlGal1p) in the galactose regulatory mechanism are conserved between *S. cerevisiae* and *K. lactis*, but the molecular mechanisms that occur as a result of the molecular interaction between these regulators are different such that KlGal1p has the dual function as galactokinase as well as a galactose-sensing protein or bears enzymatic and regulatory functions (Venkat et al. 2010), whereas these functions are split into two proteins, Gal1p and Gal3p in *Sacchraromyces* (Rubio-Texeira 2005) (Table 1.4). Moreover, the basal expression of the *LAC/GAL* genes is relatively high in *Kluyveromyces*, probably due to a *GAL4* positive autoregulatory loop and the extent to which glucose repression controls expression also seems quite strain specific (Lane and Morrissey 2010). The stain with different degrees of glucose repression depends on differences in KlGal4p binding sites, which differ by two bases in the *KlGAL4* promoter from some repressing and non-repressing strain (Kuzhandaivelu et al. 1992).

*1.2.3.2 Pentose sugars*

*S. cerevisiae* can neither ferment nor assimilate xylose and arabinose (Hahn-Hagerdal et al. 2007; Fonseca et al. 2007). Several yeasts that are able to ferment xylose to ethanol such as *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus* also ferment L-arabinose (Mishra and Singh 1993). However, very little information exists about L-arabinose transport in natural arabinose-utilizing yeasts. *Candida shehatae* possesses an L-arabinose/proton symporter (Lucas and van Uden 1986). *Pichia guilliermondii* and *Candida arabinofermentans* have low-affinity facilitated diffusion and high affinity but less-specific arabinose/$H^+$ symporters (Fonseca et al. 2007). *K. marxianus* possesses a single, high-affinity active transport system for arabinose transport (Eric et al. 2009 ).

D-xylose transport has been characterized in various yeast species. Xylose is imported in *S. cerevisiae* by using high affinity transporters, Hxt4p, Hxt5p, Hxt7p and Gal2p (Hamacher et al. 2002; Lee et al. 2002; Sedlak and Ho 2004)). *P. stipitis* transports xylose through a low-affinity proton symporter, which is shared between glucose and xylose. This transporter was inhibited by glucose (Agbogbo and Coward-Kelly 2008). *K. marxianus* shows facilitated diffusion low-affinity transport activity when grown on xylose under microaerobic conditions, and both a low-affinity and an active high-affinity transport activity when grown on xylose under fully aerobic conditions (Stambuk et al. (2003). Xylose metabolism in yeast proceeds mostly via an oxidative-reductive pathway. Xylose is first reduced to xylitol by an NAD(P)H-linked xylose reductase (*XYL1*), followed by xylitol oxidation to xylulose by an NAD-linked xylitol dehydrogenase (*XYL2*). As these oxidoreductases have different cofactor specificities, the conversion process of xylose into xylulose yields one $NADP^+$ and one NADH. NADPH can be regenerated by the pentose-phosphate pathway, whereas NADH can be reoxidized via the respiratory chain under aerobic condition. Under anaerobic conditions without an electron acceptor such as acetoin or fufural, cells cannot maintain a redox balance and ferment xylose. With regard to the L-arabinose metabolization by yeast, the pathway is quite similar to the xylose pathway. Both pathways share a partial overlap. This pathway consists of two $NAD^+$-linked oxidations and two NADPH-

linked reductions, resulting in a redox cofactor imbalance under anaerobic conditions (Dien et al. 1996).

*1.2.3.3 Disaccharides and oligosaccharides*

Disacchrarides may be transported either with or without hydrolization to the component monosaccharides across the yeast plasma membrane (Table 1.5)

Lactose utilization has been extensively studied in *K. lactis*, results of which allow to assume that the fundamental aspects are conserved in *K. marxianus* (Rubio-Texeira 2006; Schaffrath and Breunig 2000). As mentioned above, one of common features of *K. lactis* and *K. marxianus* is their capacity to utilize lactose as a carbon source, a trait that is absent in *S. cerevisiae*. The ability to utilize lactose is conferred by two genes, *LAC12*, which encodes a lactose permease required for lactose uptake into cells, and *LAC4*, which encodes a β-galactosidase that hydrolyses lactose to the monomers glucose and galactose. As in *S. cerevisiae*, galactose is further metabolised to glucose-6-P via the Leloir pathway. The evolutionary history of the *LAC12–LAC4* gene pair is not clear but their regulation is integrated with the Gal4p/Gal80p system that is well-studied in *S. cerevisae* (Lane and Morrissey 2010).

Sucrose is readily assimilated and fermented by yeasts and is abundant in many natural, complex growth media like molasses and sugar cane juice. Some strains of *S. cerevisiae* have been reported to possess a sucrose proton symporter (Santos et al. 1982), but predominantly first convert sucrose to glucose and fructose. This is accomplished at the yeast cell envelope by a periplasmic invertase. The sugar is hydrolyzed in most cases by an extranal invertase, which is generally repressed by glucose. In *K. lactis*, the gene *KlINV1* encoding invertase has been cloned and characterized. It shows high amino acid sequence homology with that from *S. cerevisiae* and with the inulinase, a fructofuranosidase that hydrolyzes sucrose, from *K. marxianus* (Georis et al. 1999).

Melibiose is a disaccharide that is formed by galactose and glucose linked together with a β-1,6 glycosidic bond. It can be formed by invertase-mediated hydrolysis of raffinose, which produces melibiose and fructose. Melibiose can be broken down into its component saccharides by the enzyme α-galactosidase.

Raffinose is a trisaccharide composed of galactose, fructose, and glucose, and is formed by attaching a fructose molecule to glucose molecule in melibiose with an α-1,2 bond. Invertase enzyme can break the α-1,2 bond of raffinose and creates melibiose and fructose just as it splits the same link in sucrose to create glucose and fructose. The fructose molecule can be transferred into cells and metabolized.

**Table 1.4** Specific gene of the yeast *GAL* regulon

| Gene | Category[a] | Function | $UAS_G$[b] | $URS_G$[c] |
|---|---|---|---|---|
| *(A) GAL/MEL regulon from S. cerevisiae* | | | | |
| *MEL1* | C | α-Galactosidase; EC 3.2.1.22 | 1 | 1 |
| *GAL2* | C | Galactose permease | 2 | 0 |
| *GAL1* | C/R | Bifunctional sensor inducer/galactokinase; EC 2.7.1.6 | 4 | 1 |
| *GAL7* | C | Galactose-1-phosphate uridylyltransferase; EC 2.7.7.12 | 2 | 0 |
| *GAL10* | C | Uridine diphosphoglucose 4-epimerase; EC 5.1.3.2 | 4 | 0[d] |
| *GAL4* | R | Transcriptional activator | 0 | 1 |
| *GAL80* | R | Gal4p repressor | 1 | 0 |
| *GAL3* | R | Gal80 repressor (sensor/inducer) | 1 | 1 |
| *(B) GAL/LAC regulon from K. lactis* | | | | |
| *LAC12* | C | Lactose/galactose permease | 4 | 0 |
| *LAC4* | C | β-Galactosidase; EC 3.2.1.23 | 4 | 0 |
| *KlGAL1* | C/R | Bifunctional sensor inducer/galactokinase; EC 2.7.1.6 | 4 | 1 |
| *KlGAL7* | C | Galactose-1-phosphate uridylyltransferase; EC 2.7.7.12 | 2 | 0 |
| *KlGAL10* | C | Uridine diphosphoglucose 4-epimerase; EC 5.1.3.2 | 4 | 0[d] |
| *KlGAL4* | R | Transcriptional activator | 1 | 0 |
| *KlGAL80* | R | Gal4p repressor | 2 | 0 |

Because of a more generalized role of *PGM2/GAL5* in carbon metabolism, this gene has not been included in this table.

[a] Catabolic (C) or regulatory (R) function.

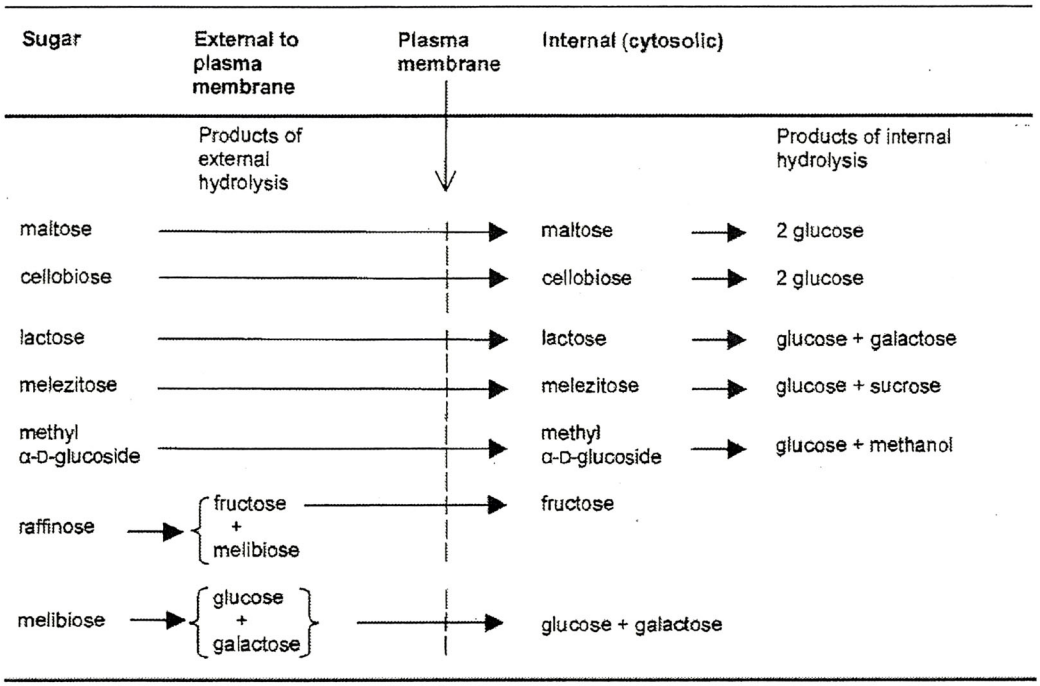
[b] Confirmed Gal4p-binding sites (upstream activating sequence, $UAS_G$) are described in

[c] Confirmed Mig1p-binding sites (upstream repressor sequence, $URS_G$) are described in

[d] $URS_G$ located in the *GAL1–GAL10* intergenic region is closest to *GAL1*.

From: Rubio-Texeira, 2005

**Table 1.5** Location of hydrolysis of oligosaccharides in most yeasts




From: Barnett, 1981

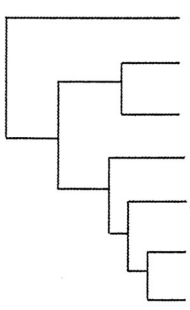
### 1.2.4 *Kluyveromyces marxianus*

*K. marxianus*, a NCY, is described as a homothallic (hemiascomycetous yeast), is phylogenetically related to *S. cerevisiae*, and is a sister species to the better-known *Kluyveromyces lactis* (Lachance 1998; Llorente et al. 2000). *K. lactis* and *K. marxianus* have a major common feature that is the capacity to assimilate lactose and to use it as a carbon source but this trait is absent in *S. cerevisiae*. In contrast to *K. lactis*, which is a model for non-conventional yeasts (Fukuhara 2006; Schaffrath and Breunig 2000), the sister species *K. marxianus* has little accumulated knowledge, no complete genome sequence, no type strain adopted as a reference for basic research purposes. While, *K. marxianus* has been more widely adopted by industry because of some of its qualities, such as an extremely rapid growth rate, thermotolerance and a broad substrate spectrum (Fonseca et al. 2008).

Since there are few reviews of *K. marxianus* and recent review from Lane and Morrissey (2010) has been published, this review is summarized into the following subsection.

### *1.2.4.1 Taxonomy and phylogeny of K. marxianus*

Application of technique from Kurtzman and colleagues (Kurtzman and Robnett 1998) through using a variation in the D1/D2 region of the large subunit (25S) rDNA as the benchmark for categorizing yeast and understanding the relatedness between strains, resulted in major re-organisation and reclassification within the *Saccharomyces* complex/clade, with yeasts previously classified as part of the genus *Kluyveromyces* strongly affected. The highly polyphyletic nature of the original *Kluyveromyces* genus required the renaming of most of its species and the genus now retains just six species (Kurtzman 2003; Lachance 2007) (Fig. 1.5).



| | Lactose assimilation | Inulinase | $\mu_{max}$ | Glucose fermentation | Maximum temperature |
|---|---|---|---|---|---|
| *Saccharomyces cerevisiae* | - | - | 0.37 | + | 35°C |
| *Kluyveromyces aestaurii* | - | - | n/a | + | 35°C |
| *Kluyveromyces nonfermentans* | - | - | n/a | - | 42°C |
| *Kluyveromyces wikerhamii* | - | - | 0.43 | + | 37°C |
| *Kluyveromyces lactis* | + | - | 0.50 | + | 37°C |
| *Kluyveromyces marxianus* | + | + | 0.60 | + | 52°C |
| *Kluyveromyces dobzhanskii* | - | - | n/a | + | 35°C |

From: Lane and Morrissey, 2010

**Fig. 1.5—Relationship of *K. marxianus* to other yeasts.** The tree shows the phylogenetic relationships between the species in the genus *Kluyveromyces* with *Saccharomyces cerevisiae* included for comparison. Some key traits are also presented, highlighting notable features of *K. marxianus* that differentiate this yeast from other *Kluyveromyces* species. Individual strains within a species may show some variation but the general traits listed apply to the species.

Both *Kluyveromyces* and *Saccharomyces* genera are part of the ''Saccharomyces'' complex, itself a subclade within the Saccharomycotina or hemiascomycetes. A whole-genome duplication (WGD) event that occurred 100 million years ago (Wolfe and Shields 1997) caused the *Saccharomyces* complex is itself divided. Genera and species in the *Saccharomyces* complex are defined by whether they emerged pre- or post-WGD, with *Saccharomyces sensu stricto* species post-WGD and *Kluyveromyces* species, pre-WGD. After WGD event, the evolution

that is important for *S. cerevisiae* was presented that are an additional copy of genes and genes had flexibility to evolve new functions. From these, genes that are homologous between *Saccharomyces* and *Kluyveromyces* have functionally diverged and, in some cases, functional conservation is present with ''less-related'' yeasts outside the *Saccharomyces* complex.

Genome structure and ploidy are variable within yeasts. The ploidy status of *K. marxianus* is less clear. Although, the conventional study revealed that the species was haploid (Johannsen 1980; Steensma et al. 1998). Recently, the widely used strain *K. marxianus* CBS 6566 is a diploid (Ribeiro et al. 2007). On the other hand, *K. marxianus*, which are prevalent in research and industrial settings, seems to be both haploid and diploid forms (Hong et al. 2007; Pecota et al. 2007; Nonklang et al. 2008) and some other strains are indicated to be haploid.

*1.2.4.2 Sugar metabolism and physiology*

The ability to utilize particular sugars, and the pathways used to generate energy from sugars, are defining features of different yeasts and, until the advent of molecular tools, was a major classification tool for yeasts (Lane and Morrissey 2010). It is fascinating that although components of the core pathways of glycolysis and the TCA cycle are largely conserved, regulation differs dramatically between yeast species (Flores et al. 2000). These differences in regulatory mechanisms give rise to a number of peculiarities that are commonly referred to as the Crabtree, Kluyver, Pasteur and Custer effects, reflecting the scientists who first described them (Table 1.6). These effects are still not completely understood. The post-WGD, most genes in the lineage leading to *Saccharomyces* are returned to single copy, but many of genes involved in glycolysis and sugar metabolism were retained to duplicate copies. The capability of *Saccharomyces* to rapidly utilize glucose by fermentation to ethanol, was assumed that due to an increased glycolytic flux arisen from metabolic adaptation after WGD (van Hoek and Hogeweg 2009).

*K. marxianus*, like *S. cerevisae*, is a respiro-fermentative yeast, and it can generate energy either via the TCA cycle by oxidative phosphorylation or by fermentation to ethanol (Lane and Morrissey 2010). Crabtree effect mechanism is

still not fully understood but probably arises from a combination of enzyme saturation under high glycolytic flux, glucose repression of TCA enzymes and expression of specific redox balancing metabolic reactions (Merico et al. 2007). *K. marxianus* was classified as Crabtree-negative yeast (as opposed to Crabtree-positive yeast), which do not produce ethanol under aerobic conditions and are respiratory unlimited even at high glucose concentrations (van Dijken et al. 1993).

**Table 1.6** Regulatory phenomena in sugar metabolism of yeasts

| Phenomenon | Description | Comments | Examples |
|---|---|---|---|
| Pasteur effect | Activation of sugar consumption rate by anaerobiosis | Only observable in resting or nutrient-starved cells. | *S. cerevisiae* |
| Crabtree effect | Suppression of respiration by high glucose | Cells continue to ferment irrespective of oxygen availability due to glucose repressing/inactivating respiratory enzymes or due to the inherent limited respiratory capacity of cells. | *S. cerevisiae* *Sch. Pombe* |
| Custers effect | Transient inhibition of fermentation by anaerobiosis | Oxygen stimulates ethanol production due to a lack of intracellular $NAD^+$ following secretion of acetate | *Dekkera and Brettanomyces spp.* |
| Kluyver effect | Anaerobic fermentation of glucose, but not of other sugars, although they may be aerobically assimilated | This phenomenon may be linked to sugar transport limitations or altered activity of pyruvate decarboxylase | *C. utilis* |

From: Walker, 1998

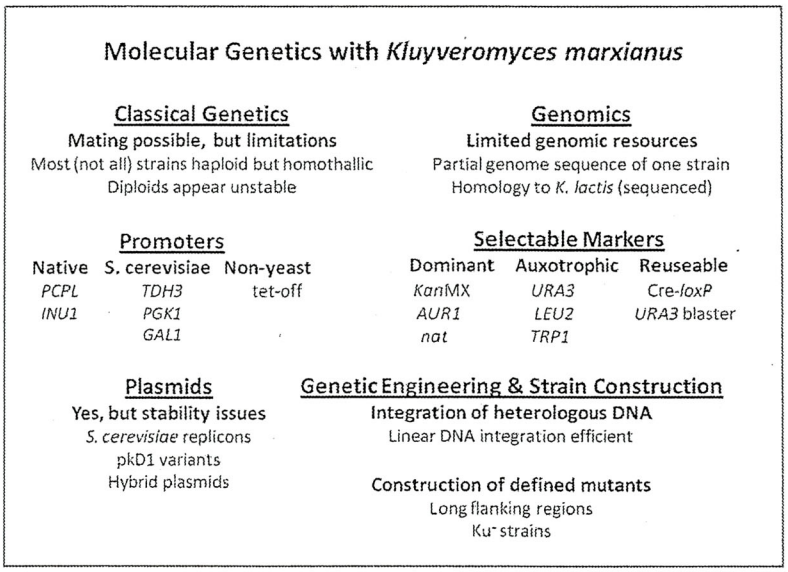
*1.2.4.3 Industrial exploitation*

The development of biotechnological applications with *K. marxianus* has been motivated by a number of it advantages including a broader variety of substrates and at higher temperatures, its higher specific growth rates (Fonseca et al. 2008). The underlying interest in *K. marxianus* is undoubtedly driven by applications in the biotechnology industry. Commercially, the most important current application is production of native enzymes, such as inulinase, β-galactosidases and pectinases (Lane and Morrissey 2010). Inulinase, which hydrolyses a plant fructan called inulin, is of particular interest, as this enzyme is not commonly found in other yeasts or fungi. Its expression is induced by inulin or sucrose, and the enzyme can be excreted to the culture medium or remain associated to the cell wall (Rouwenhorst et al. 1988). The β-galactosidase activity of *K. marxianus* has been exploited for some time, where the yeast was used to treat lactose-containing waste from the cheese industry (Lane and Morrissey 2010). The biomass is used as animal feed or converted to extract and used in the food processing industry. Recently *K. marxianus*, a thermotolerant yeast, has been received much attention for ethanol production at high temperatures (Fonseca et al. 2008). Besides, the treatment of dairy waste streams, the biosorption and bioaccumulative properties of *K. marxianus* are utilized in bioremediation of textile dyes and copper from wastewaters, and the yeast is used to treat waste and paper sludge.

*1.2.4.4 Molecular and genetic tools*

In spite of traditional biotechnology gain a lot of knowledge to improve processes and to identify and select more efficient strains, molecular and genetic approaches are required for understanding of microorganism and achieving of industrial process. Although molecular research with *K. marxianus* lags behind *K. lactis*, tools are now becoming available that will facilitate strain engineering. The current status of these tools is illustrated in Fig 1.6 (for more detail, see in Lane and Morrissey 2010). One major limitation is the lack of comprehensive genome sequence information. There is only availability of partial genome sequence from

strain CBS 712 (Llorente et al. 2000) and comparison to the completed *K. lactis* genome sequence (Dujon et al. 2004).




From: Lane and Morrissey, 2010

**Fig. 1.6—Molecular tools and resources for *K. marxianus*.** A summary is presented of the status of the key molecular reagents that are available, or required, to facilitate molecular genetics and strain improvement in *K. marxianus*.

Transformation of foreign DNA into *K. marxianus* can be achieved by electroporation or by LiAc procedures using protocols adapted from *S. cerevisiae* and *K. lactis* (Iborra 1993; Zhang et al. 2003). To obtain a stable strain for making mutants lacking a specific gene or introducing a new trait into a strain, integration of DNA fragments into the chromosome is required. Integration of a DNA fragment into the genome exploits the DNA repair processes in the cell and can be random or targeted (Aylon and Kupiec 2004; Daley et al. 2005). The following of introduction of linear DNA fragment flanked by homology sequences to a region of genomic DNA, the process of homologous recombination can direct allele replacement and introduction of the new DNA fragment into that specific genomic locus. Another mechanism known as non-homologous end joining (NHEJ), which mediated by the Ku70/Ku80 heterodimer, responses for random integration of DNA into the genome. The frequency at which any species or strain integrates DNA into a specific target

site is largely determined by the relative efficiency of the HR and NHEJ systems, which compete for binding to free DNA ends (Lane and Morrissey 2010).

In contrast to *S. cerevisiae*, which HR is highly effective and efficiencies of close to 100 % (Baudin et al. 1993), the efficiency in *K. lactis* and *K. marxianus* can be extremely low. To overcome this, screening of large number of candidate transformants or improvements in gene-targeting efficiency are requires. There are a number of strategies that can be taken to improve gene-targeting efficiencies (Kooistra et al. 2004) such as increasing the length of homologous flanking sequence, manipulating the concentration of DNA, or adding of salmon sperm carrier DNA. The most striking results, however, were obtained when NHEJ was inactivated by deletion of the *KU80* gene (Lane and Morrissey 2010). While, autonomously replicating plasmids for *Kluyveromyces* are generally quite limited.

The dominant markers such as *kan*MX, *AUR1* or *nat*, for resistance to G418, aureobasidin A and nurseothricin, respectively, can be used a selectable marker (Hashida-Okado et al. 1998; Ribeiro et al. 2007). Additionally, auxotrophic strains of different *K. marxianus* can be created e.g. auxotrophs strains for uracil, leucine, tryptophan, lysine and adenine. Multiple selectable markers or reusable marker, such as URA3 blaster and Cre-*loxP*, is required for constructing a sequential gene knockouts or complement mutant.

Using a variety of promoters for heterologous gene expression has performed in *K. marxianus*. These are the promoters from *S. cerevisiae* such as *PGK1* (Ball et al. 1999; Pecota et al. 2007), *TDH3* (Nonklang et al. 2008) or *GAL1* (Almeida et al. 2003), native *K. marxianus* such as inulinase promoter *INU1* (Bergkamp et al. 1993), and non-yeast promoter system that is of interest is the tetracycline promoter, which was shown to give very good on/off regulation (no expression occur in the presence of tetracycline) in *S. cerevisiae* (Gari et al. 1997) and *K. marxianus* (Pecota and Da Silva 2005).

