CHARACTERIZATION AND IMPROVEMENT OF THERMOTOLERANT ETHANOLOGENIC MICROBES FOR LOW-COST BIOETHANOL PRODUCTION FROM VARIOUS FEEDSTOCKS

(種々の原料からの低コストバイオエタノール生産のための耐熱性エタノール生産性微生物の特性と改良)

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase gene
Ara	arabinose
ATP	Adenosine triphosphate
CBP	Consolidated bioprocessing
CGM	corn gluten meal
CGF	corn gluten feed
CO_2	carbon dioxide
2-DOG	2-deoxyglucose
dH_2O	distilled water
DNA	deoxyribonucleic acid
ED	Entner–Doudoroff pathway
EDA	2-keto-3-deoxy-gluconate adolase
EDD	6-phosphogluconate dehydratase
EMP	Embden-Meyerhof-Parnas pathway
ENO	enolase
FBPA	fructose bisphosphate aldolase
$FeSO_4$	ferrous sulfate
FOS	fructose oligosaccharides
Gal	galactose
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
Glc	glucose
GLK	Glucokinase
GRAS	generally recognized as having a safe
H^+	hydrogen ions
HK	hexokinase
H_2O_2	hydrogen peroxide
HMF	5-hydroxymethylfurfural
HPLC	High-performance liquid chromatography
KMYPD	Yeast-peptone-glucose medium for *Kluyveromyces marxianus*

LIST OF ABBREVIATIONS

(Continued)

LB	Luria-Bertani
Man	mannose
Mbar	millibar
MnSOD	Manganese-dependent superoxide dismutase
n/a	not available
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
$O_2^{\bullet}$	superoxide radical
OD	optical density
$OH^{\bullet}$	hydroxyl radical
OPT	putative glutathione transporter gene
PDC	pyruvate decarboxylase
PFK	phosphofructokinase
PGI	phosphoglucoisomerase
PGK	phosphoglycerate kinase
PGM	phosphoglyceromutase
PGL	phosphogluconolastonase
PYK	pyruvate kinase
RKI	Ribose-5-phosphate isomerase gene
ROS	reactive oxygen species
rpm	round per minute
SD	standard deviation
SSF	simultaneous saccharification and fermentation
SSCF	simultaneous saccharification and co-fermentation
TCA	tricarboxylic acid cycle
T_{max}	maximum temperature
TPI	triose phosphate isomerase
UV	ultraviolet
YP	yeast extract and peptone

LIST OF ABBREVIATIONS

(Continued)

YPAra	Yeast-peptone-arabinose medium
YPD	Yeast-peptone-glucose medium
YPGal	Yeast-peptone-galactose medium
YPXyl	Yeast-peptone-xylose medium
YPMan	Yeast-peptone-mannose medium
ZMYPD	Yeast-peptone-glucose medium for *Zymomonas mobilis*
ZWF	Glucose-6-phosphate dehydrogenase

CHAPTER 1

General Introduction and Literature Review

1.1 General introduction

Bioethanol is one of alternative and renewable fuels made from various plant biomasses by ethanologenic microorganisms. U.S. and Brazil are not only the petroleum-producing countries but also the largest and the second largest bioethanol producers, respectively. The two countries account for 86% of global bioethanol production and are the major bioethanol exporters. To address energy security, the governments of Asian countries, especially Thailand, are also promoting bioethanol production program. The bioethanol can reduce the dependence of fossil fuel and can also shift from foreign energy imports to domestically produced energy. Moreover, bioethanol production can contribute to boosting agricultural and rural development by creating new job opportunities and new income sources for farmers and rural residences. At present, major feedstocks for bioethanol production