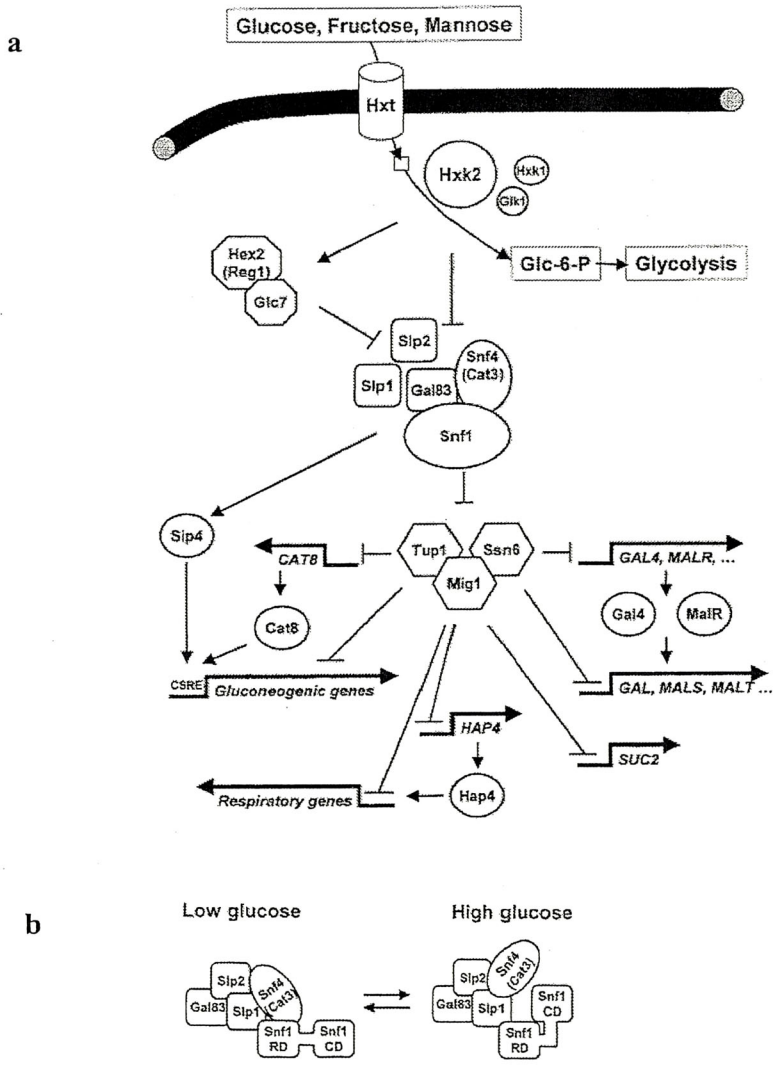
### 1.2.5 Glucose repression

Glucose is considered the preferred carbon source of yeast, because the presence of this hexose inhibits the utilization of other carbon sources. Yeast

exercises this preference in part by repressing the transcription of genes required for the utilization of the alternative carbon sources. Target genes of this glucose repression pathway include the *SUC* genes encoding invertase, *GAL* and *MAL* genes involved in utilization of galactose and maltose, and the nonfermentable carbon sources (encoding proteins involved in mitochondrial biogenesis, respiration, the citric acid cycle, etc.) are also repressed by glucose. Other fermentable carbon sources (e.g. fructose, galactose, and maltose) can exert repressive effects on members of this set of target genes as well, though they are generally less potent than glucose.

The canonical glucose repression pathway in *S. cerevisiae* involves binding of transcriptional repressors to the promoters of affected genes; these repressors, the Mig1 (Nehlin and Ronne 1990 ) and Mig2 (Luftiyya and Johnston 1996) zinc-finger proteins, require the Ssn6 and Tup1 corepressors for activity (Keleher et al. 1992; Vallier and Carlson 1994). As illustrated in Fig. 1.7a, its central components are the Mig1 transcriptional repressor complex, the Snf1-protein kinase complex and protein phosphatase 1. Mig1 is localized in the nucleus in glucose-grown cells, and upon removal of glucose it is rapidly phosphorylated and translocated from the nucleus to the cytoplasm (De Vit et al. 1997). Derepression requires the Snf1 protein kinase complex (Carlson et al. 1981, 1999). This heterotrimeric complex includes the Snf1 catalytic subunit, which has a catalytic domain and an autoregulatory domain. Activation of the kinase in response to glucose limitation is apparently accompanied by a conformational change of the kinase complex (Jiang and Carlson 1996) (Fig. 1.7b). The other components of the complex are a regulatory subunit (Snf4) involved in responding to glucose, and an oligomerization factor (Sip1, Sip2, or Gal83) that influences the target specificity of the complex (Schmidt and McCartney 2000). The Snf1 kinase phosphorylates Mig1 (Treitel et al. 1998; Smith et al. 1999), resulting in its dissociation from promoter DNA and thus permitting transcription of target genes. The activity of the Snf1 kinase is regulated by glucose; in the presence of the hexose the catalytic domain is inactive due to intramolecular interaction with the autoregulatory domain. As glucose concentrations decline, the Snf4 protein liberates the Snf1 catalytic domain, which is then able to phosphorylate Mig1. The process is reversed by the Glc7-Reg1

protein phosphatase, which restores the inactive conformation of the Snf1 catalytic and autoregulatory domains at high glucose concentrations.




From: Rolland et al., 2002

**Fig. 1.7—The main glucose-repression pathway.** **a** Simplified schematic representation of mediators and targets of the main catabolite-repression pathway. Repression is exerted by the complex Mig1/Ssn6/Tup1 on different gene families including family-specific transcriptional activators such as Gal4 (galactose utilization), MalR (maltose utilization), Hap4 (respiratory genes) and Cat8 (gluconeogenic genes). The Snf1 kinase associated with one of the regulatory subunits Sip1, Sip2 or Gal83 and the activating subunit Snf4 has a negative effect on the activity of the repression complex. During growth on glucose, Snf1 activity is inhibited by different upstream regulators which include the hexose kinases and the Glc7 phosphatase. The Snf1 kinase complex is also required for activation of Sip4 which is required in concert with Cat8 for the derepression of the gluconeogenic genes. **b** Glucose-induced conformational change of the Snf1-protein kinase complex.

In this study, I focused on the capability of *K. marxianus* DMKU3-1042 in utilization and fermentation of various sugars including mono-, di-, tri-, and poly-saccharides presented in various feedstocks such as sugar cane, molasses, lignocellulose and inulin-rich plants, which are valuable sources for bioethanol under a high temperature. Feedstrocks consist of mixed sugars together with glucose, which causes the glucose effect (glucose repression) that obstructs the utilization of other carbon sources. Thus, glucose effect on assimilation of other sugars in *K. marxianus* was examined not only by the addition of glucose but also by the addition of 2-deoxyglucose. For further consideration of the glucose effect, the expression of genes responsible for utilization of other sugars such as *HXK2, GAL1*, *XYL1* and *INU1* was analyzed. Glucose effect on the production and secretion of inulinase was also examined.

# CHAPTER 2

## Growth and Ethanol Fermentation Ability on Hexose and Pentose Sugars and Glucose Effect under Various Conditions in Thermotolerant Yeast *Kluyveromyces marxianus*

## 2.1 Abstract

Ethanol fermentation ability of the thermotolerant yeast *Kluyveromyces marxianus*, which is able to utilize various sugars including glucose, mannose, galactose, xylose, and arabinose, was examined under shaking and static conditions at high temperatures. The yeast was found to produce ethanol from all of these sugars except for arabinose under a shaking condition but only from hexose sugars under a static condition. Growth and sugar utilization rate under a static condition were slower than those under a shaking condition, but maximum ethanol yield was slightly higher. Even at 40°C, a level of ethanol production similar to that at 30°C was observed except for galactose under a static condition. Glucose repression on utilization of other sugars was observed, and it was more evident at elevated temperatures. Consistent results were obtained by the addition of 2-deoxyglucose. The glucose effect was further examined at a transcription level, and it was found that *KmGAL1* for galactokinase and *KmXYL1* for xylose reductase for galactose and xylose/arabinose utilization, respectively, were repressed by glucose at low and high temperatures, but *KmHXK2* for hexokinase was not repressed. We discuss the possible mechanism of glucose repression and the potential for utilization of *K. marxianus* in high-temperature fermentation with mixed sugars containing glucose.

## 2.2 Introduction

*Kluyveromyces marxianus* is a close relative of *Kluyveromyces lactis*, a model Crabtree-negative yeast that has been extensively investigated (Gonzalez-Siso et al. 2000, 2009; Schaffrath and Breunig 2000; van Ooyen et al. 2006). The major common feature of *K. lactis* and *K. marxianus* is the capacity to assimilate lactose as a carbon source, a feature that is absent in *Saccharomyces cerevisiae*. In contrast to *K. lactis*, there is no complete genome sequence, no type strain adopted as a reference for basic research purposes, and little accumulation of information for *K. marxianus*, though some heterologous protein production systems in the yeast become available (Lane and Morrissey 2010). Nevertheless, *K. marxianus* has a number of advantages over *K. lactis* or *S. cerevisiae*, including a broad substrate spectrum, thermotolerance, and high growth rate (Fonseca et al. 2008), and is thus an alternative to *S. cerevisiae* as an ethanol producer.

Because of the increasing concern regarding petroleum costs and global warming, worldwide has been shown the production of bioethanol as a renewable energy source, especially from renewable resources (Hahn-Hagerdal et al. 2006). Nowadays, the most commonly utilized renewable fuel is ethanol mainly from sugar cane and starch, and thus, the consumption of these biomasses has dramatically increased. The utilization of cornstarch for bioethanol production may cause a serious problem in competition with cornstarch as food. In the near future, it is expected that a source for low-cost ethanol production will be lignocellulosic biomass such as agricultural and forestry residues. Lignocellulosic materials are composed of three main polymers: cellulose (~47% of dry weight), hemicelluloses (~30% of dry weight), and lignin (~25% of dry weight; (Wiselogel et al. 1996; Aristidou and Penttila 2000). Cellulose is a homopolymer of glucose (Glu), while hemicellulose is composed of hexose sugars—glucose, mannose (Man), and galactose (Gal), and pentose sugars—xylose (Xyl) and arabinose (Ara; Saha 2003). Hemicellulose is easily hydrolyzed to its constituent monosaccharides compared to cellulose (Aristidou and Penttila 2000; Zaldivar et al. 2001; Perez et al. 2002).

Considering sugar constituents in hemicellulose, the development of microbes that can utilize and convert a variety of sugars into ethanol is required. Besides, thermotolerant microbes applicable for high-temperature fermentation that efficiently produce ethanol at elevated temperatures are expected to have a potential for reducing cooling costs (Banat et al. 1998). High-temperature fermentation also has advantages of efficient simultaneous saccharification and fermentation, a continuous shift from fermentation to distillation, reducing risk of contamination, and suitability for application in tropical countries (Anderson et al. 1986; Banat et al. 1998; Limtong et al. 2007) Additionally, glucose effect that reduces synthesis of enzymes for catabolism of alternative carbon sources should be considered. A significant reduction of a large number of cellular functions as the glucose effect has been reported in *S. cerevisiae* (Gancedo 1998). The regulatory mechanism for D-galactose utilization is at least partially conserved between *K. lactis* and *S. cerevisiae*, though the molecular interactions between regulators are different (Rubio-Texeira 2005; Venkat et al. 2010). Several glucose-repressed genes are known to be controlled by Mig1p, a transcription factor involved in glucose repression, directly or indirectly via repression of a transcription activator (Gancedo 1998). On the other hand, the regulation of genes for a D-xylose-inducible pathway, including an NAD(P)H-linked xylose reductase (Xyl1p) and an NAD-linked xylitol dehydrogenase (Xyl2p), and for an L-arabinose-inducible pathway partially overlapping with the D-xylose-inducible pathway (Fig. 2.1) has not been elucidated in yeast.

*K. marxianus* DMKU3-1042 is a microorganism that possesses the capability for assimilation of many different kinds of sugars at high temperatures (Nonklang et al. 2008). Nevertheless, there are no reports on glucose repression at a high temperature in *K. marxianus*.

In this study, the potential of *K. marxianus* DMKU3-1042 for utilization of and ethanol production from sugars present in hemicellulose hydrolysate was for the first time examined under shaking and static conditions at high temperatures. This strain can grow and produce ethanol at a high temperature (Limtong et al. 2007) and is the most thermotolerant among strains available (Nonklang et al. 2008; Abdel-Banat et al. 2010). We therefore consider this strain to be a suitable candidate

for high-temperature ethanol fermentation. Glucose repression on assimilation of other sugars was also tested not only by the addition of glucose but also by the addition of 2-deoxyglucose (2-DOG). To further consider the repression, the expression of genes responsible for utilization of mannose, galactose, xylose, or arabinose was analyzed. This is the first study to reveal that the extent of glucose repression is different at different temperatures and on different sugars in *K. marxianus*. This study also provides valuable information for application of *K. marxianus* and indicates its usefulness for high-temperature ethanol fermentation.

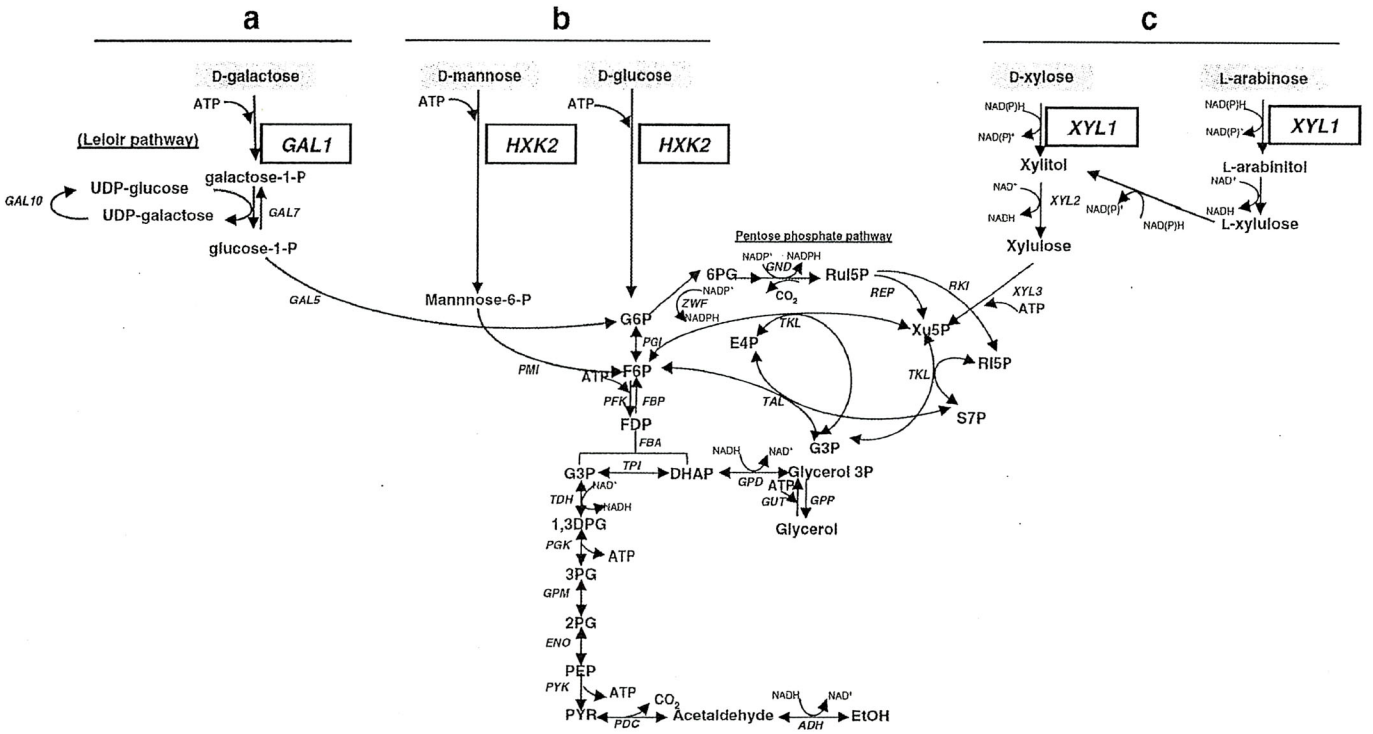



**Fig. 2.1—Schematic diagram representing sugar metabolisms to ethanol in yeast and selected genes, *GAL1*, *HXK2*, and *XYL1* for expression analysis**. Three genes were involved in galactose, mannose/glucose, and xylose/arabinose catabolic pathways, respectively. **a** Galactose was converted to galactose-1-phosphate by galactokinase encoded by *GAL1* and then subsequently converted to glucose-6-phosphate via the Leloir pathway, which consists of four catalytic steps. **b** After glucose or mannose phosphorylation by hexokinase encoded by *HXK2*, glucose was converted to be glucose-6-phosphate, an intermediate in glycolysis, whereas mannose was converted to be mannose-6-phosphate and then was isomerized to fructose-6-phosphate by phosphomannose isomerase. **c** D-xylose and L-arabinose metabolisms in yeast share a partial overlap between their catabolic pathways. Xylose metabolism proceeds mostly via two oxidative–reductive reactions. Xylose is firstly reduced to xylitol by an NAD(P)H-linked xylose reductase encoded by *XYL1*, followed by xylitol oxidation to xylulose by an $NAD^+$-linked xylitol dehydrogenase encoded by *XYL2*. Arabinose is also converted to L-arabinitol by *XYL1*. This metabolism pathway is quite similar to that of the xylose metabolism and consists of two $NAD^+$-linked oxidative reactions and two NADPH-linked reductive reactions.

## 2.3 Materials and methods

### 2.3.1 Strain and media

The yeast strain used in this study was *K. marxianus* DMKU3-1042 strain, one of the isolates from soil and water samples obtained around sugar cane plantations and sugar factories in Thailand (Limtong et al. 2007). Culture was carried out in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) as used for preparation of the inoculum. Different carbon sources were used, singly or in combination with 20 g/l of glucose. For investigating the utilization of an individual sugar, YP medium (10 g/l yeast extract and 20 g/l peptone) supplemented with 20 g/l of Glc, Man, Gal, Xyl, or Ara was used. These media were designated as YPD, YPMan, YPGal, YPXyl, and YPAra, respectively. The YP medium with 20 g/l glucose in combination with 20 g/l of Man, Gal, Xyl, or Ara was used for experiments with mixed sugars that were named YPDMan, YPDGal, YPDXyl, and YPDAra, respectively. To examine the effect of antimycin A, 5 μM antimycin A was added.

### 2.3.2 Cultivation conditions

For the inoculum preparation, the yeast strain was cultivated in 20 ml of YPD medium in a 100-ml Erlenmeyer flask at 30°C with orbital shaking at 160 rpm for 18 h. The preculture was inoculated into a 300-ml Erlenmeyer flask containing 100 ml fresh medium of YP containing Glc, Man, Gal, Xyl, or Ara or containing Glc combined with others sugars at initial $OD_{660}$ values of 0.1 and 1 for shaking and static conditions, respectively. Incubation conditions for the shaking condition were standardized on the rotary shaker with 160 rpm at 30°C, 40°C, and 45°C, whereas for the static condition, flasks were constantly stood at 30°C and 45°C.

### 2.3.3 Analytical methods

Cell density was measured turbidimetrically at 660 nm. To determine sugar and ethanol concentrations in culture media, cultures were sampled and subjected to a low-speed centrifugation. The supernatant was frozen and kept at -20°C until analyzed. Quantitative analysis of sugar and ethanol was performed by high-performance liquid chromatography (Hitachi, Japan). A Gel pack column GL-C610-S (Hitachi, Japan) was used together with a refractive index detector (Model L-2490, Hitachi) at 60°C with 0.3 ml/min eluent of deionized water.

### 2.3.4 Analysis of glucose repression by using glucose analog 2-DOG

Cells grown in YPD medium for 18 h were collected. After washing the cells with deionized water, the suspended cells ($1\times10^7$cells/ml) were 10-fold sequentially diluted and then spotted onto agar plates of YPMan, YPGal, YPXyl, and YPAra with or without 0.01% 2-DOG. YPD plates were used as a control. These plates were incubated at 30°C, 40°C, and 45°C for 48 h.

### 2.3.5 RT-PCR analysis

Total RNA from cells, which had been grown in various media as described below, was isolated by the hot phenol method (Aiba et al. 1981). Isolated RNA was treated with RNase-free DNase I (Qiagen, Japan) for 15 min, and then DNase I was inactivated at 75°C for 10 min. For each experimental condition, the three genes *KmHXK2*, *KmGAL1*, and *KmXYL1* for hexokinase, galactokinase, and xylose reductase, respectively, were used to determine the effect of glucose repression. *KmACT1* for actin was used as an internal control. Cells were first grown in YPD medium until the exponential phase at 30°C, washed two times and resuspended with YP medium, and subsequently inoculated at 5% into YPD, YPMan, YPGal, YPXyl, or YPAra for a single sugar condition or into YPDMan,

YPDGal, YPDXyl, or YPDAra for a mixed sugars condition and further incubated for 6 h (mid-exponential phase) at 30°C or 45°C. The concentration of RNA was estimated spectrophotometrically at 260 nm. RT-PCR analysis was performed using an mRNA Selective RT-PCR kit (Takara, Japan) with 0.1 μg of total RNA as a template and the primer sets for RT-PCR (Table 2.1). For detection of an internal control, 0.01 μg of total RNA was used as a template. RT-PCR was performed on a Takara PCR thermal cycler MP (Takara Biomedicals, Japan). After RT reaction had been performed at 40°C for 15 min, PCR consisting of denaturing at 82°C for 1 min, annealing for 1 min at a fixed temperature, 5 degrees lower than $T_m$, which was calculated by the rule of thumb method, and extension at 72°C for 1 min was carried out using two primers for each gene. The PCR products after 20, 25, 30, and 35 cycles or 20, 23, 26, 29, and 32 cycles for each gene were analyzed by 0.9% agarose gel electrophoresis. Intensity of bands of RT-PCR products was quantitatively determined using the UN-SCAN-IT gel™ automated digitizing system (Silk Scientific, USA). Under our conditions, the RNA-selective RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

**Table 2.1** Primers used for RT-PCR in this study

| Name | Gene | Sequence 5′→3′ | Length (bp) |
|---|---|---|---|
| RT-KmHXK2-F | *KmHXK2* | AAGAAGCCACCAGCCAGA | 542 |
| RT-KmHXK2-R | | ACATCGTGGCCTTCGACA | |
| RT-KmGAL1-F | *KmGAL1* | AGGTCGCCAGGTAGAGTG | 529 |
| RT-KmGAL1-R | | CGTCCTTACCGCAGATAG | |
| RT-KmXYL1-F | *KmXYL1* | CTCGCACCAACAGTTACC | 571 |
| RT-KmXYL1-R | | AGGCGACTGGCTTGATAC | |
| RT-KmACT1-F | *KmACT1* | ACGTTGTTCCAATCTACGCC | 491 |
| RT-KmACT1-R | | AGAAGATGGAGCCAAAGCAG | |

## 2.4 Results

### 2.4.1 Cell growth and ethanol production in YP medium containing a single sugar under a shaking condition at different temperatures

To examine the potential for utilization of sugars found in hemicellulose hydrolysate in *K. marxianus* DMKU3-1042, cells were grown in a medium containing Glc, Man, Gal, Xyl, or Ara under a shaking condition at different temperatures, and cell growth and concentrations of sugar and ethanol in the medium were monitored (Fig. 2.2; Table 2.2). Cell growth was observed as sugar reduced on all sugars tested at 30°C, 40°C, and 45°C, indicating that the strain can use these sugars for growth at high temperatures. In YPD, YPMan, and YPGal, ethanol was increased roughly in anti-parallel with decrease in sugar at the beginning of cultivation. The growth was gradually decreased with increase in temperature, but ethanol accumulation was nearly the same except for a delay at 45°C. In YPXyl, ethanol and xylitol were accumulated. No ethanol accumulation, however, was detected in YPAra. YPXyl and YPAra showed a longer lag phase and a lower maximum growth rates.

At 30°C, Glc, Man, and Gal were completely consumed at 12 h, while Xyl and Ara utilization was completed at 72 and 96 h, respectively. The maximum ethanol yield was the highest in YPD and YPGal followed by that in YPMan. In YPXyl, the largest amount of ethanol production was 2.5 g/l at 72 h, and xylitol production was 4.3 g/l at 48 h. The maximum ethanol yield in YPXyl was 3.6 times lower than those in YPD and YPGal. The maximum sugar utilization rate and the maximum growth rate were the highest in YPGal, and the specific growth rate and the specific sugar utilization rate at 6 h were the highest in YPD.

At 40°C, Glc, Man, and Gal utilization was nearly completed at 12 h, whereas Xyl was completely consumed within 72 h. Ara was not completely utilized within 96 h. The amount of ethanol production was the largest at 12 h in YPD, YPMan, and YPGal. In YPXyl, xylitol accumulation was 7.0 g/l, which was higher than the amounts at 30°C and 45°C. The maximum ethanol yield was highest

in YPGal, which was higher than those at 30°C and 45°C. The specific growth and sugar utilization rates at 6 h were slightly lower than those at 30°C in YPMan and YPGal.

At 45°C, Glc and Man were completely utilized at 24 h, whereas Gal was completely utilized at 48 h. Xyl and Ara were not completely utilized within 96 h. Interestingly, the ethanol level in YPGal was stable until the end of fermentation, whereas the level in other media at all temperatures was gradually reduced with incubation. The specific growth rate and sugar utilization speed were greatly reduced in YPD, YPMan, and YPGal compared to those at 30°C or 40°C.

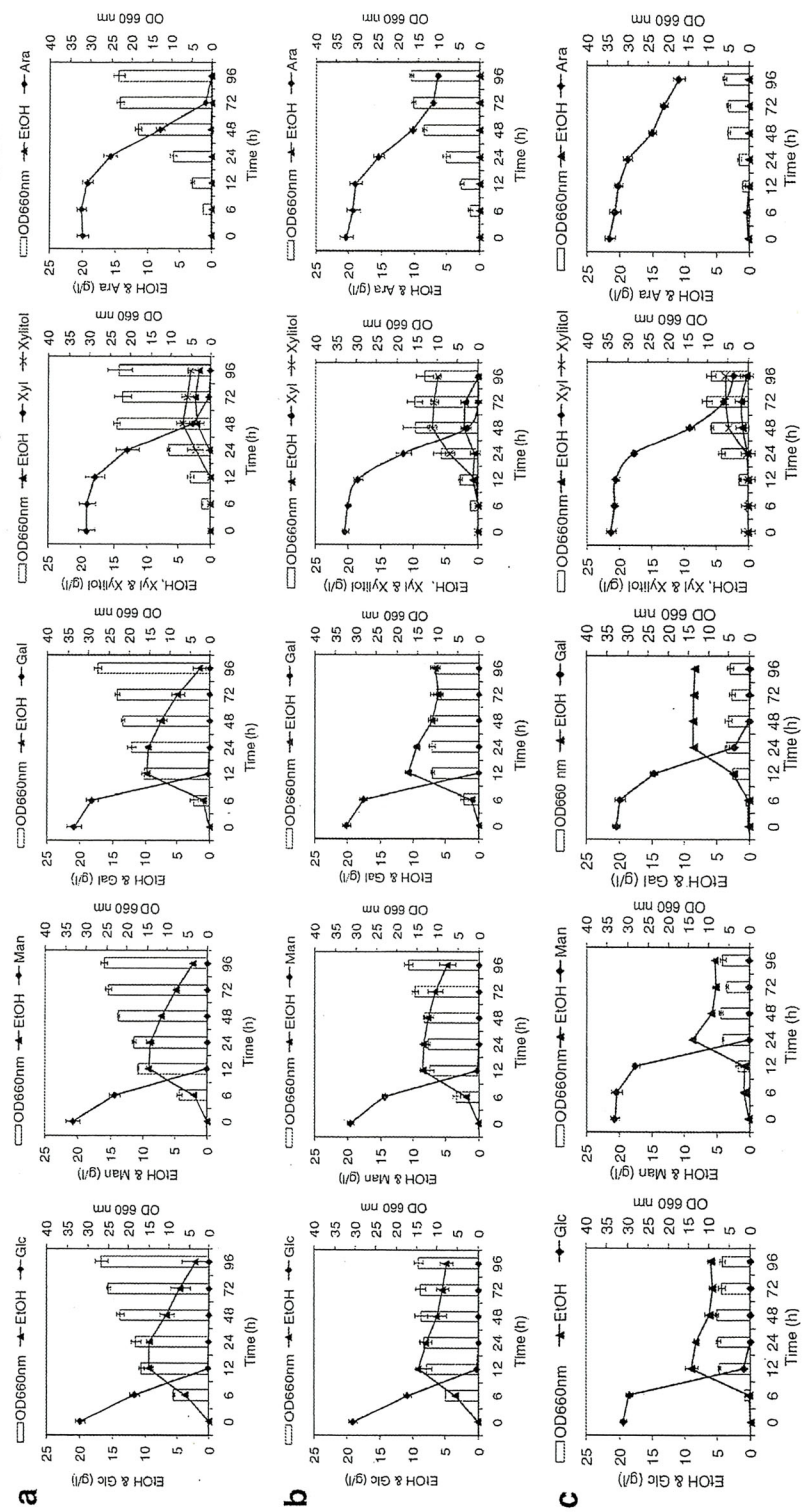



**Fig. 2.2**—Growth and metabolite profiles of *K. marxianus* DMKU3-1042 grown in YP medium containing 20 g/l of glucose (YPD), mannose (YPMan), galactose (YPGal), xylose (YPXyl), or arabinose (YPAra) at 30°C (**a**), 40°C (**b**), and 45°C (**c**) under a shaking condition (160 rpm). Initial $OD_{660}$ was adjusted to 0.1. *Bars* represent the ±SD for three independent experiments.

**Table 2.2** Parameters in YP medium containing a single sugar under a shaking condition (160 rpm) at various temperatures

| Medium | Temperature (°C) | Max. $Y_{p/s}$ (g/g) | Time of fermentation (h)[a] | $\mu_{x/s}$ ($h^{-1}$) at 6 h | Max. $\mu_{x/s}$ ($h^{-1}$) | $\gamma_s$ (g/l h) at 6 h | Max. $\gamma_s$ (g/l h) |
|---|---|---|---|---|---|---|---|
| YPD | 30 | 0.47±0.02 | 12 | 1.5±0.06 | 1.5 (6)±0.06 | 1.4±0.02 | 1.9 (12)±0.12 |
| | 40 | 0.48±0.03 | 12 | 1.3±0.01 | 1.3 (6)±0.01 | 1.4±0.02 | 1.8 (12)±0.11 |
| | 45 | 0.48±0.07 | 12 | 0.20±0.02 | 1.0 (12)±0.02 | 0.15±0.03 | 2.9 (12)±0.12 |
| YPMan | 30 | 0.44±0.01 | 12 | 1.2±0.10 | 1.7 (12)±0.11 | 1.1±0.06 | 2.4 (12)±0.17 |
| | 40 | 0.44±0.01 | 12 | 0.87±0.16 | 1.1 (12)±0.15 | 0.86±0.02 | 2.4 (12)±0.06 |
| | 45 | 0.42±0.01 | 24 | 0.03±0.02 | 0.42 (12)±0.09 | 0.05±0.14 | 1.5 (24)±0.05 |
| YPGal | 30 | 0.47±0.05 | 12 | 0.66±0.17 | 2.1 (12)±0.04 | 0.46±0.03 | 3.0 (12)±0.28 |
| | 40 | 0.53±0.01 | 12 | 0.57±0.16 | 1.3 (12)±0.14 | 0.43±0.01 | 2.9 (12)±0.09 |
| | 45 | 0.42±0.01 | 48 | 0.15±0.01 | 0.51 (12)±0.09 | 0.09±0.19 | 1.0 (24)±0.05 |
| YPXyl | 30 | 0.13±0.02 | 72 | 0.34±0.02 | 1.1 (48)±0.05 | 0.01±0.01 | 0.84 (48)±0.04 |
| | 40 | 0.11±0.01 | 48 | 0.29±0.01 | 0.54 (48)±0.09 | 0.08±0.03 | 0.82 (48)±0.04 |
| | 45 | 0.06±0.01 | 72 | 0.07±0.00 | 0.37 (24)±0.06 | 0.09±0.08 | 0.72 (48)±0.05 |
| YPAra | 30 | – | – | 0.34±0.02 | 0.84 (48)±0.05 | 0.00±0.01 | 0.65 (48)±0.04 |
| | 40 | – | – | 0.34±0.09 | 0.46 (48)±0.04 | 0.17±0.01 | 0.44 (48)±0.02 |
| | 45 | – | – | 0.08±0.00 | 0.22 (48)±0.01 | 0.13±0.02 | 0.31 (48)±0.01 |

Values in parenthesis represent cultivation times

*Max.* $Y_{p/s}$ maximum ethanol yield, $\mu_{x/s}$ specific growth rate, *Max.* $\mu_{x/s}$ maximum growth rate, $\gamma_s$ specific sugar utilization rate, *Max.* $\gamma_s$ maximum sugar utilization rate, ± SD from three independent experiments

[a] Time required for the maximum ethanol concentration to be reached

### 2.4.2 Cell growth and ethanol production in YP medium containing mixed sugars with Glc under a shaking condition at different temperatures

There have been a number of extensive studies on glucose repression in *S. cerevisiae* and *K. lactis*, which were mainly focused on sucrose, lactose, or galactose utilization (Dong and Dickson 1997; Gancedo 1998; Venkat et al. 2010). However, little is known about the glucose effect on Xyl or Ara utilization. We thus performed experiments to determine the effect of glucose on utilization of other sugars at different temperatures (Fig. 2.3; Table 2.3).

Utilization patterns of Glc in mixed sugars were similar to those in a single sugar of Glc at 30°C and 40°C, but utilization was delayed at 45°C. In YPDGal, YPDXyl, and YPDAra, Glc was firstly consumed at 30°C and 40°C within 12 h and within 24 h at 45°C, whereas in YPDMan, Glc was simultaneously utilized with Man and both sugars were used up until 12 h at 30°C and 40°C and until 24 h at 45°C.

At 30°C, Xyl and Ara utilization in YPDXyl and YPDAra started after 24 and 12 h, respectively, when Glc utilization was finished, but their consumption was not completed within 96 h, although both sugars were completely used within 96 h in experiments with a single sugar (Fig. 2.2a). Gal in YPDGal was utilized in parallel with Glc, but a slight delay of Gal utilization was observed compared to that in a single sugar. In YPDXyl, xylitol was accumulated as Xyl was utilized. Interestingly, the ethanol level in YPDXyl was maintained during the cultivation, whereas in combinations with other sugars, the ethanol level was gradually reduced after reaching the maximum level. This indicates that Xyl is preferable as a second sugar for ethanol production. The maximum growth rate was the highest in YPDMan.

At 40°C, Glc was utilized in a pattern similar to that at 30°C in all cases. Growth and maximum growth rate were reduced in YPDMan and YPDGal compared to those at 30°C. Xyl and Ara utilization speeds from 12 to 96 h were slower than those at 30°C, and both sugars largely remained even after 96 h. The ethanol production level in all cases was the highest at 12 h except for that in

YPDGal (24 h). Interestingly, the reduction rate of ethanol at 40°C was slightly lower than that at 30°C. The maximum ethanol yields in YPDMan and YPDGal were 0.50 and 0.49, respectively.

At 45°C, cells hardly utilized Xyl or Ara in the presence of Glc but could utilize Xyl or Ara when they were added as a single sugar (Fig. 2.2c). It thus seems that ethanol in YPDXyl or YPDAra comes only from Glc. Gal utilization in YPDGal started at 12 h and stopped at 48 h. After 48 h, Gal concentration was constant until the end of the fermentation process, and some amount of ethanol was consumed after 72 h. The utilization of Gal may somehow be limited at this temperature when Glc coexists.

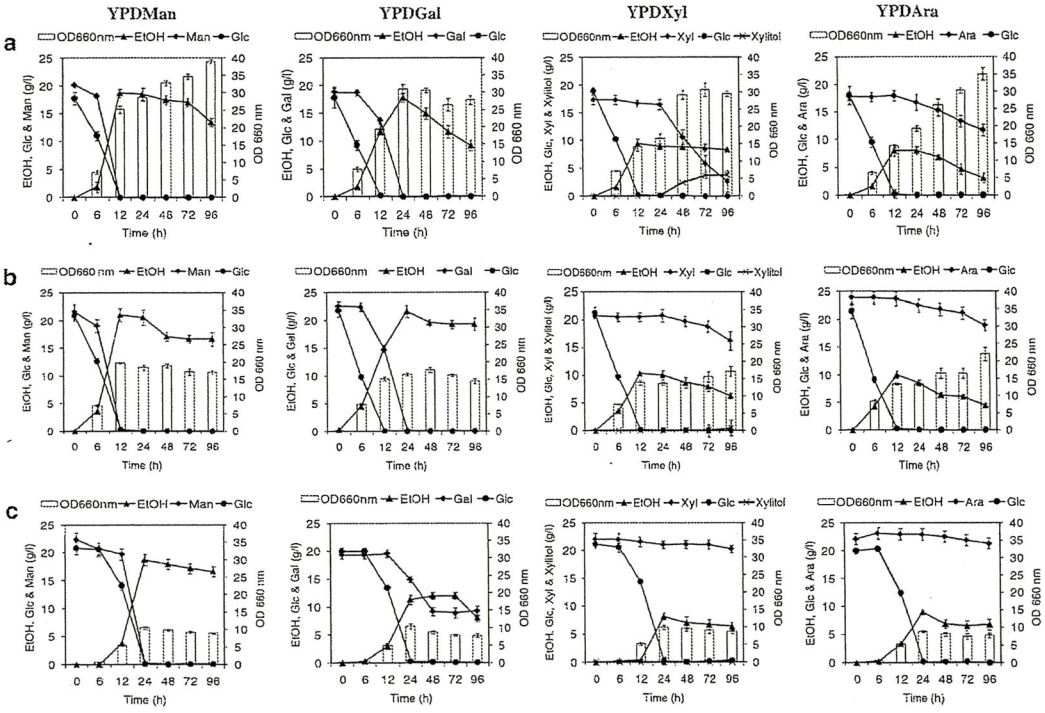



**Fig. 2.3**—Growth and metabolite profiles of *K. marxianus* DMKU3-1042 grown in YP medium containing 20 g/l glucose with 20 g/l of mannose (YPDMan), galactose (YPDGal), xylose (YPDXyl), or arabinose (YPDAra) at 30°C (**a**), 40°C (**b**), and 45°C (**c**) under a shaking condition (160 rpm). Initial $OD_{660}$ was adjusted to 0.1. *Bars* represent the ±SD for three independent experiments.

**Table 2.3** Parameters in YP medium containing mixed sugars with Glc under a shaking condition (160 rpm) at various temperatures

| Medium | Temperature (°C) | Max. $Y_{p/s}$ (g/g) | Time of fermentation (h)[a] | $\mu_{x/s}$ ($h^{-1}$) at 6 h | Max. $\mu_{x/s}$ ($h^{-1}$) | $\gamma_s$ (g/l h) at 6 h | | Max. $\gamma_s$ (g/l h) | |
|---|---|---|---|---|---|---|---|---|---|
| YPDMan | 30 | 0.49±0.02 | 12 | 1.2±0.09 | 3.0 (12)±0.15 | Glc | 1.1±0.09 | Glc | 1.8 (12)±0.12 |
| | | | | | | Man | 0.33±0.12 | Man | 3.0 (12)±0.06 |
| | 40 | 0.50±0.01 | 12 | 1.2±0.05 | 2.1 (12)±0.06 | Glc | 1.4±0.07 | Glc | 2.1 (12)±0.10 |
| | | | | | | Man | 0.43±0.03 | Man | 3.1 (12)±0.20 |
| | 45 | 0.44±0.01 | 24 | 0.09±0.01 | 0.80 (12)±0.04 | Glc | 0.04±0.05 | Glc | 1.2 (24)±0.06 |
| | | | | | | Man | 0.28±0.01 | Man | 1.6 (24)±0.09 |
| YPDGal | 30 | 0.49±0.02 | 24 | 1.3±0.12 | 1.9 (12)±0.26 | Glc | 1.4±0.14 | Glc | 1.5 (12)±0.15 |
| | | | | | | Gal | 0.01±0.07 | Gal | 1.1 (24)±0.02 |
| | 40 | 0.49±0.01 | 24 | 1.3±0.05 | 1.3 (6)±0.05 | Glc | 2.0±0.10 | Glc | 2.0 (6)±0.10 |
| | | | | | | Gal | 0.03±0.01 | Gal | 1.3 (12)±0.05 |
| | 45 | 0.40±0.01 | 48 | 0.08±0.01 | 0.72 (12)±0.02 | Glc | 0.00±0.01 | Glc | 1.1 (24)±0.03 |
| | | | | | | Gal | 0.01±0.01 | Gal | 0.48 (48)±0.02 |
| YPDXyl | 30 | 0.49±0.03 | 12 | 1.2±0.03 | 1.2 (6)±0.03 | Glc | 1.5±0.05 | Glc | 1.7 (12)±0.07 |
| | | | | | | Xyl | 0.00±0.01 | Xyl | 0.49 (48)±0.12 |
| | 40 | 0.49±0.02 | 12 | 1.3±0.02 | 1.3 (6)±0.02 | Glc | 1.9±0.09 | Glc | 1.9 (6)±0.09 |
| | | | | | | Xyl | 0.03±0.08 | Xyl | 0.22 (96)±0.08 |
| | 45 | 0.37±0.02 | 24 | 0.06±0.01 | 0.79 (12)±0.07 | Glc | 0.10±0.06 | Glc | 1.2 (24)±0.11 |
| | | | | | | Xyl | 0.01±0.01 | Xyl | 0.07 (12)±0.04 |
| YPDAra | 30 | 0.45±0.04 | 12 | 1.1±0.08 | 1.3 (12)±0.08 | Glc | 1.4±0.13 | Glc | 1.6 (12)±0.13 |
| | | | | | | Ara | 0.02±0.01 | Ara | 0.16 (72)±0.01 |
| | 40 | 0.47±0.01 | 12 | 1.4±0.08 | 1.4 (6)±0.08 | Glc | 2.1±0.13 | Glc | 2.1 (6)±0.13 |
| | | | | | | Ara | 0.00±0.01 | Ara | 0.19 (96)±0.01 |
| | 45 | 0.47±0.02 | 24 | 0.08±0.08 | 0.78 (12)±0.08 | Glc | 0.00±0.01 | Glc | 1.3 (12)±0.08 |
| | | | | | | Ara | 0.00±0.01 | Ara | 0.03 (48)±0.01 |

Values in parenthesis represent cultivation times

*Max.* $Y_{p/s}$ maximum ethanol yield, $\mu_{x/s}$ specific growth rate, ± SD from three independent experiments, *Max.* $\mu_{x/s}$ maximum growth rate, $\gamma_s$ specific sugar utilization rate, *Max.* $\gamma_s$ maximum sugar utilization rate

[a] Time required for the maximum ethanol concentration to be reached

### 2.4.3 Cell growth and ethanol production in YP medium containing a single sugar under a static condition at different temperatures

*K. marxianus* is classified as a facultatively fermentative and Crabtree-negative yeast (van Dijken et al. 1993) and is thus unable to grow under strictly anaerobic conditions. Occurrence of ethanol formation in the organism is almost exclusively linked to oxygen limitation (Visser et al. 1990; van Dijken et al. 1993; Bellaver et al. 2004). Therefore, in order to investigate the effect of oxygen limitation on the utilization and metabolic profiles of individual sugars in DMKU3-1042, cells were cultured in YP medium containing one of the five sugars under a static condition at 30°C and 45°C (Fig. 2.4; Table 2.4). Growth, sugar consumption speed and ethanol production speed under a static condition were found to be lower than those under a shaking condition. However, the maximum ethanol yields tended to be higher, especially in YPD, YPMan, and YPGal, than those under a shaking condition and remained constant throughout the process.

At 30°C, cells completely consumed Glc and Man within 24 h and completely consumed Gal within 48 h. The maximum ethanol yields in YPD, YPMan, and YPGal were 0.50, 0.47, and 0.54, respectively. The time required to reach maximum ethanol level under a static condition and the maximum growth rate were longer and lower, respectively, than those under a shaking condition in all cases.

At 45°C, growth, sugar utilization speed and ethanol yield in YPD, YPGal, and YPMan were reduced from those at 30°C. The maximum ethanol yields in YPD, YPGal, and YPMan were also slightly higher than those under a shaking condition.

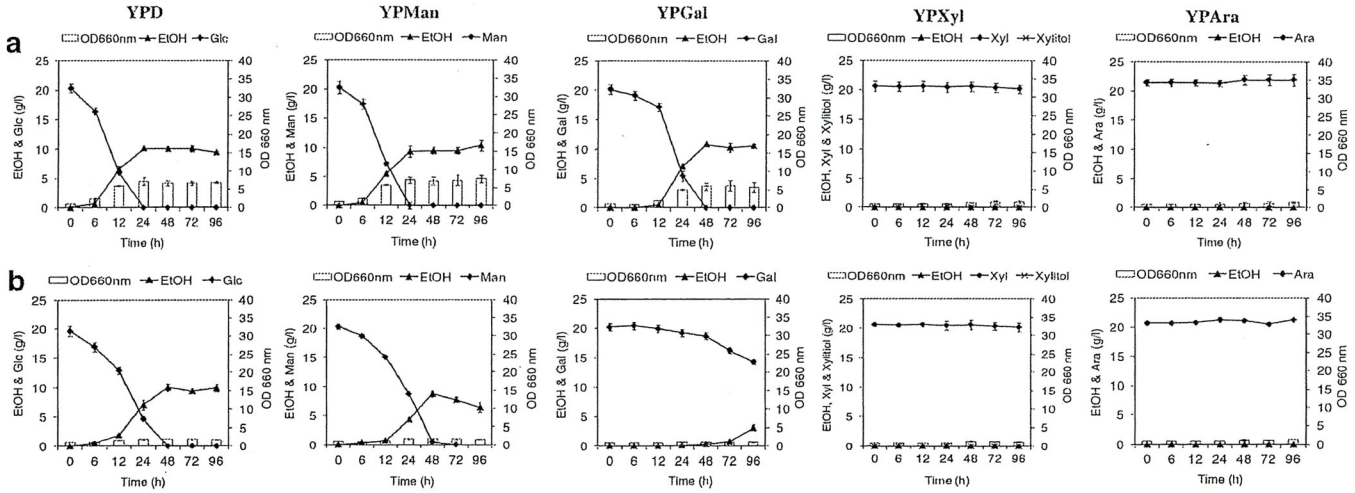



**Fig. 2.4**—Growth and metabolite profiles of *K. marxianus* DMKU3-1042 grown in YP medium containing 20 g/l of glucose (YPD), mannose (YPMan), galactose (YPGal), xylose (YPXyl), or arabinose (YPAra) at 30°C (**a**) and 45°C (**b**) under a static condition (0 rpm). Initial $OD_{660}$ was adjusted to 1. *Bars* represent the ±SD for three independent experiments.

**Table 2.4** Parameters in YP medium containing a single sugar under a static condition (0 rpm) at two different temperatures

| Medium | Temperature (°C) | Max. $Y_{p/s}$ (g/g) | Time of fermentation (h)[a] | $\mu_{x/s}$ ($h^{-1}$) at 6 h | Max. $\mu_{x/s}$ ($h^{-1}$) | $\gamma_s$ (g/l h) at 6 h | Max. $\gamma_s$ (g/l h) |
|---|---|---|---|---|---|---|---|
| YPD | 30 | 0.50±0.01 | 24 | 0.23±0.04 | 0.59 (12)±0.04 | 0.67±0.02 | 1.7 (12)±0.06 |
| | 45 | 0.51±0.02 | 48 | 0.01±0.00 | 0.09 (12)±0.01 | 0.43±0.02 | 0.69 (24)±0.03 |
| YPMan | 30 | 0.47±0.04 | 48 | 0.16±0.04 | 0.63 (12)±0.03 | 0.47±0.02 | 1.7 (12)±0.09 |
| | 45 | 0.44±0.02 | 48 | 0.01±0.00 | 0.08 (12)±0.04 | 0.28±0.01 | 0.69 (48)±0.02 |
| YPGal | 30 | 0.54±0.02 | 48 | 0.03±0.01 | 0.26 (24)±0.02 | 0.17±0.01 | 0.97 (24)±0.04 |
| | 45 | 0.52±0.07 | 96 | 0.00±0.00 | 0.01 (72)±0.01 | 0.00±0.00 | 0.20 (72)±0.01 |
| YPXyl | 30 | — | — | 0.00±0.00 | 0.02 (48)±0.01 | — | — |
| | 45 | – | – | 0.00±0.00 | 0.01 (12)±0.01 | – | – |
| YPAra | 30 | — | — | 0.00±0.00 | 0.03 (96)±0.03 | — | — |
| | 45 | – | – | 0.00±0.00 | 0.01 (12)±0.01 | – | – |

Values in parenthesis represent cultivation times

*Max.* $Y_{p/s}$ maximum ethanol yield, $\mu_{x/s}$ specific growth rate, ± SD from three independent experiments, *Max.* $\mu_{x/s}$ maximum growth rate, $\gamma_s$ specific sugar utilization rate, *Max.* $\gamma_s$ maximum sugar utilization rate

[a] Time required for the maximum ethanol concentration to be reached

This organism hardly consumed Xyl and Ara under this condition at either temperature despite the fact that they were utilized under a shaking condition. We assume that their utilization requires respiratory activity in mitochondria. To examine the assumption, we used antimycin A as a respiratory chain inhibitor at cytochrome $bc_1$ complex as shown in Fig. 2.5. Cells could not grow on YPXyl and YPAra plates in the presence of antimycin A at 30°C and 45°C but grow on YPD plates. This result suggests that utilization of pentose sugars requires respiratory activity to sustain cofactor balance and ATP level.

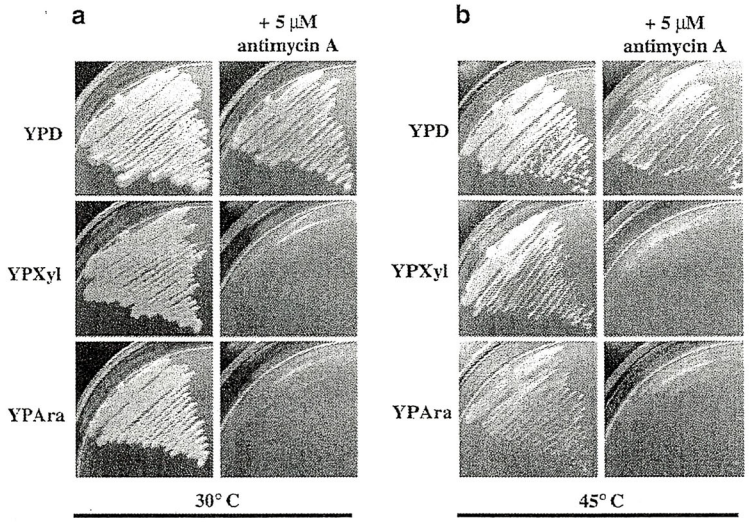



**Fig. 2.5**—Antimycin A effect on xylose (YPXyl) and arabinose (YPAra) utilization at 30°C and 45°C. Cells grown on YPD plate for 2 days at 30°C was streaked on YPXyl, YPAra, and YPD plates with or without 5 μM antimycin A and then incubated at 30°C (**a**) and 45°C (**b**) for 2 days. Data were reproduced by two independent experiments.

### 2.4.4 Cell growth and ethanol production in YP medium containing mixed sugars with Glc under a static condition at different temperatures

Glucose effect on other sugars utilization was examined under a static condition at 30°C and 45°C (Fig. 2.6; Table 2.5). Results of experiments in YPDXyl and YPDAra are not shown because cells were not able to consume Ara or Xyl under a static condition (data not shown, see Fig. 2.4).

At 30°C, Glc in the presence of other sugars was completely consumed within 24 h. The maximum sugar utilization rate of Glc in YPDMan was slightly lower than that in YPD. In YPDMan, Glc and Man were simultaneously utilized and completely consumed at nearly the same time. The Man and Gal utilization rates in YPDMan and YPDGal were lower than those under a shaking condition (Figs. 2.2a, 2.3a) and those in YPMan and YPGal, respectively, under a static condition (Fig. 2.4a). Gal utilization started after nearly complete consumption of Glc, indicating to glucose repression on Gal utilization. The maximum ethanol yields in YPDMan and YPDGal were 0.54 and 0.53, respectively.

At 45°C, the maximum sugar utilization rate of Glc in media of both mixed sugars was reduced. Man in YPDMan was simultaneously utilized with Glc but could not be used up within 96 h. Gal utilization in YPDGal started from 24 h after complete consumption of Glc, but its utilization was largely reduced compared to that at 30°C. The maximum ethanol yields in YPDMan and YPDGal were 0.47 and 0.40, respectively, which were slightly reduced from those at 30°C.

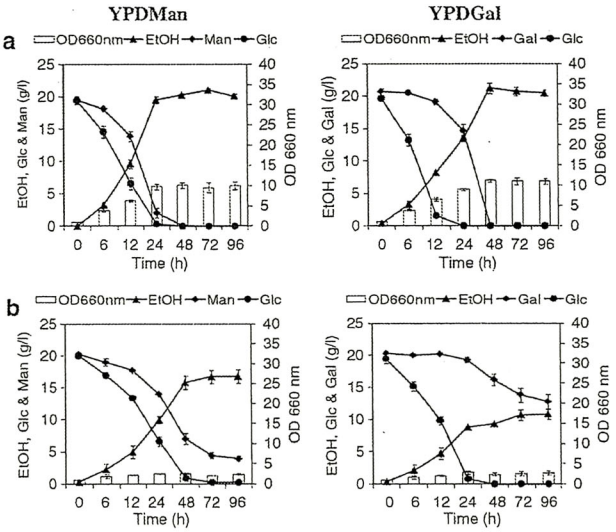



**Fig. 2.6**—Growth and metabolite profiles of *K. marxianus* DMKU3-1042 grown in YP medium containing 20 g/l glucose with 20 g/l of mannose (YPDMan) or galactose (YPDGal) at 30°C (**a**) and 45°C (**b**) under a static condition (0 rpm). Initial $OD_{660}$ was adjusted to 1. *Bars* represent the ±SD for three independent experiments.

**Table 2.5** Parameters in YP medium containing mixed sugars with Glc under a static condition (0 rpm) at two different temperatures

| Medium | Temperature (°C) | Max. $Y_{p/s}$ (g/g) | Time of fermentation (h)[a] | $\mu_{x/s}$ ($h^{-1}$) at 6 h | Max. $\mu_{x/s}$ ($h^{-1}$) | $\gamma_s$ (g/l h) at 6 h | | Max. $\gamma_s$ (g/l h) | |
|---|---|---|---|---|---|---|---|---|---|
| YPDMan | 30 | 0.54±0.02 | 72 | 0.47±0.06 | 0.47 (6)±0.06 | Glc | 0.80±0.09 | Glc | 1.3 (12)±0.02 |
| | | | | | | Man | 0.24±0.03 | Man | 0.98 (24)±0.02 |
| | 45 | 0.47±0.03 | 96 | 0.13±0.05 | 0.13 (6)±0.05 | Glc | 0.51±0.05 | Glc | 0.60 (12)±0.07 |
| | | | | | | Man | 0.20±0.05 | Man | 0.58 (48)±0.06 |
| YPDGal | 30 | 0.53±0.02 | 48 | 0.48±0.03 | 0.48 (6)±0.03 | Glc | 1.1±0.13 | Glc | 2.0 (12)±0.15 |
| | | | | | | Gal | 0.03±0.01 | Gal | 1.2 (48)±0.09 |
| | 45 | 0.40±0.03 | 96 | 0.12±0.06 | 0.12 (6)±0.06 | Glc | 0.72±0.06 | Glc | 0.90 (12)±0.04 |
| | | | | | | Gal | 0.05±0.06 | Gal | 0.26 (48)±0.05 |

Values in parenthesis represent cultivation times

*Max.* $Y_{p/s}$ maximum ethanol yield, $\mu_{x/s}$ specific growth rate, ± SD from three independent experiments, *Max.* $\mu_{x/s}$ maximum growth rate, $\gamma_s$ specific sugar utilization rate, *Max.* $\gamma_s$ maximum sugar utilization rate

[a] Time required for the maximum ethanol concentration to be reached

### 2.4.5 Analysis of glucose repression with 2-DOG

To further understand the glucose repression in *K. marxianus*, the effect of 2-DOG as an analog of glucose on the utilization of other sugars at different temperatures was examined. Cells were spotted onto agar plates of YPMan, YPGal, YPXyl, and YPAra with and without 0.01% 2-DOG and incubated at three different temperatures (Fig. 2.7). In the absence of 2-DOG, cells on YPMan and YPGal grew better than did YPXyl and YPAra at all temperatures tested except for YPMan at 45°C. When 2-DOG was added to the medium, cell growth in YPGal, YPXyl, and YPAra, but not that in YPMan, tended to decrease. Notably, the effect of 2-DOG was enhanced as temperature increased. These results are consistent with those presented in Figs. 2.3 and 2.6 and thus confirm the existence of glucose repression on Gal, Xyl, and Ara utilization in the organism.

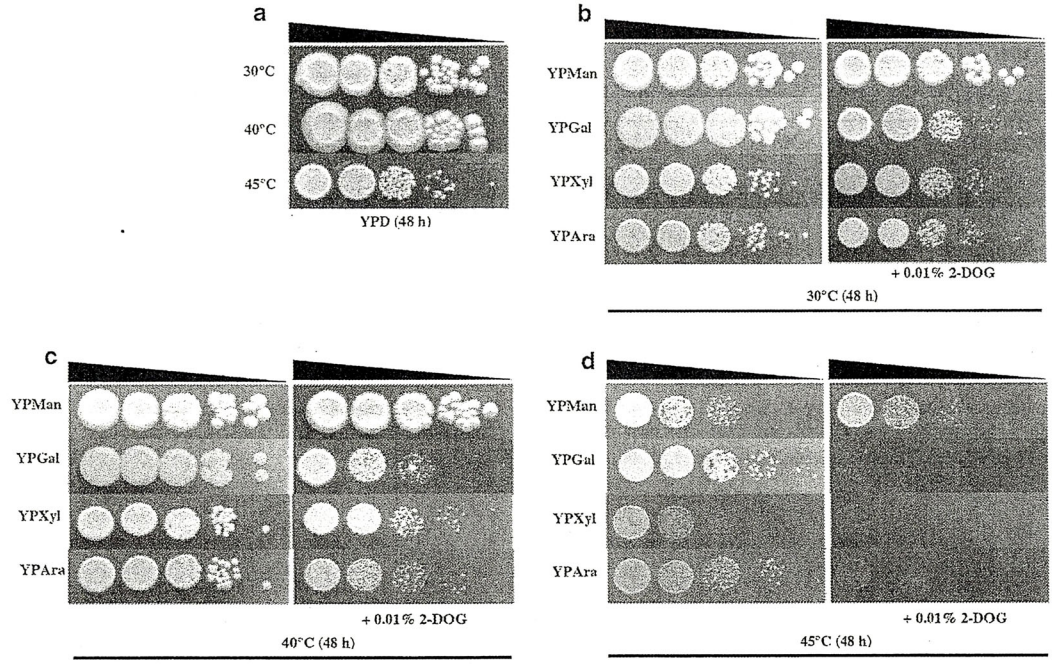



**Fig. 2.7**—2-Deoxyglucose (2-DOG) effect on other sugars utilization at different temperatures. Cells grown in YPD medium for 18 h at 30°C were harvested and washed with deionized water, and the cell suspension (1 x$10^7$cells/ml) was 10-fold sequentially diluted and then spotted onto YPMan, YPGal, YPXyl, and YPAra plates with or without 0.01% 2-DOG. YPD plates were used as a control (**a**). All plates were incubated at 30°C (**b**), 40°C (**c**), and 45°C (**d**) for 48 h. Data were reproduced by two independent experiments.

### 2.4.6 Expression of *KmHXK2*, *KmGAL1*, and *KmXYL1* and glucose effect on expression of *KmGAL1* and *KmXYL1*

The catabolism pathways of the five sugars tested are shown in Fig. 2.1. Hexokinase 2, galactokinase, and xylose reductase function in phosphorylation of D-mannose and D-glucose, phosphorylation of D-galactose, and xylitol production, respectively.

To examine the expression of *KmHXK2* for hexokinase 2, *KmGAL1* for galactokinase, and *KmXYL1* for xylose reductase, we performed RT-PCR with total RNAs from cells grown until the mid-exponential phase in YP medium containing different sugars or different sugars with Glc at two different temperatures under shaking conditions (Fig. 2.8). The band intensity was converted to relative values by

comparison with that of *KmACT1* as an internal control. The values thus reflect the expression level of each gene tested.

The expression profiles indicated that in YPD, YPMan, and YPDMan, *KmHXK2* was similarly expressed at both 30°C and 45°C, but its expression was slightly lower at 45°C in YPD. Expressional regulation of the gene as the glucose repression was not observed. *KmGAL1* was downregulated in YPD at both temperatures and induced about 2-fold in the presence of Gal. Glucose repression on *KmGAL1* was observed in YPDGal at 30°C (Fig. 2.8a, c) and was more evident at 45°C (Fig. 2.8b, c). *KmXYL1* expression in YPD at 30°C and 45°C could not be detected, but it was clearly induced in the presence of Xyl or Ara at both temperatures. Its induction levels in YPXyl and YPAra at 30°C were about 2.8 and 3.8 times higher, respectively, than those at 45°C. The ratios of *KmXYL1* expression in YPXyl versus that in YPDXyl and in YPAra versus that in YPDAra at 30°C were reduced by 5.7- and 6.8-fold, respectively, and those at 45°C were reduced by 2.4- and 5.1-fold, respectively. Therefore, these results suggest that *KmGAL1* and *KmXYL1* are subjected to glucose repression.

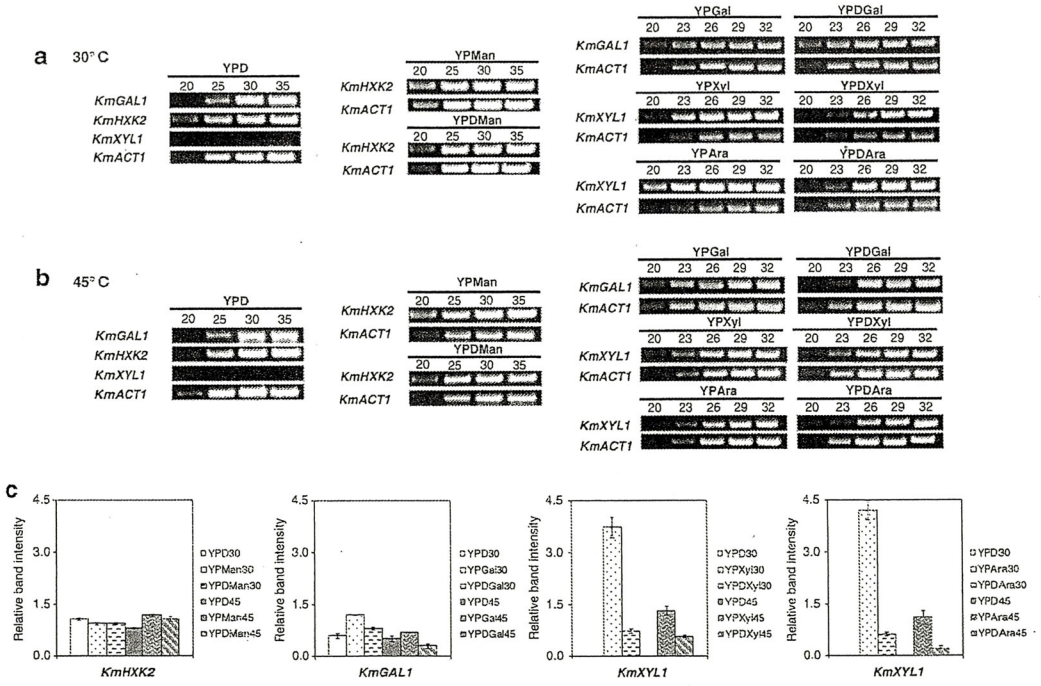



**Fig. 2.8** Expression of *KmHXK2*, *KmGAL*, and *KmXYL1* genes in YP medium containing a single sugar or a sugar in combination with Glc. Cells grown in YPD medium for 18 h at 30°C were inoculated into batch culture, which was conducted in 100-ml Erlenmeyer flasks in 30-ml YP medium containing 20 g/l of glucose (YPD), mannose (YPMan), galactose (YPGal), arabinose (YPAra), or xylose (YPXyl) or 20 g/l of glucose with 20 g/l of mannose (YPDMan), galactose (YPDGal), xylose (YPDXyl), or arabinose (YPDAra). Cells were further cultivated at 30°C (**a**) or 45°C (**b**) for 6 h under a shaking condition (160 rpm). Total RNA was then isolated and subjected to RT-PCR. RT-PCR was performed with primers specific to *KmGAL1*, *KmHXK2*, and *KmXYL1*. After reverse transcriptase reaction, PCR products of 20, 25, 30, and 35 cycles or 20, 23, 26, 29, and 32 cycles were subjected to agarose gel electrophoresis and stained with ethidium bromide. **c** Band intensity was analyzed by using UN-SCAN-IT gel™ automated digitizing system (Silk Scientific). Relative values of intensity of bands in **a** and **b** were determined as a ratio of intensity of each band for each gene to that for *KmACT1*. *Bars* represent the ±SD for three independent experiments.

## 2.5 Discussion

In this study, we evaluated the capability of *K. marxianus* DMKU3-1042 for utilization of various sugars as constituents of hemicellulose or ethanol fermentation at high temperatures and under different aeration conditions. Additionally, its glucose effect on utilization of other sugars was examined because glucose coexists with other sugars in various biomasses. It was found that the yeast

was efficiently capable of assimilating Glc, Man, and Gal up to 45°C under shaking and static conditions except for Gal at high temperatures under a static condition. Xyl and Ara were also utilized under a shaking condition, though the utilization speed was slower than those for the three hexoses and gradually decreased as temperature increased. These pentoses, however, could not be utilized under a static condition. These findings indicate high capabilities of the organism for utilization of various sugars at relatively high temperatures and of hexoses under a static condition.

The inability of the organism to utilize the two pentoses under a static condition is possibly due to incapability of NADH oxidation by respiratory chain in mitochondria. Consistent with this, antimycin A hampered cell growth (Fig. 2.5), and disruption of components or synthesis of components of the respiratory chain caused defective growth in YPXyl or YPAra (unpublished data). The utilization of Gal at 45°C under a static condition was greatly reduced compared to that at 30°C. We have no clear explanation for this phenomenon, but the initial steps including the import of Gal and its conversion to glucose 6-phosphate may require more energy than those for Glc or Man (De Bruijne et al. 1988; Van Leeuwen et al. 1991) or the ATP level may influence the activity of the $H^+$-galactoside symporter (Van den Broek et al. 1987).

Ethanol yield from Gal was higher than those from Glc and Man (Fig. 2.2; Table 2.2), and slight glucose repression was observed in Gal utilization even at 40°C (Figs. 2.3 and 2.6; Tables 2.3 and 2.5). Although Xyl and Ara utilization was retarded in the presence of Glc, especially at 45°C, alleviation of the retardation was observed at 40°C (Figs. 2.3, 2.6). Unlike *S. cerevisiae,* this strain shows no glucose repression in sucrose utilization (unpublished data). In addition, reduction of ethanol level at elevated temperatures under a shaking condition was lower than that at 30°C (Figs. 2.2, 2.3). This phenomenon is possibly due to the reduction of expression of *KmADH4* for alcohol dehydrogenase 4, which is induced by ethanol and thought to be involved in ethanol degradation (Lertwattanasakul et al. 2007). *KmADH4* is expressed in the stationary phase, in which glucose is completely consumed and ethanol concentration becomes the highest. The transcription level of *KmADH4*,

however, is low at 45°C compared to that at 30°C. Ethanol evaporation is another factor to be considered, though it had a minor effect under this condition because efficient evaporation occurs under the conditions of a higher concentration of ethanol and higher temperature (Abdel-Banat et al. 2010).

It is likely that Man and Glc uptake in *K. marxianus* is performed by the same low-affinity glucose transporter (Gasnier 1987). When the two sugars were present together, only slight delay of Man utilization was observed (Figs. 2.3, 2.6). This may be due not to glucose repression but to the $K_m$ value for Man being higher than that for Glc as in the case of *S. cerevisiae* (Reifenberger et al. 1997). Consistent with this, no growth retardation was observed in YPMan in the presence of 2-DOG (Fig. 2.7).

Glucose repression was observed as a delay of utilization of Gal, Xyl, and Ara when Glc was present. One of the possible targets responsible for the repression is the process for sugar uptake. Three symporters in *K. marxianus* appear to be sensitive to carbon catabolite inactivation (De Bruijne et al. 1988), but no detailed report on Xyl and Ara transport systems in *K. marxianus* is available. Hexose transporters sensitive to glucose repression as in *S. cerevisiae* and *Pichia stipitis* (Kilian and van Uden 1988; Hamacher et al. 2002; Lee et al. 2002; Sedlak and Ho 2004; Agbogbo and Coward-Kelly 2008) would function in the transport of these pentoses. These sugars were utilized under a shaking condition but not under a static condition (Fig. 2.4), which is known as the Kluyver effect (Fukuhara 2003). Because of a cofactor imbalance in Xyl and Ara metabolic pathways (Dien et al. 1996; van Maris et al. 2006) and low ATP productivity compared to that of hexose, ethanol production was not observed in YPAra and was retarded in YPXyl with accumulation of xylitol (Fig. 2.2).

*KmGAL1* as a representative gene in Gal utilization was examined as a target for the glucose repression. *KmGAL1* had no canonical sequence for Mig1p but had five putative binding sequences for GAL4p within a 1-kb upstream region from its translation initiation site (data not shown). On the other hand, *ScGAL1* in *S. cerevisiae* and *KlGAL1* in *K. lactis* possess one and four canonical sequences for Mig1p and Gal4p, respectively (Rubio-Texeira 2005). *KmGAL1* was repressed in YPDGal, the repression being significant at a high temperature (Fig. 2.8), indicating

the possibility that *KmGAL1* has a Mig1p-binding site(s) that is a sequence different from those for Mig1p in *S. cerevisiae*. The significant effect at a high temperature might be due to strong binding of Mig1p compared to that at a low temperature or an unknown factor(s) might be involved.

When cells were harvested for expression analysis at 6 h, when Xyl and Ara had hardly been utilized in any of the media tested (Figs. 2.2 and 2.3), *KmXYL1* was strongly induced and glucose repression upon its induction was observed (Fig. 2.8). *KmXYL1*, however, has no Mig1p-binding sequence at its upstream region, suggesting the involvement of another glucose repression factor in its repression. Jeffries and Van Vleet (2009) reported that *PsXYL1*, *PsXYL2*, and *PsXYL3* involved in *P. stipitis* Xyl metabolism, which are upregulated by Xyl under aerobic or oxygen-limited conditions, are downregulated by Glc. However, there are no data for their expression regulation under the condition with mixed sugars. Bicho et al. (1988) reported that Glc represses activities of xylose reductase and xylitol dehydrogenase in *P. stipitis* and *Pachysolen tannophilus* in a medium containing Glu and Xyl. However, it is not clear whether glucose repression occurs at the transcription level or at the post-transcription level. Therefore, the mechanism of glucose repression of genes responsible for Xyl or Ara metabolism in yeast is still not clear. It is possible that there are other regulatory pathways instead of Mig1p for the glucose repression of *KmXYL1*.

Our findings allow us to conclude that *K. marxianus* DMKU3-1042 has high potential for utilization of hexoses and pentoses derived from hemicellulose. This study has revealed for the first time the extent of glucose repression on different sugars at different temperatures and has provided valuable information for application of *K. marxianus* and for high-temperature ethanol fermentation, which will help to improve the efficiency of its utilization for biotechnological purposes. Its relatively weak glucose repression also encourages us to apply it for conversion of biomass containing various sugars.

# CHAPTER 3

# Utilization Capability of Sucrose, Raffinose and Inulin and Its Less-Sensitiveness to Glucose Repression in Thermotolerant *Kluyveromyces marxianus* DMKU3-1042

## 3.1 Abstract

*Kluyveromyces marxianus* possesses a useful potential to assimilate a wide variety of substrates at a high temperature, but the negative effect by coexisting glucose is critical for utilization of biomass containing various sugars. Such a negative effect on the activity of inulinase, which is the sole enzyme to hydrolyze sucrose, raffinose and inulin, has been demonstrated in *K. marxianus* without analysis at the gene level. To clarify the utilization capability of sucrose, raffinose and inulin and the glucose effect on inulinase in *K. marxianus* DMKU3-1042, its growth and metabolite profiles on these sugars were examined with or without glucose under a static condition, in which glucose repression evidently occurs. Consumption of sucrose was not influenced by glucose or 2-deoxyglucose. On the other hand, raffinose and inulin consumption was hampered by glucose at 30°C but hardly hampered at 45°C. Unlike *S. cerevisiae*, increase in glucose concentration had no effect on sucrose utilization. These sugar-specific glucose effects were consistent with the level of inulinase activity but not with that of the *KmINU1* transcript, which was repressed in the presence of glucose via KmMig1p. This inconsistency may be due to sufficient activity of inulinase even when glucose is present. Our results encourage us to apply *K. marxianus* DMKU3-1042 to high-temperature ethanol fermentation with biomass containing these sugars with glucose.

## 3.2 Introduction

Glucose-mediated negative control in the budding yeast *Saccharomyces cerevisiae* is a model system for transcriptional repression (Ronne 1995; Entian and Schüller 1997; Gancedo 1998). This control, called glucose repression, physiologically occurs when glucose coexists as one of carbon sources, by which cells shut down the transcription of a specific set of genes for respiration, gluconeogenesis and the metabolism of alternative carbon sources, which may allow cells to perform rational energy consumption.

*ScSUC2* in *S. cerevsiae* is exclusively and strongly regulated by glucose. Results of extensive genetic analyses with mutants defective in glucose repression and derepression and with extragenic suppressors as well as results of protein-protein interaction studies have led to an understanding of the regulation mechanism of *ScSUC2* (Johnston and Carlson 1992; Entian and Schüller 1997), in which two glucose specific effectors, ScMig1p and ScMig2p, are vitally involved (Nehlin and Ronne 1990; Luftiyya and Johnston 1996). *KlINV1* for invertase in *Kluyveromyces lactis* is also under the control of glucose repression, but in contrast to that of *ScSUC2*, its repression is independent of KlMig1p (Georis et al. 1999).

Invertase secreted from *S. cerevisiae* cells resides mainly in the cell wall to perform its physiological function, cleavage of sucrose molecules diffusible into the cell wall (Nam et al. 1993). Such specific localization of invertase may be ecologically beneficial for efficient scavenging of hydrolyzed products. Similarly, the cell-wall retention of inulinase may be advantageous for sucrose utilization in *K. marxianus*. However, this may not be the case for raffinose or inulin utilization because both sugar molecules hardly penetrate into the cell wall (Phelps 1965; Scherrer et al. 1974) and must therefore be hydrolyzed outside the cell wall.

Production of inulinase has been extensively investigated in *K. marxianus* (Cruz-Guerrero et al. 1995; Kalil et al. 2001; Singh et al. 2007). The investigation was mainly focused on optimization of its production under various conditions including operating parameters such as pH, temperature, agitation and aeration in addition to the culture medium, but the results were not sufficient to provide a clear picture of its regulation mechanism. As a consequence, conflicting

opinions regarding expression of the enzyme have accumulated. It was demonstrated that inulinase synthesis is under the control of induction by its substrate with catabolic repression in *K. fragilis* and *K. bulgaricus* (Grootwassink and Fleming 1980; Grootwassink and Hewitt 1983), of induction without catabolic repression in *K. marxianus* UCD (FST) 55-82 (Parekh and Margaritis 1985) or of induction with catabolic repression in *K. marxianus* CBS 6556 (Rouwenhorst et al. 1988). On the other hand, other strains in the same species exhibit no induction by a substrate (Cruz-Guerrero et al. 1995; Schwan et al. 1997). Furthermore, Gupta et al. (1994) reported that glucose is responsible for catabolic repression, whereas sucrose and fructose act as weaker inducers than inulin in *K. fragilis*. However, all of these reports focused on the enzymatic activity of inulinase in the culture medium or cell wall fraction but not on expression at the transcriptional level. *KmMIG1* has been cloned and characterized in *K. marxianus* SGE11 (Cassart et al. 1997), revealing that its physiological role is similar to that of *ScMIG1* in *S. cerevisiae*; that is, KmMig1p represses the expression of *KmINU1* as a counterpart of *ScSUC2* in *S. cerevisiae* and was shown to be fully functional when expressed in *S. cerevisiae*.

Aiming at the realization of high-temperature fermentation as a beneficial and economical technology, utilization capability of various sugars derived from hemicellulose and ethanol productivity have been shown in thermotolerant *K. marxianus* DMKU3-1042 at a relatively high temperature (Rodrussamee et al. 2011). The effect of glucose repression on sugar utilization in the organism, which becomes a critical point for application of biomass containing various sugars, has been shown to be more evident under a static condition. In this study, to determine the regulation mechanism of inulinase via glucose in *K. marxianus* DMKU3-1042, we compared the fermentation capabilities of its substrates, sucrose, raffinose and inulin, in the presence and absence of glucose at different temperatures under a static condition, and we examined the effects of glucose on the transcripts of *KmINU1* and *KmMIG1* and on the production and secretion of inulinase. Detailed analyses reveal that *K. marxianus* DMKU3-1042 is useful for high temperature fermentation with biomass constituted of these sugars and glucose.

## 3.3 Materials and methods

### 3.3.1 Materials

Oligonucleotide primers were synthesized by Proligo Japan (Tokyo). Other chemicals were all of analytical grade.

### 3.3.2 Strains, media and culture conditions

Yeast strains used in this work were *K. marxianus* DMKU3-1042 strain, which has been deposited in the NITE Biological Resource Center (NBRC) under the deposit number NITE BP-283 (Limtong et al. 2007), and *S. cerevisiae* BY4743 (MATa/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*). Media used were YP (1% w/v yeast extract and 2% w/v peptone) supplemented with different carbon sources: YPD, with 2% w/v glucose; YPSuc, with 2% w/v sucrose; YPRaf, with 2% w/v raffinose; YPInu, with 2% w/v inulin; YPGal, with 2% w/v galactose; YPFrt, with 2% fructose; YPDSuc, with 2% w/v glucose and 2% w/v sucrose; YPDRaf, with 2% w/v glucose and 2% w/v raffinose; YPDInu, with 2% w/v glucose and 2% w/v inulin; and YPDGal, with 2% w/v glucose and 2% w/v galactose. If required, 0.01% w/v 2-deoxyglucose (2-DOG) was added to the medium. Cells grown in YPD medium at 30°C for 18 h were inoculated into a 100-ml batch culture medium in a 300-ml Erlenmeyer flask and incubated under a static condition at 30°C or 45°C. The culture flasks were shaken to make cell density homogeneous before samples were taken for measurement as times indicated.

### 3.3.3 Analytical methods

Cell growth was determined by means of periodical optical density (660 nm) measurement. Concentrations of glucose, ethanol, sucrose, raffinose, inulin, fructose, melibiose and galactose during fermentation were determined at 35°C by an HPLC system consisting of an L-2130 Pump, L-2490 Refractive Index Detector, L-2200 Autosampler, L-2350 Column oven, and Hitachi Model D-2000 Elite HPLC System Manager, equipped with a GL-C610-S Gelpack® column (Hitachi Chemical, Tokyo, Japan) using distilled water from an RFD240NA Water Distillation Apparatus (Aquarius, ADVENTEC®, Japan) as a mobile phase at a flow rate of 0.3 ml/min.

To examine production and distribution of inulinase, inulinase activity was measured at 50°C as described previously (Rouwenhorst et al. 1988) except that the initial rate of reducing sugar released was determined by the colorimetric 3,5-dinitrosalicylic acid method (Miller 1959). Cells were grown at 30°C or 45°C as described above and the culture at 6 h was subjected to a low-speed centrifugation to separate supernatant and precipitate fractions. The latter was suspended in 0.1 M acetate buffer (pH 4.5). Both fractions, called supernatant and cell fractions, were then used for inulinase assay and measurement of cell dry weight. One unit of inulinase activity was defined as the amount of enzyme catalyzing the liberation of 1 μmol of fructose $min^{-1}$ at pH 4.5 and 50°C. Specific enzyme activities are expressed per milligram of cell dry weight.

### 3.3.4 RT-PCR analysis

Cells grown in YPD medium for 18 h were subsequently inoculated at 5% into YPD, YPSuc, YPRaf, YPInu, YPDSuc, YPDRaf or YPDInu, and after 4 h of incubation at 30°C or 45°C, total RNAs were isolated by the hot phenol method. RT-PCR analysis was performed as described previously (Lertwattanasakul et al. 2007; Sootsuwan et al. 2007). Primers used for KmINU1, KmMIG1 and KmACT1 were 5'-GTACAACCCAGCAGCCA-3' for KmINU1-213 and 5'-

GCTTGGAGTCGGAGGAG-3' for KmINU1-784, 5'-CGGACGCATACTGGGGA-3' for KmMIG1-160 and 5'-ACCGAGTGGAGGGTTGT-3' for KmMIG1-707, and 5'-ACGTTGTTCCAATCTACGCC-3' for KmACT1-5 and 5'-AGAAGATG-GAGCCAAAGCAG-3' for KmACT1-3. Relative band intensities were determined using scanned images and UN-SCAN-IT software (Silk Scientific, Orem, UT, U.S.A.). Under our conditions, the RNA-selective RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

### 3.3.5 Database search

Homology searching was performed by FASTA and BLAST in GenBank, NCBI, DDBJ, EMBL, and SWISS-PROT databases. Comparisons of nucleotide and amino acid sequences were conducted by Genetyx (Software Development, Tokyo). The *KmINU1* sequence obtained from *K. marxianus* DMKU3-1042 has been submitted to the DDBJ database under the accession number AB621573.

## 3.4 Results

### 3.4.1 Glucose effect on utilization of Suc, Raf or Inu

To determine whether there is a glucose effect on utilization of Suc, Raf or Inu in *K. marxianus* DMKU3-1042, its growth was compared on YPSuc, YPRaf and YPInu with or without Glc at 30°C or 45°C under a static condition (Figs. 3.1 and 3.2; Tables 3.1 and 3.2). Growth on YPGal was also tested as a positive control for glucose repression.

In the absence of Glc, Suc and Raf were rapidly consumed and were completely consumed within the first 12 h at 30°C, whereas Inu was consumed at a relatively slow rate (0.66 g/l h at 6 h) (Fig. 3.1a; Table 3.1). The maximum growth

level on Raf was low compared to that on the other two sugars because of the production of unmetabolizable melibiose. The rate of ethanol production on Suc was low at 45°C due to the slow uptake of Glc and Frt following hydrolysis of Suc (Fig. 3.1b). The utilization of Gal was very slow with a delay of about 6 h compared to that under a shaking condition at 30°C (Rodrussamee et al. 2011) and hardly occurred at 45°C. The consumption of Suc and Inu at 45°C was slightly faster than that at 30°C and the consumption of Raf at 45°C was slower than that at 30°C (Fig. 3.1b; Table 3.1).

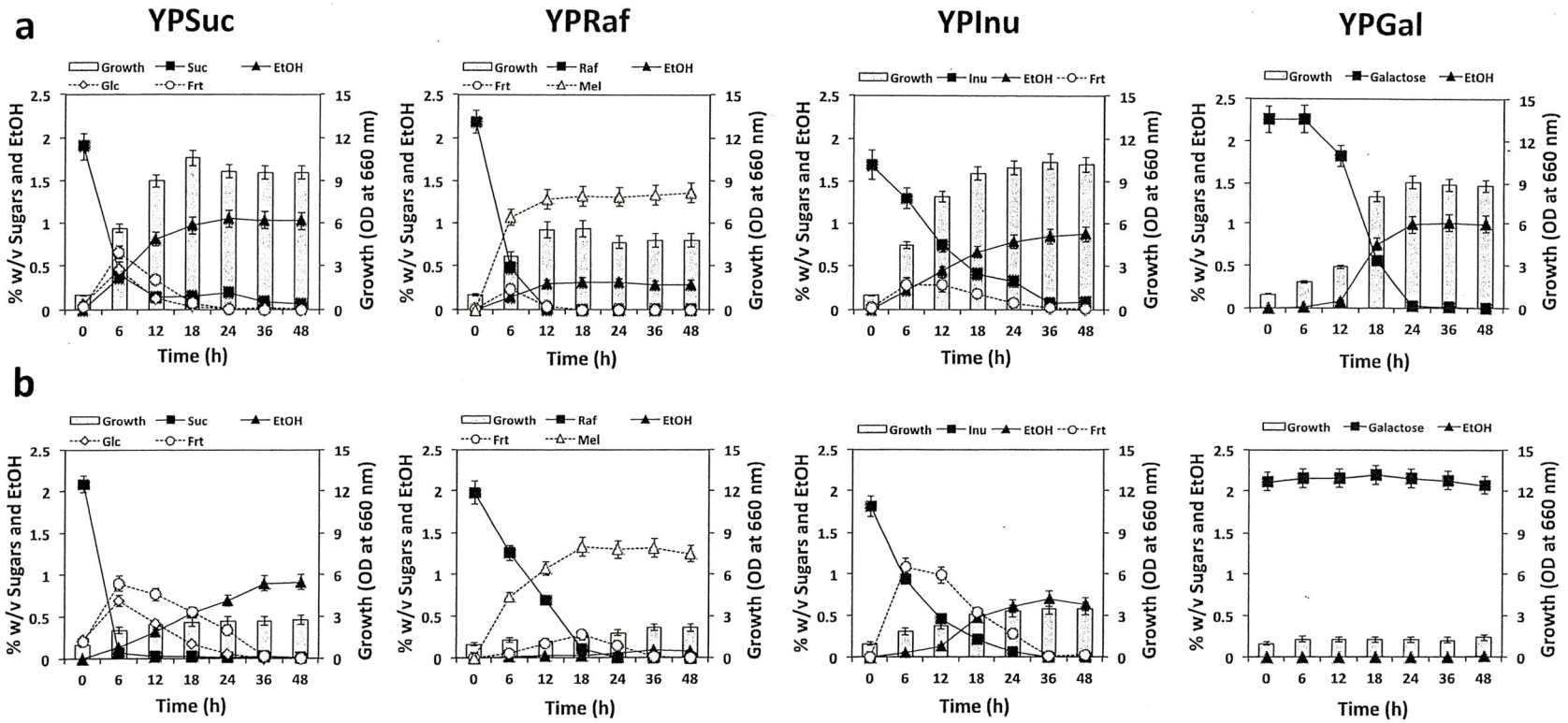



**Fig. 3.1**—Static batch fermentation of sucrose, raffinose or inulin in the absence of glucose. Cells grown in YPD medium at 30°C for 18 h were inoculated into batch culture, which was conducted in 300-ml Erlenmeyer flask containing 100 ml of YP medium containing 2% glucose (YPD), sucrose (YPSuc), raffinose (YPRaf), inulin (YPInu) or galactose (YPGal) at 30°C (**a**) and 45°C (**b**) under a static condition as time indicated. Initial $OD_{660}$ was adjusted to 1.0. *Bars* represent the ±SD for three independent experiments.

**Table 3.1** Parameters in YP medium containing a single sugar under a static condition at 30°C and 45°C

| Medium | Temperature (°C) | Max. $Y_{p/s}$ (g/g) | Time of fermentation (h)[a] | $\mu_{x/s}$ ($h^{-1}$) at 6 h | Max. $\mu_{x/s}$ ($h^{-1}$) | $\gamma_s$ (g/l h) at 6 h | Max. $\gamma_s$ (g/l h) |
|---|---|---|---|---|---|---|---|
| YPSuc | 30 | 0.55±0.04 | 24 | 0.77±0.07 | 0.77 (6)±0.07 | 2.5±0.02 | 2.5 (6)±0.02 |
| | 45 | 0.44±0.05 | 48 | 0.18±0.10 | 0.18 (6)±0.10 | 3.4±0.03 | 3.4 (6)±0.03 |
| YPRaf | 30 | 0.14±0.03 | 18 | 0.44±0.04 | 0.44 (6)±0.04 | 2.9±0.04 | 2.9 (6)±0.04 |
| | 45 | 0.04±0.05 | 24 | 0.05±0.09 | 0.06 (24)±0.10 | 1.2±0.01 | 1.2 (6)±0.01 |
| YPInu | 30 | 0.52±0.04 | 48 | 0.57±0.11 | 0.57 (6)±0.11 | 0.66±0.07 | 0.93 (12)±0.08 |
| | 45 | 0.58±0.01 | 18 | 0.14±0.08 | 0.14 (6)±0.08 | 1.4±0.10 | 1.4 (6)±0.10 |
| YPGal | 30 | 0.45±0.03 | 36 | 0.14±0.06 | 0.85 (18)±0.12 | 0.0±0.00 | 2.1 (18)±0.09 |
| | 45 | – | – | – | – | – | – |

Values in parenthesis represent cultivation times

*Max.* $Y_{p/s}$ maximum ethanol yield, $\mu_{x/s}$ specific growth rate, *Max.* $\mu_{x/s}$ maximum growth rate, $\gamma_s$ specific sugar utilization rate, *Max.* $\gamma_s$ maximum sugar utilization rate, ± SD from three independent experiments

[a] Time required for the maximum ethanol concentration to be reached

The rate of Suc utilization in the presence of Glc was almost the same as that in the absence of Glc at both temperatures (Fig. 3.2a, b; Table 3.2). The maximum ethanol yield from a mixture of Suc and Glc at 30°C was higher than that at 45°C, and the ethanol level was maintained until the end of the fermentation period examined. However, growth at 45°C was reduced to about 30% of that at 30°C. At 30°C, the rates of Raf and Inu utilization were reduced by 3 fold and 6 fold, respectively, in the presence of Glc (Table 3.2). Raf was consumed simultaneously with Glc, but the consumption of Inu was delayed after depletion of Glc. Both sugars were consumed much faster at 45°C than at 30°C. Almost no glucose repression was found in the utilization of Raf and Inu. This is presumably due to the availability of inulinase enzyme for hydrolytic reaction at a high temperature (see below). The effects of glucose repression on Gal and Raf utilization in *K. marxianus* DMKU3-1042 were found to be significant but weaker than that and similar to that, respectively, in *S. cerevisiae* at 30°C (data not shown).

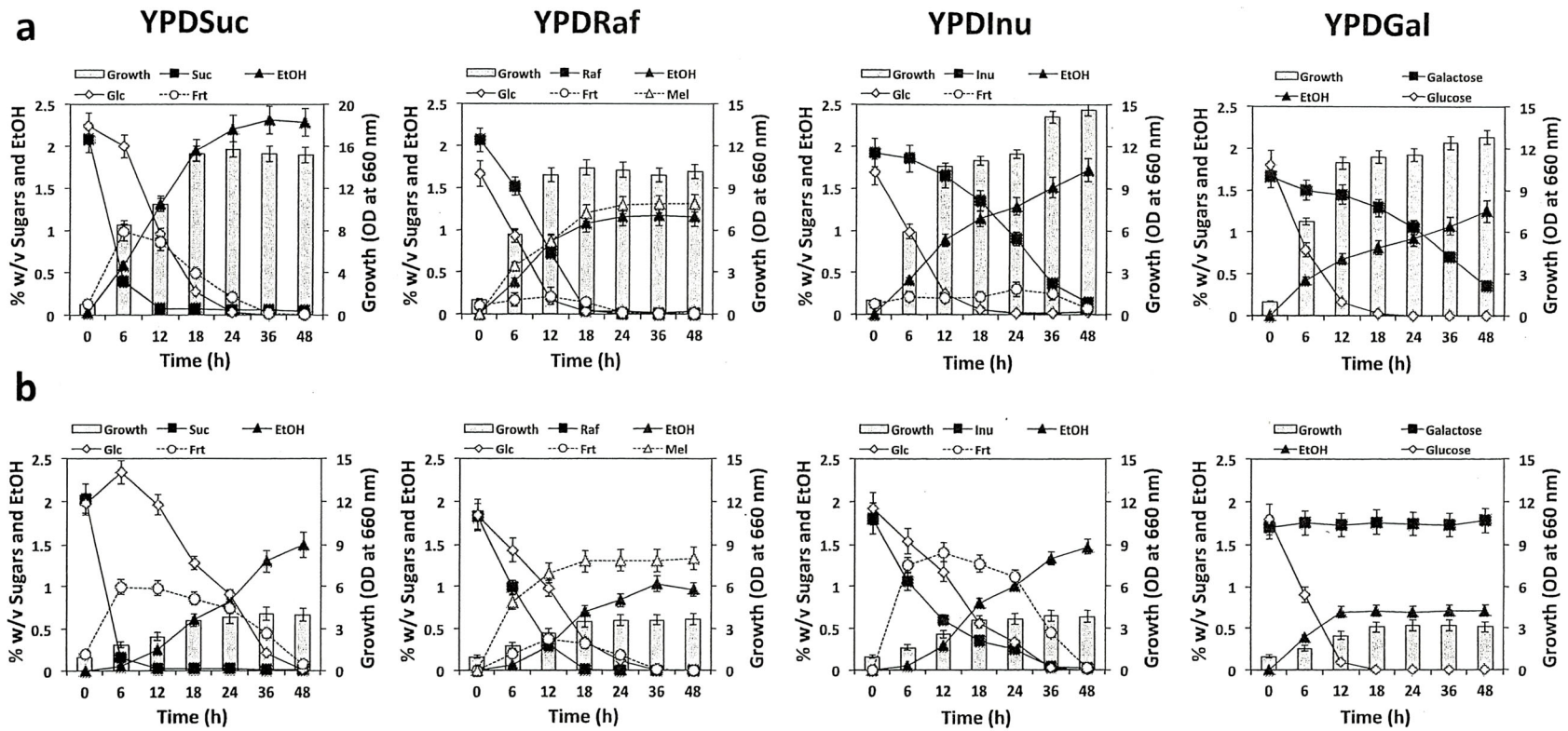



**Fig. 3.2**—Static batch fermentation of sucrose, raffinose or inulin in the presence of glucose. Cells grown in YPD medium at 30°C for 18 h were inoculated into batch culture, which was conducted in 300-ml Erlenmeyer flask containing 100 ml of YP medium containing 2% sucrose (YPSuc), raffinose (YPRaf), inulin (YPInu) or galactose (YPGal) with 2% glucose at 30°C (**a**) and 45°C (**b**) under a static condition as time indicated. Initial $OD_{660}$ was adjusted to 1.0. *Bars* represent the ±SD for three independent experiments.

**Table 3.2** Parameters in YP medium containing mixed sugars with Glc under a static condition at 30°C and 45°C

| Medium | Temperature (°C) | Max. $Y_{p/s}$ (g/g) | Time of fermentation (h)[a] | $\mu_{x/s}$ ($h^{-1}$) at 6 h | Max. $\mu_{x/s}$ ($h^{-1}$) | $\gamma_s$ (g/l h) at 6 h | | Max. $\gamma_s$ (g/l h) | |
|---|---|---|---|---|---|---|---|---|---|
| YPDSuc | 30 | 0.53±0.02 | 36 | 1.3±0.05 | 1.3 (6)±0.05 | Glc | 0.42±0.09 | Glc | 1.7 (12)±0.04 |
| | | | | | | Suc | 2.8±0.05 | Suc | 2.8 (6) ±0.05 |
| | 45 | 0.37±0.04 | 48 | 0.15±0.08 | 0.19 (18)±0.05 | Glc | −0.6±0.03 | Glc | 1.1 (18)±0.03 |
| | | | | | | Suc | 3.1±0.12 | Suc | 3.1 (6)±0.12 |
| YPDRaf | 30 | 0.31±0.05 | 36 | 0.78±0.11 | 0.78 (6)±0.11 | Glc | 1.2±0.10 | Glc | 1.3 (12)±0.05 |
| | | | | | | Raf | 0.93±0.08 | Raf | 1.3 (12)±0.01 |
| | 45 | 0.28±0.01 | 36 | 0.14±0.04 | 0.15 (12)±0.03 | Glc | 0.68±0.09 | Glc | 1.0 (18)±0.02 |
| | | | | | | Raf | 1.4±0.10 | Raf | 1.4 (6)±0.10 |
| YPDInu | 30 | 0.48±0.02 | 48 | 0.81±0.09 | 0.81 (6)±0.09 | Glc | 1.2±0.03 | Glc | 1.2 (12)±0.04 |
| | | | | | | Inu | 0.11±0.06 | Inu | 0.75 (24)±0.03 |
| | 45 | 0.40±0.02 | 48 | 0.11±0.03 | 0.16 (12)±0.01 | Glc | 0.63±0.04 | Glc | 1.0 (18)±0.07 |
| | | | | | | Inu | 1.2±0.01 | Inu | 1.2 (6)±0.01 |
| YPDGal | 30 | 0.36±0.04 | 48 | 0.96±0.03 | 0.96 (6)±0.03 | Glc | 1.7±0.05 | Glc | 1.7 (6)±0.05 |
| | | | | | | Gal | 0.27±0.03 | Gal | 0.29 (36)±0.02 |
| | 45 | 0.21±0.03 | 18 | 0.13±0.01 | 0.14 (12)±0.01 | Glc | 1.5±0.06 | Glc | 1.5 (6)±0.06 |
| | | | | | | Gal | – | Gal | – |

Values in parenthesis represent cultivation times

*Max.* $Y_{p/s}$ maximum ethanol yield, $\mu_{x/s}$ specific growth rate, *Max.* $\mu_{x/s}$ maximum growth rate, $\gamma_s$ specific sugar utilization rate, *Max.* $\gamma_s$ maximum sugar utilization rate, ± SD from three independent experiments

[a] Time required for the maximum ethanol concentration to be reached

### 3.4.2 Effect of 2-deoxyglucose (2-DOG) on utilization of Suc, Raf or Inu

To further examine the glucose effect on utilization of Suc, Raf and Inu, cell growth was compared on YPSuc, YPRaf and YPInu agar plates supplemented with 2-DOG as a glucose analogue at 30°C and 45°C (Fig. 3.3a, b). At 30°C, growth was repressed by the addition of 2-DOG on Raf and Inu as on Gal, but almost no repression was observed on Suc. Interestingly, the extent of the repression was much weaker than that in *S. cerevisiae* at 30°C (Fig. 3.3c). The repressive effect was more evident at 45°C. No growth was observed on Raf or Inu in the presence of 2-DOG at 45°C. This phenomenon is presumably due to the initial uptake of 2-DOG over Frt derived from Raf or Inu to the cells at the beginning of growth, hampering the uptake of Frt. On the other hand, *K. marxianus* could grow well even in the presence of 2-DOG when a high concentration of Frt was present at the early growth phase as in the case of YPFrt (Fig. 3.3a, b).

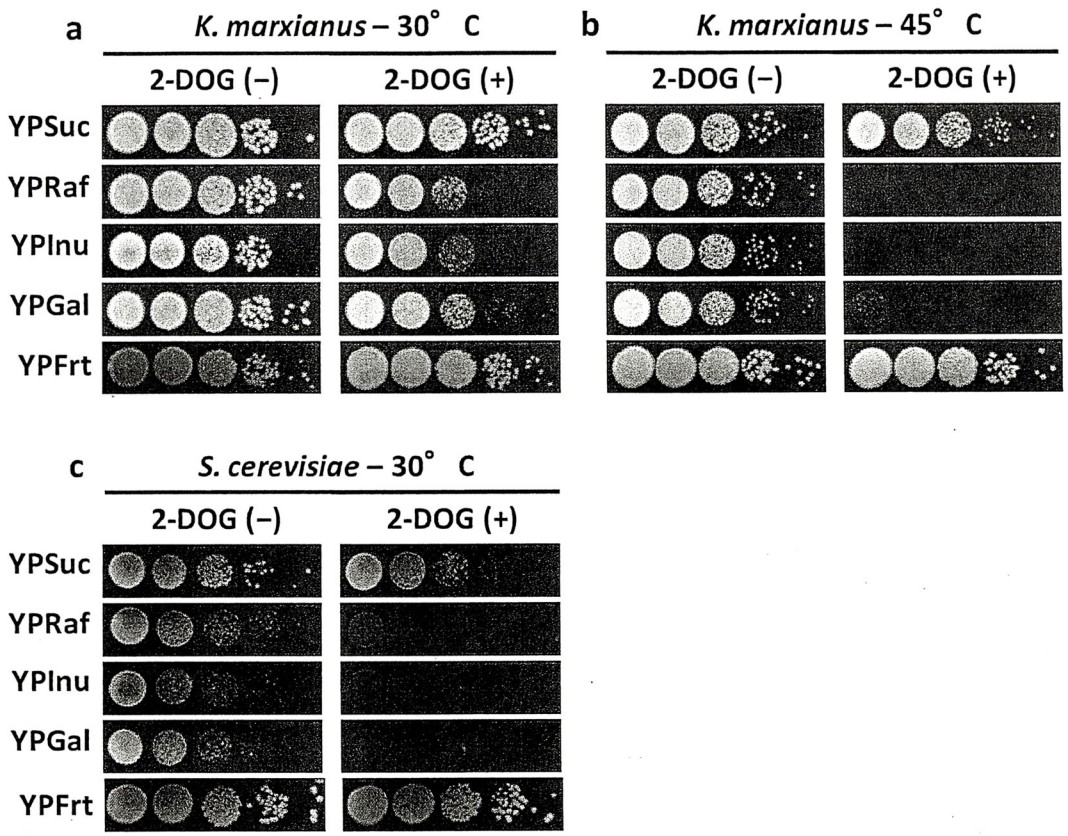



**Fig. 3.3—Effect of 2-DOG on utilization of sucrose, raffinose or inulin.** Cells were grown in YPD medium to about $10^7$ cells/ml, aliquots of 10-fold culture dilutions of cells were spotted onto agar plates containing YP medium supplemented with 2% fructose (YPFrt), 2% sucrose (YPSuc), 2% raffinose (YPRaf) or 2% inulin (YPInu) in the presence (+) or absence (–) of 0.01% 2-DOG, and the plates were incubated at 30°C (**a**) or 45°C (**b**) for 3 days. Galactose (Gal) was included as a positive control. *S. cerevisiae* was used as a reference strain for glucose repression (**c**).

To determine the mechanism behind the phenomenon described above, we performed experiments in a liquid medium of YPFrt, YPSuc, YPRaf or YPInu supplemented with 2-DOG at 30°C and 45°C. The speed of Frt uptake in YPFrt at 45°C was found to be slower than that at 30°C (Fig. 3.4). During the hydrolysis of Suc, Raf or Inu, Frt was accumulated at both temperatures and could be further utilized by the organism only at 30°C except for the case of YPSuc, where 2-DOG only slowed down the speed of Frt uptake at 45°C. However, the uptake of Frt was completely inhibited when cells were grown in YPRaf or YPInu at 45°C, and no cell growth was observed (Fig. 3.4b). Considering the fact that Raf and Inu consumption was enhanced at 45°C when Glc was added together (Fig. 3.2b), it is likely that 2-DOG was accumulated as 2-DOG-6-phosphate before hydrolysis of the sugars to prevent metabolic activities and cell growth. Taken together, the results obtained with 2-DOG for the consumption of the three sugars at 30°C were almost consistent with those obtained with Glc.

The effect of extracellular Glc concentration on glucose repression in *S. cerevisiae* has been investigated, and it has been shown that the level of repression is correlated with increase in Glc concentration (Meijer et al. 1998). To further examine the effect of Glc concentration on utilization of Suc in *K. marxianus* DMKU3-1042, we examined cell growth in 2% Suc supplemented with various concentrations of Glc under a static condition at 30°C (Fig. 3.5). Unlike *S. cerevisiae*, increase in Glc concentration from 2–8% had almost no effect on the rate of Suc consumption in the yeast.

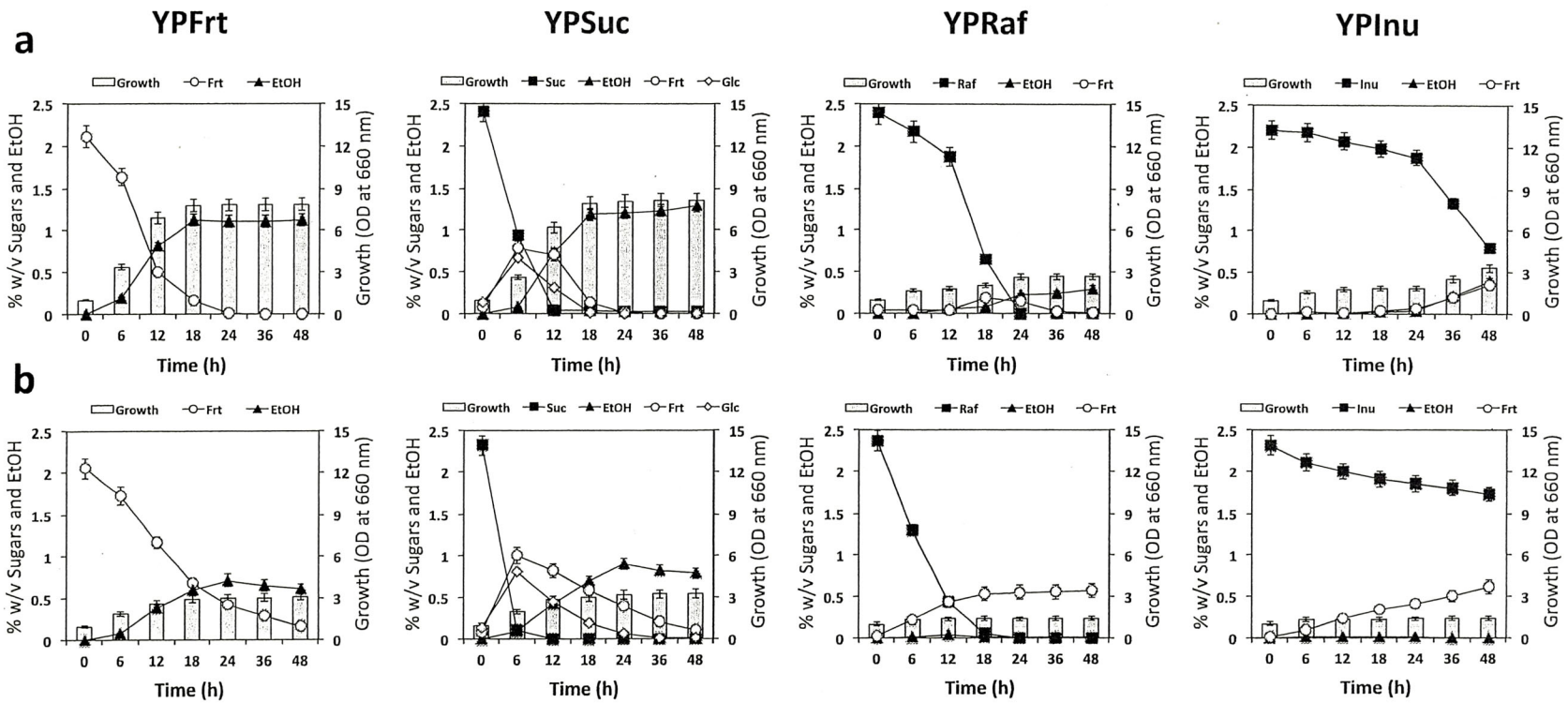



**Fig. 3.4—Effect of 2-DOG on fructose uptake.** Cells grown in YPD medium at 30°C for 18 h were inoculated into sequential batch culture, which was conducted in 300-ml Erlenmeyer flask containing 100 ml of YP medium containing 2% fructose (YPFrt), 2% sucrose (YPSuc), 2% raffinose (YPRaf) or 2% inulin (YPInu) supplemented with 0.01% 2-DOG. Cultivation was continued further at 30°C (**a**) or 45°C (**b**) under a static condition as time indicated. Initial OD660 was adjusted to 1.0. *Bars* represent the ±SD for three independent experiments.

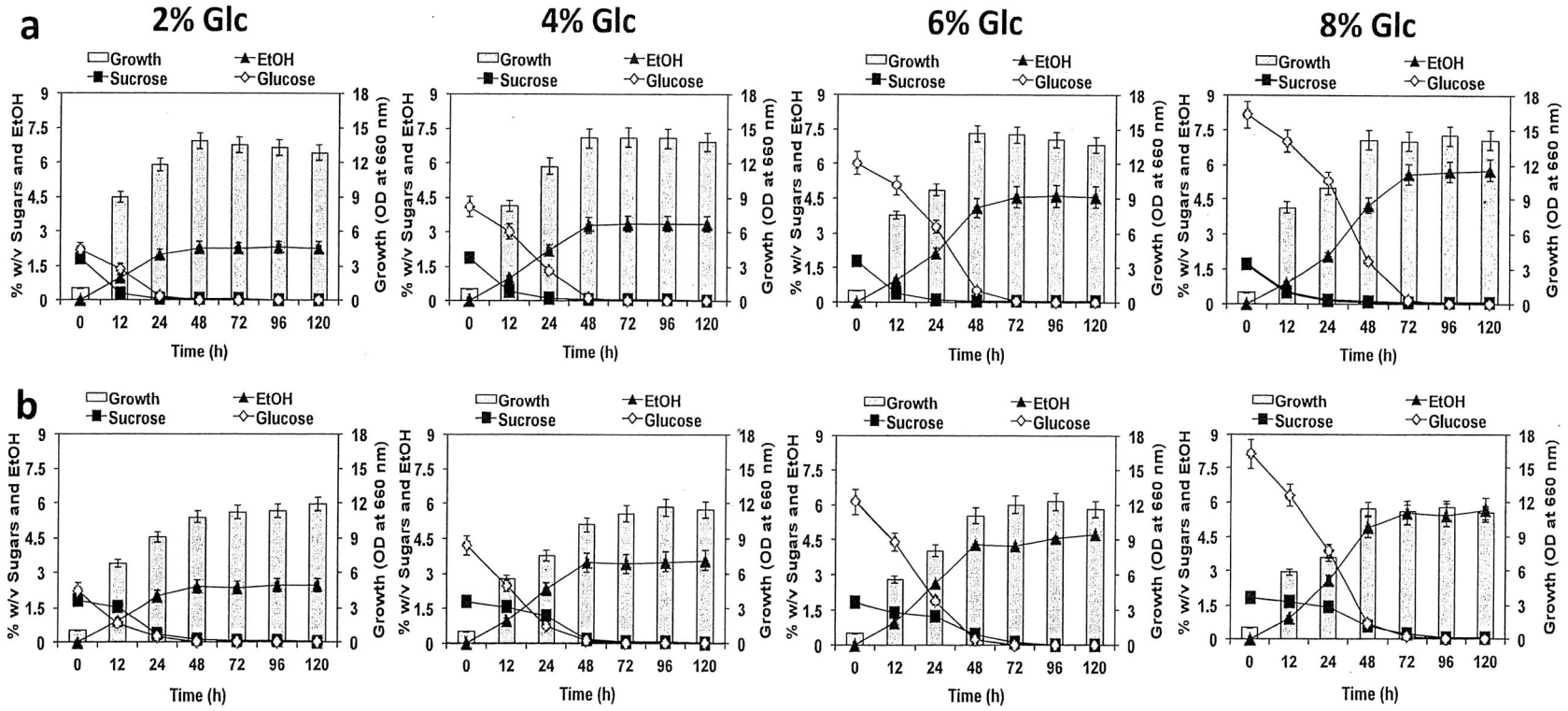



**Fig. 3.5—Effect of glucose concentrations on hydrolysis of sucrose.** Cells grown in YPD medium at 30°C for 18 h were inoculated into sequential batch culture, which was conducted in 300-ml Erlenmeyer flask containing 100 ml of YP medium supplemented with a mixture of 2% sucrose (Suc), and various concentrations of glucose (Glc) as indicated. Cultivations of *K. marxianus* (**a**) and *S. cerevisiae* (**b**) were continued further at 30°C under a static condition as time indicated. The patterns of sucrose hydrolysis at different glucose concentrations were summarized (**c**). Initial $OD_{660}$ was adjusted to 1.0. Bars represent the ±SD for three independent experiments.

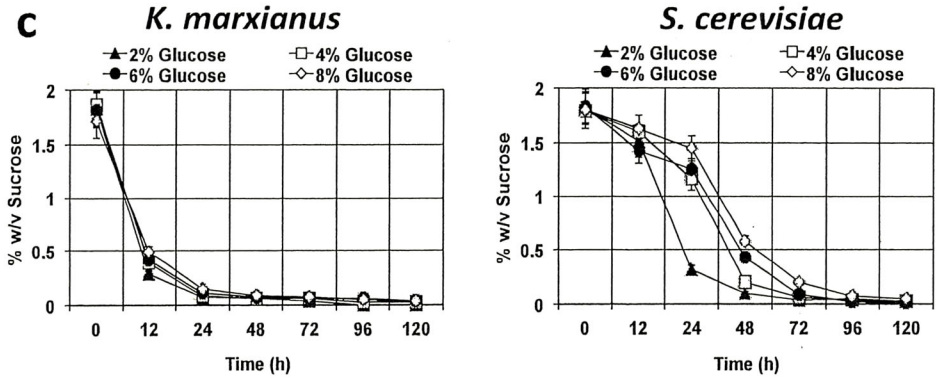



**Fig. 3.5**—Continued

### 3.4.3 Glucose effect on production and distribution of inulinase

In order to examine the glucose effect on production or distribution of inulinase, with 6-h cultures in the liquid medium of YPSuc or YPInu in the presence or absence of Glc at 30°C and 45°C as described above, we measured inulinase activity in the supernatant and cell fractions and compared total activities under different conditions or activities of the two fractions (Table 3.3). In YPD, YPSuc and YPInu media, total inulinase activities were 6.0, 6.5 and 19.2 U mg of cell dry weight$^{-1}$ at 30°C, respectively, and 11.4, 12.9 and 13.9 U mg of cell dry weight$^{-1}$ at 45°C, respectively. The tendency in difference of these values was consistent with results of RT-PCR experiments (see Fig. 3.6b) except for the case of YPInu, indicating that inulinase is induced by Inu but not by Suc at 30°C and by heat. In supernatant fractions, approximately 3-times higher inulinase activity was recovered at 45°C than that at 30°C in all media except for YPInu. The increase in total activity along with the temperature up-shift seems to reflect the increase in supernatant fraction activity, indicating facilitated secretion of inulinase at a high temperature.

Total inulinase activity in YPDInu was 3.3-times lower than that in YPInu at 30°C, but almost no such difference was observed between YPDSuc and YPSuc at both temperatures or between YPDInu and YPInu at 45°C. On the other hand, distribution of inulinase activity in supernatant fractions was hardly influenced by the addition of Glc.

**Table 3.3** Production and distribution of inulinase in static batch cultures of *K. marxianus* DMKU3-1042 in YP medium with 2% various carbon substrates

| Medium | Temperature (°C) | Total inulinase activity (U mg of cell dry $wt^{-1}$)[a] | Inulinase activity (U mg of cell dry $wt^{-1}$)[a] | |
|---|---|---|---|---|
| | | | Supernatant (%) | Cell (%) |
| YPD | 30 | 6.0 | 3.1 (52) | 2.9 (48) |
| | 45 | 11.4 | 8.8 (77) | 2.6 (23) |
| YPSuc | 30 | 6.5 | 3.1 (48) | 3.4 (52) |
| | 45 | 12.9 | 10 (78) | 2.9 (22) |
| YPInu | 30 | 19.2 | 10 (52) | 9.2 (48) |
| | 45 | 13.9 | 8.2 (59) | 5.7 (41) |
| YPDSuc | 30 | 6.4 | 4.1 (64) | 2.3 (36) |
| | 45 | 15.7 | 13 (83) | 2.7 (17) |
| YPDInu | 30 | 5.9 | 3.2 (54) | 2.7 (46) |
| | 45 | 12.4 | 9.6 (77) | 2.8 (23) |

[a] Enzyme activities were measured with sucrose as substrate with samples taken at 6 h from static batch cultures as described in Materials and methods
Values are average from two independent experiments

### 3.4.4 Alignments of conserved domains and upstream sequences of *K. marxianus* inulinase with those of glycoside hydrolase family 32 from other yeast species

*K. marxianus* DMKU3-1042 possesses only one copy of *KmINU1* encoding for inulinase as a counterpart of *ScSUC2* encoding for invertase in *S. cerevisiae* (The genome sequence will be published elsewhere.). The two enzymes belong to the glycoside hydrolase family 32 (GH32) group in carbohydrate-degrading enzymes. Comparison of primary sequences deduced from nucleotide sequences revealed that KmInu1 bears several motifs of WMNDPNG (block A), WHLY(F/Y)Q (block B), WGHA(T/V)S (block B1), FSGSMV(V/I) (block C), FRDPKVF (block D), QYECPGL (block E) and I(I/L)ELY (block G), which are conserved among invertases and inulinases in yeast GH32 enzymes (Appendix A). The conserved domains of *KmInu1* in DMKU3-1042 showed sequence identity of 100% to those of the corresponding enzyme from the other strains of *K. marxianus*. The enzyme is classified into exoinulinase on the basis of the presence of Asp in block A, whereas endoinulinase has Glu at the Asp position. The carboxyl groups of Asp in block A and Glu in block E are involved in the catalytic activity of ß-fructofuranosidases (Reddy and Maley 1996). The Glu residue in block E may act as a proton donor in the catalytic reaction, as reported for invertase from *S. cerevisiae* (Reddy and Maley 1996). The Asp residue in block D, which is conserved in all inulinases, is related to substrate recognition (Nagem et al. 2004).

However, there are conflicting data on the regulation of inulinase production among different strains of *K. marxianus*. We thus compared the upstream non-coding sequence of *KmINU1* in DMKU3-1042 with those of the corresponding genes in four other *K marxianus* strains, CBS 6556, ATCC 12424, Y1 and CBS 834 (Nucleotide sequences of *KmINU1* of CBS 4857 and IW 9801 are not available.). The inulinase gene in CBS 6556 has been reported to be repressed by Glc (Rouwenhorst et al. 1988), but there is no available information on the regulation of *KmINU1* genes in other strains. Approximately 700-bp upstream sequences of *KmINU1* from the five strains were aligned, and two putative Mig1p elements

(consensus sequence, WWWWTSYGGGG) were found in all strains (Appendix 2). These findings and evidence based on disruption of the *KmMIG1* gene (Cassart et al. 1997) suggest that the negative regulation of *KmINU1* is dependent on KmMig1p, a key effector for glucose repression as in strain CBS 6556. However, we could not compare such upstream sequences with other strains due to the lack of nucleotide sequences of *KmINU1* in databases, of which UCD (FST) 55–82 has been claimed to be free from glucose repression (Parekh and Margaritis 1985).

### 3.4.5 Glucose effect on expression of *KmINU1*

To determine whether the regulation of inulinase by Glc occurs at the transcriptional level, RT-PCR was carried out with total RNA from cells grown for 4 h at 30°C or 45°C under the same condition as that for other experiments by liquid culture (Fig. 3.6). The band intensities of *KmMIG1* and *KmINU1* were converted to relative values by comparison with that of *KmACT1* as an internal control. The values thus reflect the expression level of each gene tested. The expression profiles indicated that *KmINU1* was similarly expressed in Glc and Suc and its expression level was increased about 2-3 times in Raf and Inu at 30°C. Notably, the expression level of *KmINU1* at 45°C was more than 2-times higher than that at 30°C in all media tested. In the presence of Glc, the expression level was greatly reduced in Suc, Raf and Inu at both temperatures except for a slight reduction in Suc at 45°C. On the other hand, the expression level of *KmMIG1* was increased by the addition of glucose and was reduced at 45°C compared to that at 30°C. These expression alterations were oppositely consistent with those of *KmINU1*. Therefore, these results suggest that *KmINU1* is inducible by Raf or Inu and negatively controlled by Glc via KmMig1p in strain DMKU3-1042.

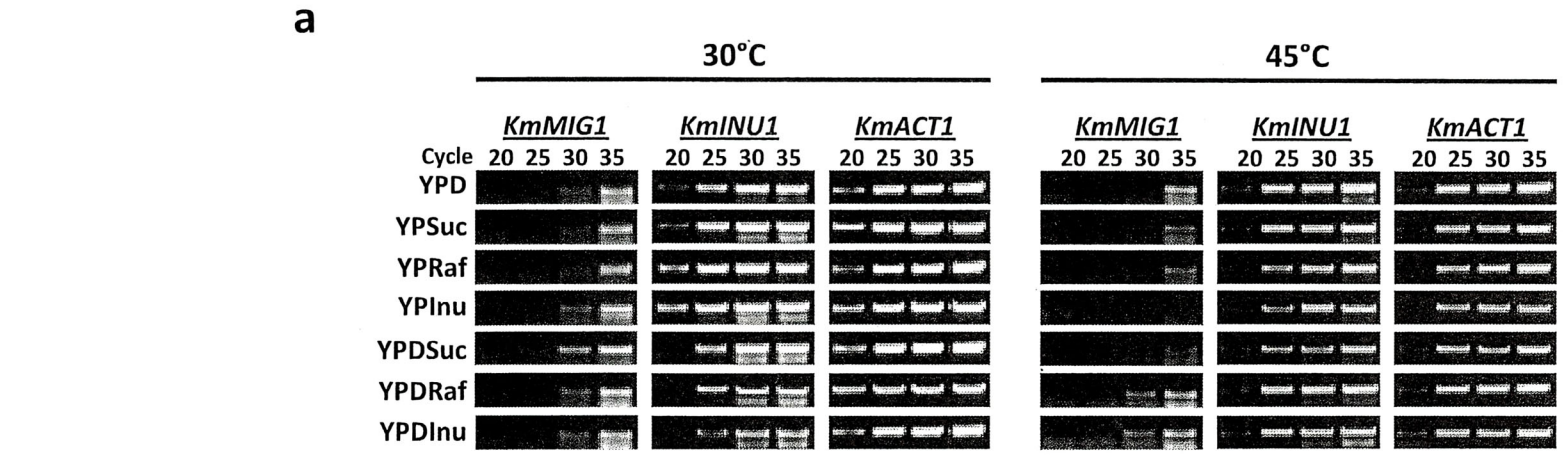



**Fig. 3.6—Expression of *KmINU1* in various sugars under a static condition.** Cells grown in YPD medium for 18 h were inoculated into YP supplemented with 2% glucose (YPD), sucrose (YPSuc), raffinose (YPRaf) or inulin (YPInu), or a mixture of these sugars with 2% glucose, and cultivated further for 4 h at 30°C or 45°C. Total RNA was then isolated and subjected to RT-PCR with primers specific to corresponding genes that amplify an approximately 500-bp DNA fragment. (**a**) After reverse transcriptase reaction, PCR products of 20, 25, 30 and 35 cycles were subjected to 0.9% agarose gel electrophoresis and stained with ethidium bromide. (**b**) Relative band intensities were determined using scanned image and UN-SCAN-IT software (Silk Scientific, Orem, UT, U.S.A.).

b

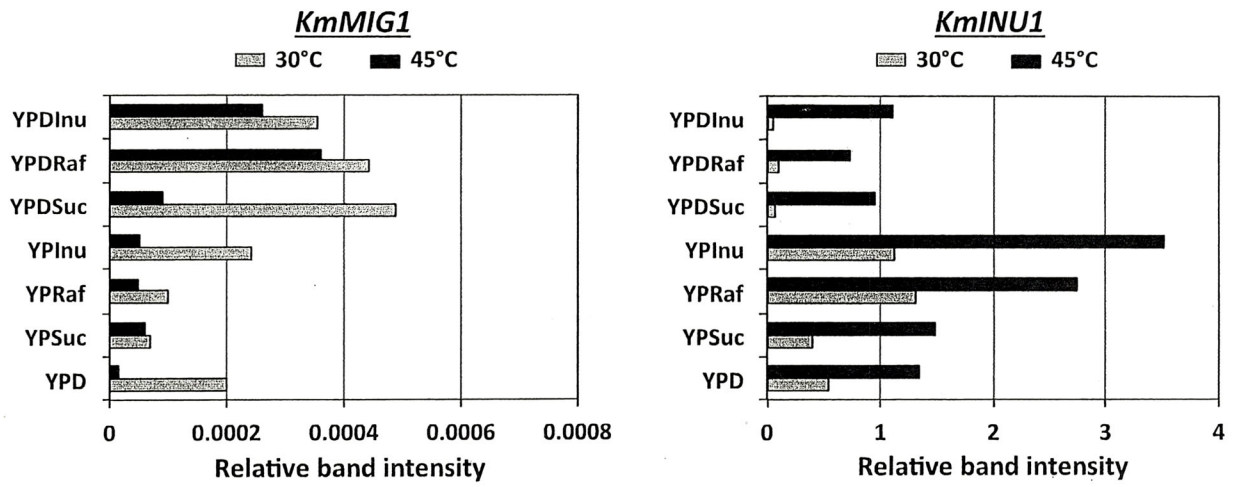



Fig. 3.6—(Continued)

## 3.5 Discussion

Results presented in this paper showed the utilization capability of Suc, Raf and Inu at a high temperature in *K. marxianus* DMKU3-1042, which is the most thermotolerant among strains available (Nonklang et al. 2008) and efficiently utilizes hexose and pentose sugars (Rodrussamee et al. 2011), as well as the glucose effects on consumption of these sugars and on the expression of *KmINU1* for inulinase responsible for their hydrolysis. This work thus also provides an insight into the fundamental mechanism of glucose repression in *K. marxianus*. The strain can assimilate the three sugars at a high temperature even under a static condition, though the respiratory yeast exhibits a sugar assimilation activity much higher under a shaking condition than that under a static condition (Rodrussamee et al. 2011). The hydrolysis and consumption of Suc, Raf or Inu in the presence of Glc were found to be preferable at a high temperature, and no detectable effect of glucose repression on Suc consumption was observed. Therefore, this strain is applicable for high-temperature ethanol fermentation with a biomass such as sugar cane juice containing mainly Suc, Glc and Frt.

Although the same inulinase is involved in the hydrolysis of Suc, Raf and Inu in *K. marxianus* DMKU3-1042, an effect of glucose repression was observed on the consumption of Raf and Inu but not on that of Suc at the low temperature (Figs. 3.1 and 3.2). The effect of sugar-specific glucose repression on consumption of sugars was consistent with that on production and secretion of inulinase, which was evaluated on the basis of inulinase activity (Table 3.3). Inconsistent results, however, were obtained by transcript analysis, revealing that *KmINU1* was down-regulated in the presence of Glc in all media tested. Coincidentally, the repression of *KmINU1* was oppositely proportional to the expressional alteration of *KmMIG1* by Glc. At the high temperature, however, no further effect of glucose repression on the consumption of Raf and Inu and on the production and secretion of inulinase was observed. Rather, the rise of temperature positively affected both production and distribution of inulinase to efficiently degrade these sugars in the strain. These results allow us to speculate that the increased inulinase production apparently

overcomes the reduction in transcript by the glucose effect. Similarly, for the phenomenon that glucose repression of the expression of *KmINU1* in YPSuc has no effect on Suc consumption, it is possible that the amount of inulinase produced under the condition of glucose repression is sufficient for cells to consume Suc as efficiently as that under the Glc-free condition.

An effect of glucose repression was observed on the utilization capability of Suc in *S. cerevisiae* but not in *K. marxianus*. The localization of Suc-hydrolyzing enzymes, invertase and inulinase in *S. cerevisiae* and *K. marxianus*, respectively, may be different or altered by cultivation conditions. In *S. cerevisiae*, almost all invertase molecules produced are retained inside the cell wall. On the other hand, a large proportion of inulinase molecules in *K. marxianus* are secreted into the culture medium (Nam et al. 1993 ). In contrast to invertase, inulinase is able to hydrolyze fructans such as inulin and levan (Kushi et al. 2000). These polysaccharides, however, are too large to enter the cell wall, and thus their hydrolysis occurs outside the cell wall (Rouwenhorst et al. 1990). We noticed that inulinase activity in the supernatant fraction at 45°C was approximately 3-times higher than that at 30°C under conditions with or without Glc except for YPInu, but the activity in the cell fraction was not altered (Table 3.3). The rise of activity in the supernatant fraction reflects the increase in *KmINU1* expression and also indicates an increase in secretion of inulinase into the culture medium. Therefore, a high temperature condition facilitates inulinase release into the culture medium presumably by change in cell wall structure as previously proposed (Rouwenhorst et al. 1988; Kushi et al. 2000).

This study has further clarified useful characteristics of *K. marxianus* DMKU3-1042 for fermentation. First, the elevation of temperature stimulates production of inulinase. This may amplify the reactivity of inulinase since the enzyme is relatively heat-resistant with optimum temperature around 50°C and 70°C for Inu and Suc as substrates, respectively (Rouwenhorst et al. 1988). Second, the elevation of temperature enhances the secretion of inulinase. Third, the consumption of these sugars is less sensitive to glucose repression. These characteristics encourage us to apply the thermotolerant yeast for high-temperature ethanol fermentation with biomass containing these sugars with Glc.

Although there are conflicting reports on the regulation of utilization of Inu among *K. marxianus* strains (Grootwassink and Fleming 1980; Grootwassink and Hewitt 1983; Parekh and Margaritis 1985; Rouwenhorst et al. 1988; Cruz-Guerrero et al. 1995; Schwan et al. 1997), our analyses revealed that the primary structure of inulinase including functional domains in different strains is highly conserved and that their inulinase genes share conserved upstream sequences including two possible Mig1 elements. Therefore, we think that the conflicting results regarding the regulation of Inu utilization are mainly due to differences in experimental conditions, including temperature, which alter the localization or activity of inulinase.

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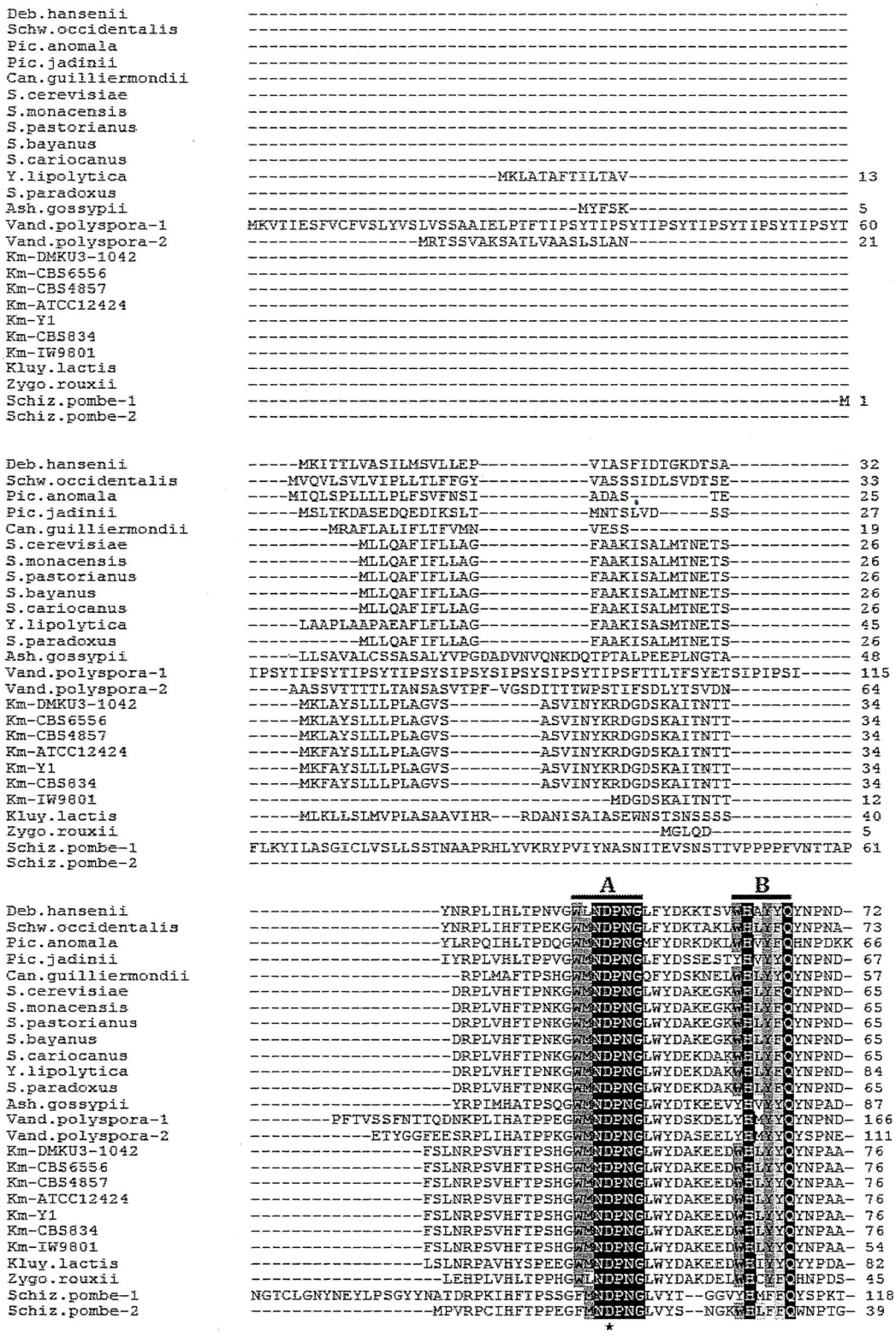
# APPENDIX A

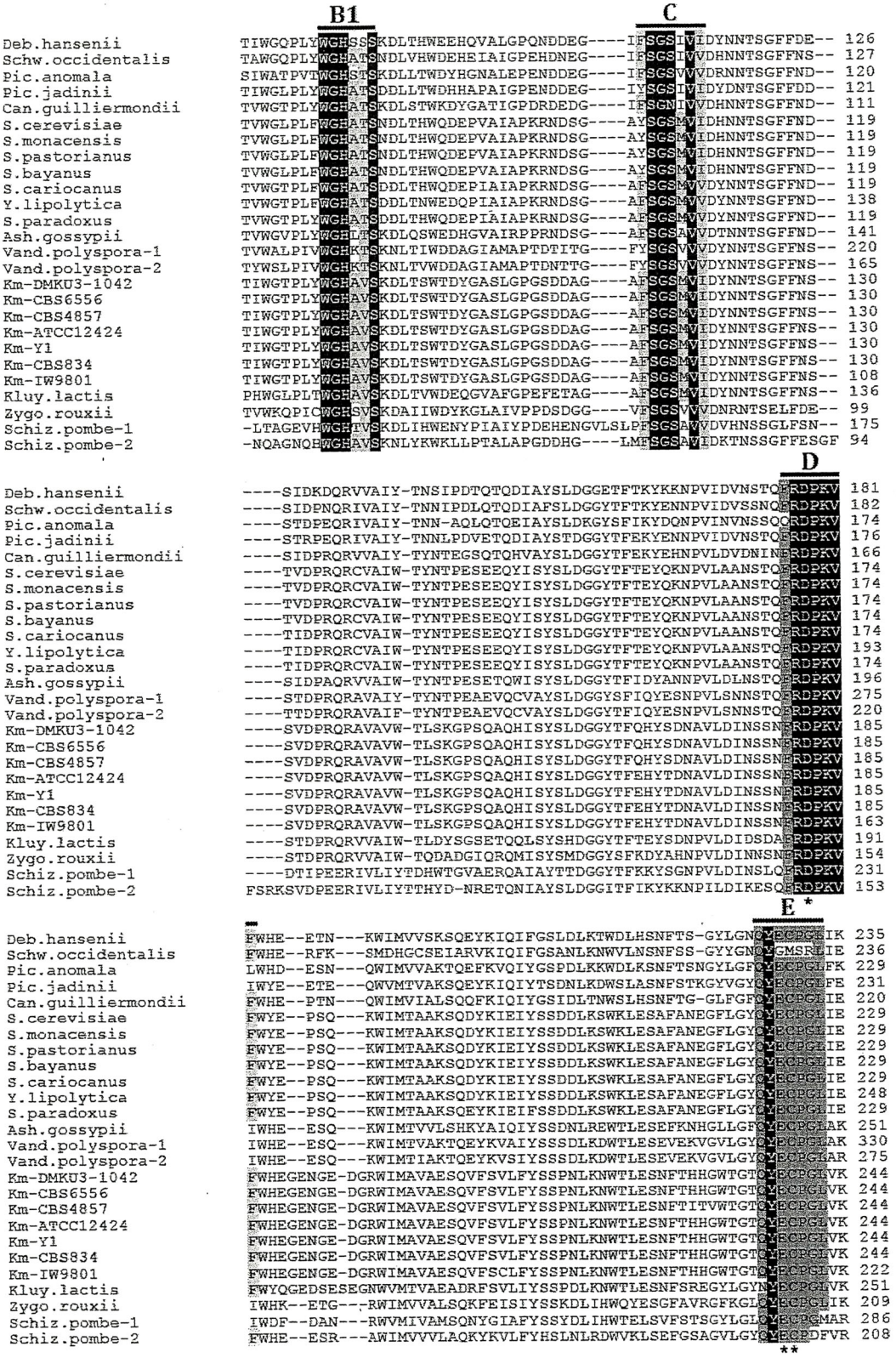
**Alignment of proteins of the glycoside hydrolase family 32 (GH32) subfamilies from yeast species**

Species are abbreviated by the following:

Deb.hansenii=*Debaryomyces hansenii*,
Schw.occidentalis=*Schwanniomyces occidentalis*,
Pic.anomala=*Pichia anomala*,
Pic.jadinii=*Pichia jadinii*,
Can.guilliermondii=*Candida guilliermondii*,
S.cerevisiae=*Saccharomyces cerevisiae*,
S.monacensis=*Saccharomyces monacensis*,
S.pastorianus=*Saccharomyces pastorianus*,
S.bayanus=*Saccharomyces bayanus*,
S.cariocanus=*Saccharomyces cariocanus*,
Y.lipolytica=*Yarrowia lipolytica*,
S.paradoxus=*Saccharomyces paradoxus*,
Ash.gossypii=*Ashbya gossypii*,
Vand.polyspora=*Vanderwaltozyma polyspora*,
Km=*Kluyveromyces marxianus*,
Kluy.lactis=*Kluyveromyces lactis*,
Zygo.rouxii=*Zygosaccharomyces rouxii*,
Schiz.pombe=*Schizosaccharomyces pombe*

Conserved residues are shaded by different intensities based on conservation level in the alignment. Residues in black show 100% conservation, residues in dark grey show ≥75% conservation, and residues in light grey show ≥50% conservation. Asterisks indicate residues previously confirmed or suspected to be part of the active site (Reddy et al. 1996). The eight conserved motifs (A, B, B1, C, D, E, F and G) are indicated at the top.


A
B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C
D
E *
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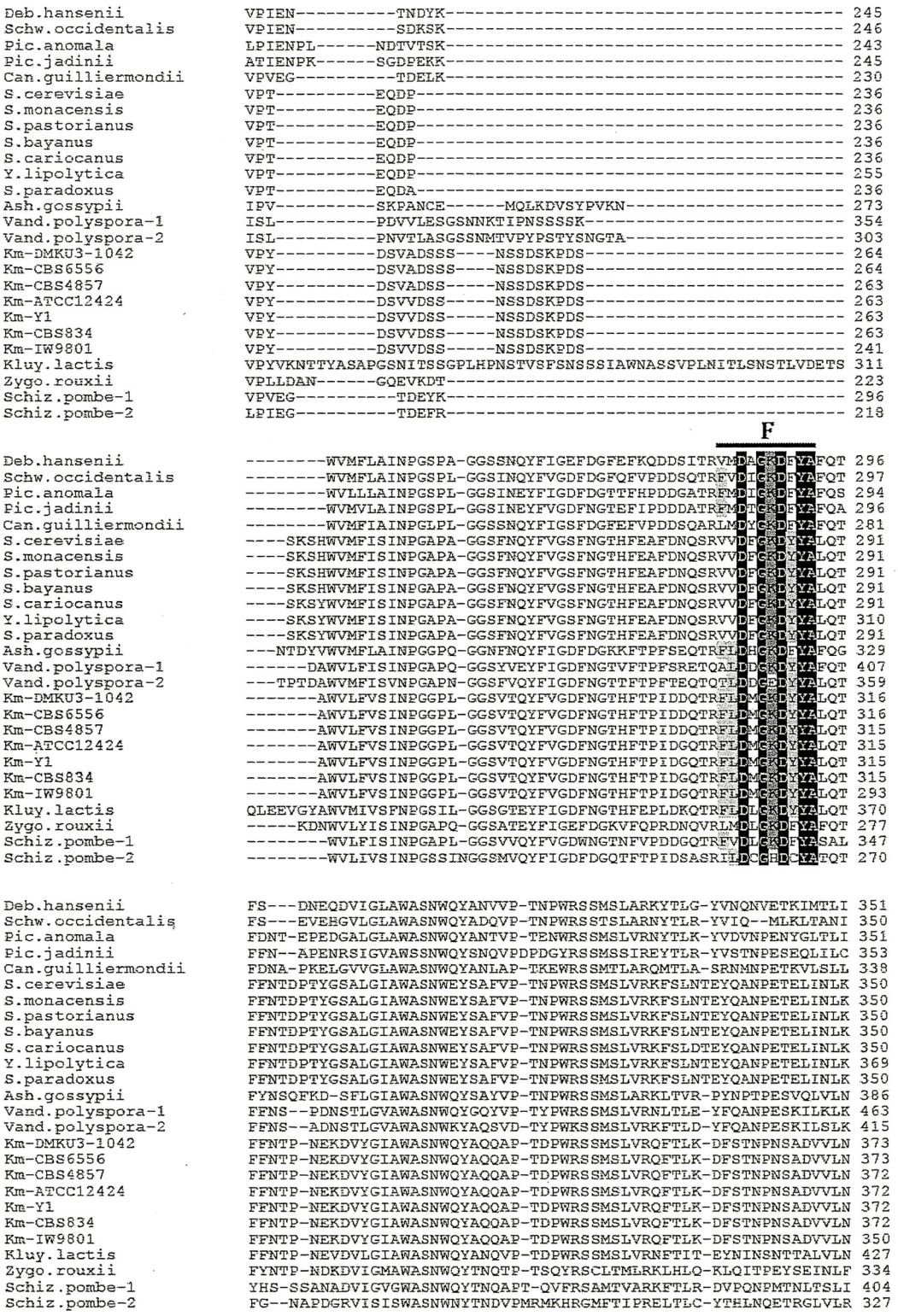
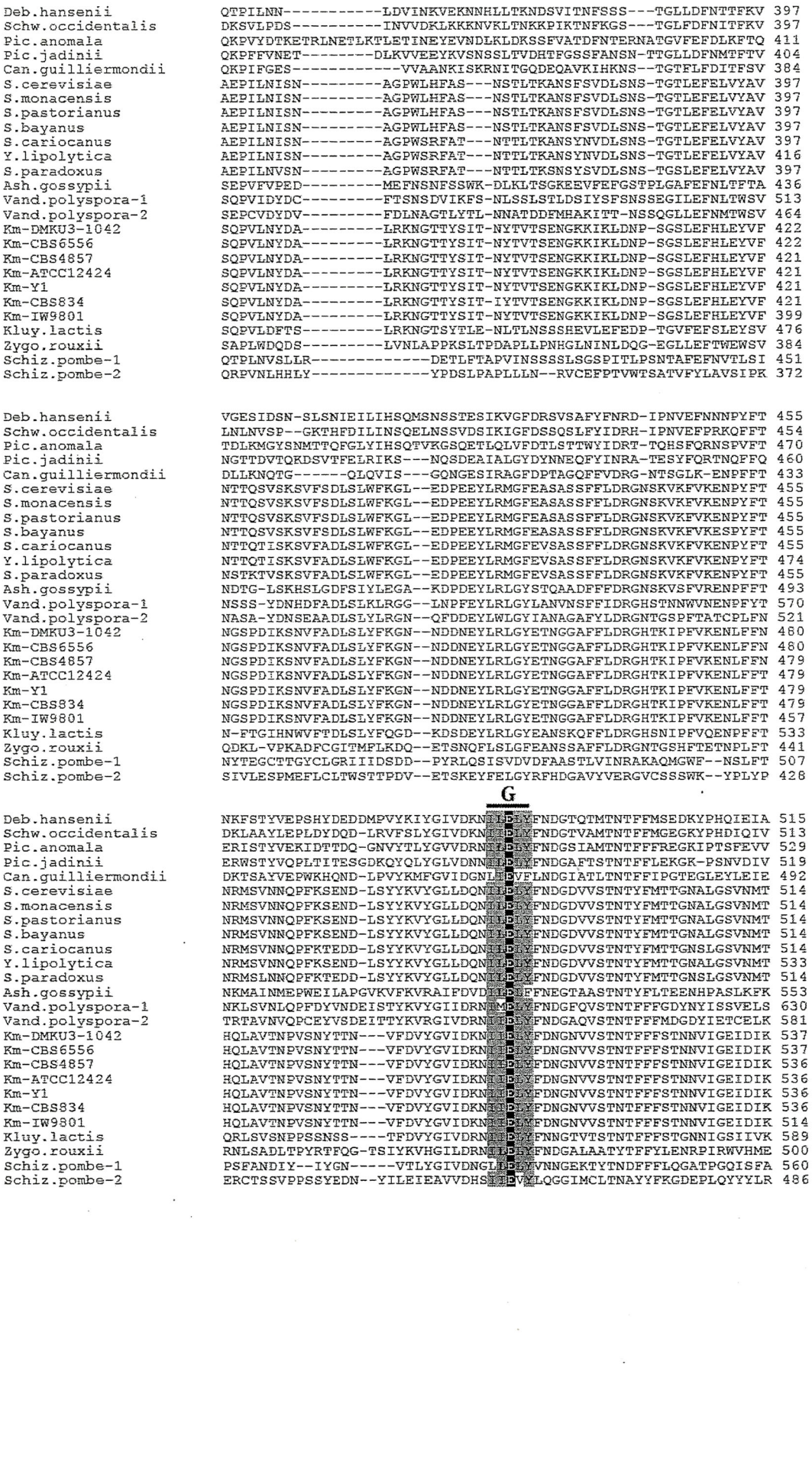
Pic.anomala
Pic.jadinii
Can.guilliermondii
S.cerevisiae
S.monacensis
S.pastorianus
S.bayanus
S.cariocanus
Y.lipolytica
S.paradoxus
Ash.gossypii
Vand.polyspora-1
Vand.polyspora-2
Km-DMKU3-1042
Km-CBS6556
Km-CBS4857
Km-ATCC12424
Km-Y1
Km-CBS834
Km-IW9801
Kluy.lactis
Zygo.rouxii
Schiz.pombe-1
Schiz.pombe-2
**


F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Schw.occidentalis DKSVLPDS----------INVVDKLKKKNVKLTNKKPIKTNFKGS---TGLFDFNITFKV 397
Pic.anomala QKPVYDTKETRLNETLKTLETINEYEVNDLKLDKSSFVATDFNTERNATGVFEFDLKFTQ 411
Pic.jadinii QKPFFVNET--------DLKVVEEYKVSNSSLTVDHTFGSSFANSN-TTGLLDFNMTFTV 404
Can.guilliermondii QKPIFGES----------VVAANKISKRNITGQDEQAVKIHKNS--TGTFLFDITFSV 384
S.cerevisiae AEPILNISN---------AGPWLHFAS---NSTLTKANSFSVDLSNS-TGTLEFELVYAV 397
S.monacensis AEPILNISN---------AGPWLHFAS---NSTLTKANSFSVDLSNS-TGTLEFELVYAV 397
S.pastorianus AEPILNISN---------AGPWLHFAS---NSTLTKANSFSVDLSNS-TGTLEFELVYAV 397
S.bayanus AEPILNISN---------AGPWLHFAS---NSTLTKANSFSVDLSNS-TGTLEFELVYAV 397
S.cariocanus AEPILNISN---------AGPWSRFAT---NTTLTKANSYNVDLSNS-TGTLEFELVYAV 397
Y.lipolytica AEPILNISN---------AGPWSRFAT---NTTLTKANSYNVDLSNS-TGTLEFELVYAV 416
S.paradoxus AEPILNVSN---------AGPWSRFAT---NTTLTKSNSYSVDLSNS-TGSLEFELVYAV 397
Ash.gossypii SEPVFVPED---------MEFNSNFSSWK-DLKLTSGKEEVPEFGSTPLGAFEFNLTFTA 436
Vand.polyspora-1 SQPVIDYDC---------FTSNSDVIKFS-NLSSLSTLDSIYSFSNSSEGILEFNLTWSV 513
Vand.polyspora-2 SEPCVDYDV---------FDLNAGTLYTL-NNATDDFMHAKITT-NSSQGLLEFNMTWSV 464
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Km-CBS4857 SQPVLNYDA---------LRKNGTTYSIT-NYTVTSENGKKIKLDNP-SGSLEFHLEYVF 421
Km-ATCC12424 SQPVLNYDA---------LRKNGTTYSIT-NYTVTSENGKKIKLDNP-SGSLEFHLEYVF 421
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Km-IW9801 SQPVLNYDA---------LRKNGTTYSIT-NYTVTSENGKKIKLDNP-SGSLEFHLEYVF 399
Kluy.lactis SQPVLDFTS---------LRKNGTSYTLE-NLTLNSSSHEVLEFEDP-TGVFEFSLEYSV 476
Zygo.rouxii SAPLWDQDS---------LVNLAPPKSLTPDAPLLPNHGLNINLDQG-EGLLEFTWEWSV 384
Schiz.pombe-1 QTPLNVSLLR----------DETLFTAPVINSSSSLSGSPITLPSNTAFEFNVTLSI 451
Schiz.pombe-2 QRPVNLHHLY----------YPDSLPAPLLLN--RVCEFPTVWTSATVFYLAVSIPK 372
Deb.hansenii VGESIDSN-SLSNIEILIHSQMSNSSTESIKVGFDRSVSAFYFNRD-IPNVEFNNNPYFT 455
Schw.occidentalis LNLNVSP--GKTHFDILINSQELNSSVDSIKIGFDSSQSLFYIDRH-IPNVEFPRKQFFT 454
Pic.anomala TDLKMGYSNMTTQFGLYIHSQTVKGSQETLQLVFDTLSTTWYIDRT-TQHSFQRNSPVFT 470
Pic.jadinii NGTTDVTQKDSVTFELRIKS---NQSDEAIALGYDYNNEQFYINRA-TESYFQRTNQFFQ 460
Can.guilliermondii DLLKNQTG------QLQVIS---GQNGESIRAGFDPTAGQFFVDRG-NTSGLK-ENPFFT 433
S.cerevisiae NTTQSVSKSVFSDLSLWFKGL--EDPEEYLRMGFEASASSFFLDRGNSKVKFVKENPYFT 455
S.monacensis NTTQSVSKSVFSDLSLWFKGL--EDPEEYLRMGFEASASSFFLDRGNSKVKFVKENPYFT 455
S.pastorianus NTTQSVSKSVFSDLSLWFKGL--EDPEEYLRMGFEASASSFFLDRGNSKVKFVKENPYFT 455
S.bayanus NTTQSVSKSVFSDLSLWFKGL--EDPEEYLRMGFEASASSFFLDRGNSKVKFVKESPYFT 455
S.cariocanus NTTQTISKSVFADLSLWFKGL--EDPEEYLRMGFEVSASSFFLDRGNSKVKFVKENPYFT 455
Y.lipolytica NTTQTISKSVFADLSLWFKGL--EDPEEYLRMGFEVSASSFFLDRGNSKVKFVKENPYFT 474
S.paradoxus NSTKTVSKSVFADLSLWFKGL--EDPEEYLRMGFEVSASSFFLDRGNSKVKFVKENPYFT 455
Ash.gossypii NDTG-LSKHSLGDFSIYLEGA--KDPDEYLRLGYSTQAADFFFDRGNSKVSFVRENPFFT 493
Vand.polyspora-1 NSSS-YDNHDFADLSLKLRGG--LNPFEYLRLGYLANVNSFFIDRGHSTNNWVNENPFYT 570
Vand.polyspora-2 NASA-YDNSEAADLSLYLRGN--QFDDEYLWLGYIANAGAFYLDRGNTGSPFTATCPLFN 521
Km-DMKU3-1042 NGSPDIKSNVFADLSLYFKGN--NDDNEYLRLGYETNGGAFFLDRGHTKIPFVKENLFFN 460
Km-CBS6556 NGSPDIKSNVFADLSLYFKGN--NDDNEYLRLGYETNGGAFFLDRGHTKIPFVKENLFFN 460
Km-CBS4857 NGSPDIKSNVFADLSLYFKGN--NDDNEYLRLGYETNGGAFFLDRGHTKIPFVKENLFFN 479
Km-ATCC12424 NGSPDIKSNVFADLSLYFKGN--NDDNEYLRLGYETNGGAFFLDRGHTKIPFVKENLFFT 479
Km-Y1 NGSPDIKSNVFADLSLYFKGN--NDDNEYLRLGYETNGGAFFLDRGHTKIPFVKENLFFT 479
Km-CBS834 NGSPDIKSNVFADLSLYFKGN--NDDNEYLRLGYETNGGAFFLDRGHTKIPFVKENLFFT 479
Km-IW9801 NGSPDIKSNVFADLSLYFKGN--NDDNEYLRLGYETNGGAFFLDRGHTKIPFVKENLFFT 457
Kluy.lactis N-FTGIHNWVFTDLSLYFQGD--KDSDEYLRLGYEANSKQFFLDRGHSNIPFVQENPFFT 533
Zygo.rouxii QDKL-VPKADFCGITMPLKDQ--ETSNQFLSLGFEANSSAFFLDRGNTGSHFTETNPLFT 441
Schiz.pombe-1 NYTEGCTTGYCLGRIIIDSDD--PYRLQSISVDVDFAASTLVINRAKAQMGWF--NSLFT 507
Schiz.pombe-2 SIVLESPMEFLCLTWSTTPDV--ETSKEYFELGYRFHDGAVYVERGVCSSSWK--YPLYP 428
G
Deb.hansenii NKFSTYVEPSHYDEDDMPVYKIYGIVDKNILELYFNDGTQTMTNTFFMSEDKYPHQIEIA 515
Schw.occidentalis DKLAAYLEPLDYDQD-LRVFSLYGIVDKNIIELYFNDGTVAMTNTFFMGEGKYPHDIQIV 513
Pic.anomala ERISTYVEKIDTTDQ-GNVYTLYGVVDRNILELYFNDGSIAMTNTFFFREGKIPTSFEVV 529
Pic.jadinii ERWSTYVQPLTITESGDKQYQLYGLVDNNILELYFNDGAFTSTNTFFLEKGK-PSNVDIV 519
Can.guilliermondii DKTSAYVEPWKHQND-LPVYKMFGVIDGNLIEVFLNDGIATLTNTFFIPGTEGLEYLEIE 492
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S.monacensis NRMSVNNQPFKSEND-LSYYKVYGLLDQNILELYFNDGDVVSTNTYFMTTGNALGSVNMT 514
S.pastorianus NRMSVNNQPFKSEND-LSYYKVYGLLDQNILELYFNDGDVVSTNTYFMTTGNALGSVNMT 514
S.bayanus NRMSVNNQPFKSEND-LSYYKVYGLLDQNILELYFNDGDVVSTNTYFMTTGNALGSVNMT 514
S.cariocanus NRMSVNNQPFKTEDD-LSYYKVYGLLDQNILELYFNDGDVVSTNTYFMTTGNSLGSVNMT 514
Y.lipolytica NRMSVNNQPFKSEND-LSYYKVYGLLDQNILELYFNDGDVVSTNTYFMTTGNALGSVNMT 533
S.paradoxus NRMSLNNQPFKTEDD-LSYYKVYGLLDQNILELYFNDGDVVSTNTYFMTTGNSLGSVNMT 514
Ash.gossypii NKMAINMEPWEILAPGVKVFKVRAIFDVDILELFFNEGTAASTNTYFLTEENHPASLKFK 553
Vand.polyspora-1 NKLSVNLQPFDYVNDEISTYKVYGIIDRNIMELYFNDGFQVSTNTFFFGDYNYISSVELS 630
Vand.polyspora-2 TRTAVNVQPCEYVSDEITTYKVRGIVDRNIIELYFNDGAQVSTNTFFFMDGDYIETCELK 581
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Km-CBS834 HQLAVTNPVSNYTTN---VFDVYGVIDKNIIELYFDNGNVVSTNTFFFSTNNVIGEIDIK 536
Km-IW9801 HQLAVTNPVSNYTTN---VFDVYGVIDKNIIELYFDNGNVVSTNTFFFSTNNVIGEIDIK 514
Kluy.lactis QRLSVSNPPSSNSS----TFDVYGIVDRNIIELYFNNGTVTSTNTFFFSTGNNIGSIIVK 589
Zygo.rouxii RNLSADLTPYRTFQG-TSIYKVHGILDRNIIELYFNDGALAATYTFFYLENRPIRWVHME 500
Schiz.pombe-1 PSFANDIY--IYGN-----VTLYGIVDNGLIELYVNNGEKTYTNDFFFLQGATPGQISFA 560
Schiz.pombe-2 ERCTSSVPPSSYEDN--YILEIEAVVDHSIIEVYLQGGIMCLTNAYYFKGDEPLQYYYLR 486

```
Deb.hansenii              SNVDG-QFELQSLLIRELNM------- 534
Schw.occidentalis         IDTEEPLFELESWIIRELNK------- 533
Pic.anomala               CDSEKSFITIDELSVRELARK------ 550
Pic.jadinii               ASSSK------EAYHRGPAD------- 533
Can.guilliermondii        SSSDAIHIVELEVKELKLRATS----- 514
S.cerevisiae              TGVDN-LFYIDKFQVREVK-------- 532
S.monacensis              TGVDN-LFYIDKFQVREVK-------- 532
S.pastorianus             TGVDN-LFYIDKFQVREVK-------- 532
S.bayanus                 TGVDN-LFYIDKFQVREVK-------- 532
S.cariocanus              -------------------------
Y.lipolytica              TGVDN-LFYIDKFQVREVK-------- 551
S.paradoxus               TGVDD-LFYIDKFQVREVK-------- 532
Ash.gossypii              TSVDN-VFIVNELSLRQLTF------- 572
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Kluy.lactis               SGVDD-VYEIESLKVNQFYVD------ 609
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Schiz.pombe-2             VPTGASLAKSGMQPLLNNRPHS----- 508
```
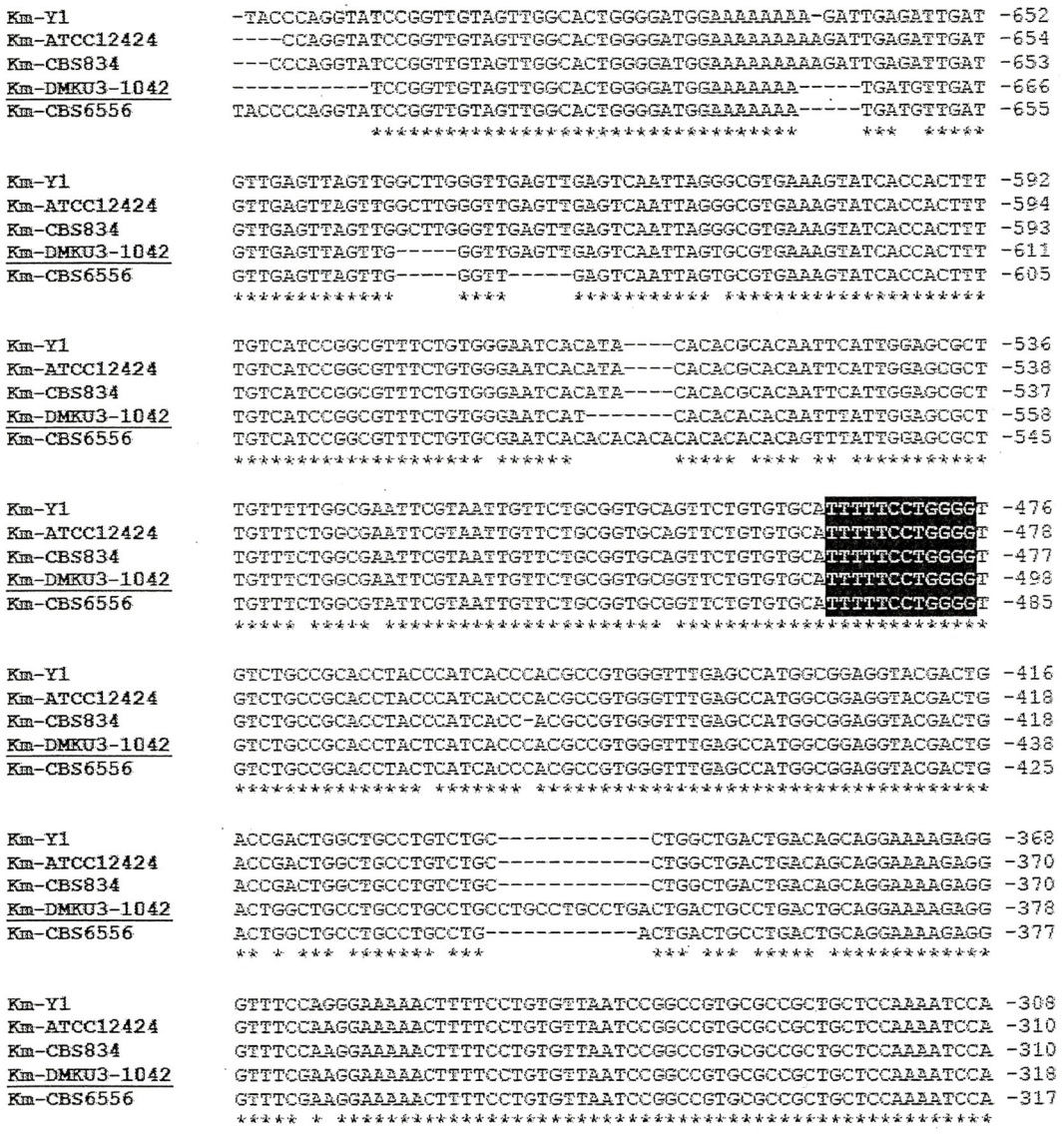
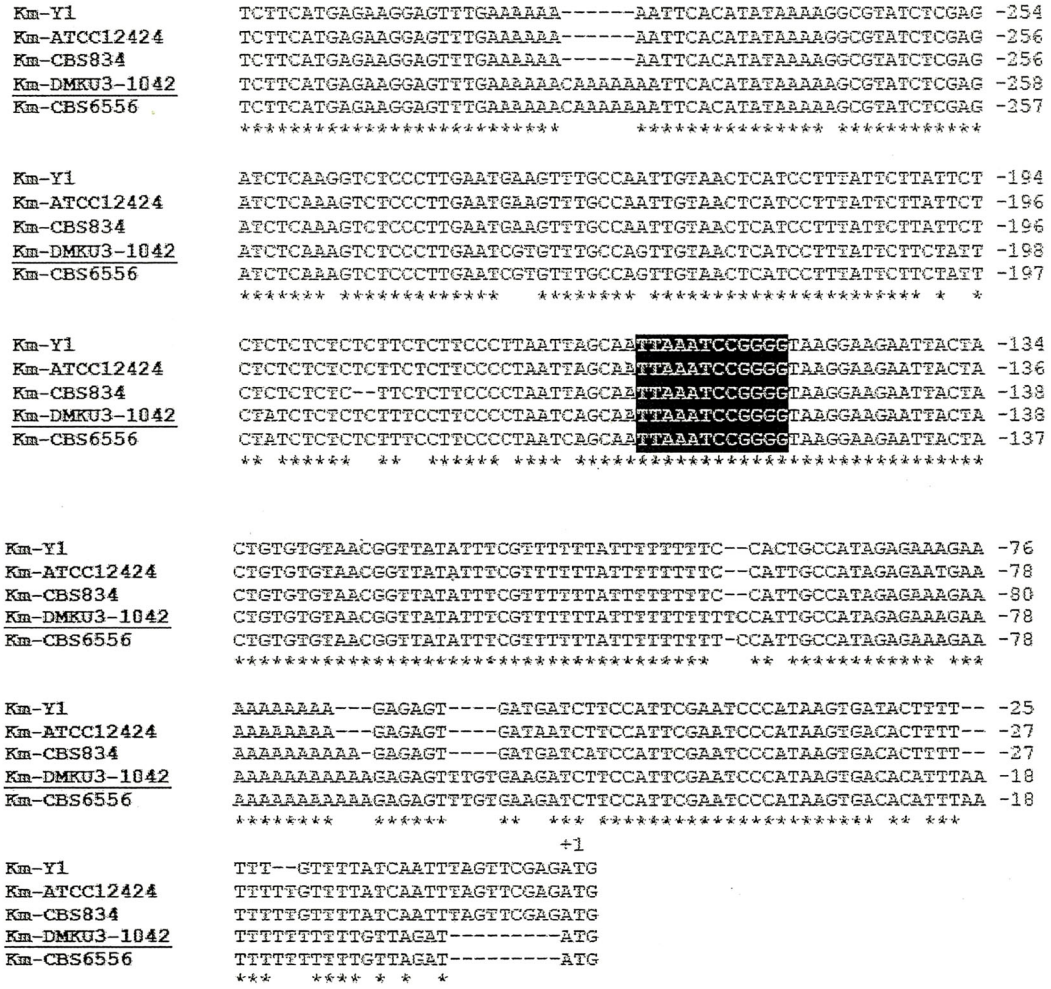

# APPENDIX B

## Alignment of upstream sequences of inulinase genes from various strains of *K. marxianus*

Km = *Kluyveromyces marxianus.* Strains names are indicated after Km-. Asterisks indicate conserved nucleotides. The putative binding sites of KmMig1p are shaded.

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Km-CBS6556 CTATCTCTCTCTTTCCTTCCCCTAATCAGCAATTAAATCCGGGGTAAGGAAGAATTACTA -137
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Km-CBS6556 CTGTGTGTAACGGTTATATTTCGTTTTTTATTTTTTTTT-CCATTGCCATAGAGAAAGAA -78
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+1
Km-Y1 TTT--GTTTTATCAATTTAGTTCGAGATG
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Km-CBS6556 TTTTTTTTTTGTTAGAT---------ATG

# SUMMARY

## Ethanol Fermentation Ability on Various Sugars and Glucose Repression in Thermotolerant Yeast *Kluyveromyces marxianus*

## (耐熱性酵母 *Kluyveromyces marxianus* における多様な糖に対するエタノール発酵能とグルコース抑制)

Alternatives to petroleum-derived fuels are being aspired in order to reduce the utilization of non-renewable resources. Ethanol derived from corn grain (starch) and sugar cane (sucrose) is the most common and currently renewable fuel but is expected to become insufficient in the near future. Therefore, the establishment of a new technology for economical utilization of lignocellulosic biomass as an attractive feedstock is crucial for enough ethanol supply. Since lignocellulose consists of glucose, galactose, mannose, xylose and arabinose, microorganisms efficiently fermenting these sugars are desired. One of such microbes is *Kluyveromyces marxianus*, which has a useful potential to assimilate a wide variety of substrates, thermotolerance and high growth rate and is thus an alternative to *S. cerevisiae* as an ethanol producer.

The potential of *K. marxianus* DMKU3-1042 for utilization of and ethanol production from sugars present in hemicellulose hydrolysate was examined under shaking and static conditions at high temperatures. The yeast was found to produce ethanol from all of these sugars except for arabinose under a shaking condition but only from hexose sugars under a static condition. Growth and sugar utilization rate under a static condition were slower than those under a shaking condition, but maximum ethanol yield was slightly higher and maintain in constant level. Even at 40°C, a level of ethanol production similar to that at 30°C was observed except for galactose under a static condition. Glucose repression on assimilation of other sugars was also tested not only by the addition of glucose but also by the addition of 2-deoxyglucose (2-DOG). Consistent results were obtained that glucose repression was found on galactose, xylose, and arabinose utilization and more evident at elevated temperatures. In addition, the expression of genes

responsible for utilization of other sugars was analyzed. It was found that *KmGAL1* for galactokinase and *KmXYL1* for xylose reductase for galactose and xylose/arabinose utilization, respectively, were repressed by glucose at low and high temperatures, but *KmHXK2* for hexokinase was not repressed. The possible regulations of glucose repression of *KmGAL1* and *KmXYL1* were *KmGAL1* might contain a putative Mig1p-binding site(s) with a different sequence from those in *S. cerevisiae* and *KmXYL1* might have other regulatory pathways instead of Mig1p, respectively.

In addition to the far more abundant lignocellulosic feedstocks, inulin-rich plants have been considered for fuel ethanol production. Besides, *K. marxianas* has been used as a source of inulinase, which hydrolyses sucrose, raffinose and inulin. This enzyme has a particular interest, because this enzyme is not commonly found in other yeasts or fungi. Glucose effect on the activity of inulinase has been demonstrated in *K. marxianus* without analysis at the gene level. To clarify the utilization capability of sucrose, raffinose and inulin and the glucose effect on inulinase in *K. marxianus* DMKU3-1042, its growth and metabolite profiles on these sugars were examined with or without glucose under a static condition, in which glucose repression evidently occurs. Consumption of sucrose was not influenced by glucose or 2-DOG. On the other hand, raffinose and inulin consumption was hampered by glucose at 30°C but hardly hampered at 45°C. Unlike *S. cerevisiae*, increase in glucose concentration had no effect on sucrose utilization. These sugar-specific glucose effects were consistent with the level of inulinase activity but not with that of the *KmINU1* transcript, which was repressed in the presence of glucose via KmMig1p. This inconsistence may be due to sufficient activity of inulinase even when glucose is present.

This study provides valuable information for application of *K. marxianus* DMKU3-1042 and indicates its usefulness for high-temperature ethanol fermentation with biomass containing various sugars with glucose.

# 学位論文要旨

## 耐熱性酵母 *Kluyveromyces marxianus* における多様な糖に対するエタノール発酵能とグルコース抑制

## (Ethanol Fermentation Ability on Various Sugars and Glucose Repression in Thermotolerant Yeast *Kluyveromyces marxianus*)

非再生可能資源の使用量を減少させるために、化石燃料の代替物の開発が望まれている。穀物（でんぷん）やサトウキビ（蔗糖）から生産されるエタノールは、最も一般的な再生可能資源であるが、近い将来不足すると予想される。従って、食物残査に含まれるリグノセルロース系バイオマスを経済的に利用する新たな技術の樹立が十分なエタノール供給のために不可欠である。リグノセルロースはグルコース、ガラクトース、マンノース、キシロースやアラビノースなど含んでいることからそれらの糖を効率的に発酵する微生物の開発が望まれる。そのような微生物の１つである *Kluyveromyces marxianus* は、広い糖資化性、耐熱性、早い増殖速度などの特性をもち、*Saccharomyces cerevisiae* に替わるエタノール生産酵母となると期待されている。

*K. marxianus* DMKU3-1042 についてヘミセルロース加水分解物に含まれる種々の糖の資化性やエタノール生産性を高温振とう培養条件および高温静置培養条件で検討した。この酵母は アラビノースを除く検討した全ての糖からエタノールを生産した。静置培養条件での生育と糖資化性は、振とう培養条件のものより遅いが、最大エタノール収率は若干高く一定に保たれた。40° C 静置培養条件において、ガラクトース以外の糖で 30° C と同様なレベルのエタノール生産が観察された。他の糖の資化に対するグルコース抑制についてグルコース添加だけでなく 2-デオキシグルコースを用いて調べた。グルコース抑制がガラクトース、キシロース、アラビノースにおいて観察され、高温でより顕著であった。加えて、ガラクトースやキシロース／アラビノース資化に関与するガラクトースキナーゼ遺伝子 *KmGAL1* の発現やキシロース還元酵素遺伝子 *KmXYL1* の発現が低温並びに高温でグルコースによって抑制された。

しかし、ヘキソキナーゼ遺伝子 *KmHXK2* の発現は抑制されなかった。*KmGAL1* は Mig1p 結合配列と見なされる（但し、*S. cerevisiae* の Mig1p 結合配列とは異なる）配列をもち、*KmXYL1* は Mig1p 以外の制御系をもつかもしれない。

豊富なリグノセルロース系バイオマスに加えて、イヌリンを多く含むバイオマスもエタノール燃料生産に良い原料であると考えられている。一方、*K. marxianas* は蔗糖、ラフィノース、イヌリンを加水分解するイヌリナーゼの供給源である。この酵素は他の酵母やカビに存在しないことから興味がもたれている。 これまでイヌリナーゼ活性に対するグルコース効果が調べられているが、遺伝子レベルのものはない。そこで、*K. marxianus* DMKU3-1042 について、蔗糖、ラフィノース、イヌリンの資化能や イヌリナーゼ に対するグルコース効果を明らかにするために、それらの糖について増殖や代謝プロファイルを静置培養条件（グルコース抑制が顕著に観察される条件）でグルコース添加あるいは無添加で検討した。蔗糖の消費はグルコースや 2-デオキシグルコースによって影響を受けなかった。一方、ラフィノースやイヌリンは 30°C でグルコースによって抑制されたが、45°C では抑制されなかった。*S. cerevisiae* とは異なり、グルコースの濃度を増加しても蔗糖消費に影響は見られなかった。このような糖特異的なグルコースの影響はイヌリナーゼ酵素活性のレベルと一致したが、イヌリナーゼ酵素遺伝子転写レベルとは一致しなかった（イヌリナーゼ酵素遺伝子転写は KmMig1p を介して抑制された）。この食い違いはグルコースが存在しても十分なイヌリナーゼ酵素活性があるためと推測された。

本研究は、*K. marxianus* DMKU3-1042 の利用における貴重な情報を与えると同時に、グルコースを含む多数の糖からなるバイオマスを用いた高温エタノール発酵への同酵母の有用性を示唆する。

# LIST OF PUBLICATION

This thesis is based on the following two studies, which are referred to in chapter 2 and 3.

**1. Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus***

Nadchanok Rodrussamee, Noppon Lertwattanasakul, Katsushi Hirata, Suprayogi, Savitree Limtong, Tomoyuki Kosaka and Mamoru Yamada

Appl Microbiol Biotechnol (2011), 90(4):1573-1586

**(CHAPTER 2)**

**2. Utilization capability of sucrose, raffinose and inulin and its less-sensitiveness to glucose repression in thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042**

Noppon Lertwattanasakul, Nadchanok Rodrussamee, Suprayogi, Savitree Limtong, Pornthap Thanonkeo, Tomoyuki Kosaka, Mamoru Yamada

AMB Express (2011), In press

**(CHAPTER 3)**

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**PRESENTATION**

- The 18th Annual Meeting of the Thai Society for Biotechnololy; Biotechnology: Benefits&Bioethics; November 2-3, 2006. The Montien Riverside Hotel, Bangkok, Thailand. Title: Cloning and characterization of pediocin gene from *Pediococcocus pentosaceus*

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- JSPS-NRCT Asian Core Program 1st Joint Seminar on Capacity Building and Development of Microbial Potential and Fermentation Technology towards New Era 20-21 March, 2009 at 8th Floor, Graduate School, Kasetsart University. (Title: Ethanol production in *Kluyveromyces marxianus* from xylose).

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- The 28th International Specialised Symposium on Yeasts: Metabolic and Bioprocess Engineering for Sustainable Development 15-18 Sep 2010, Montien Riverside Hotel, Bangkok, Thailand. Title: Basic analysis of themotolerant yeast *Kluyveromyces marxianus* for high-temperature ethanol fermentation.

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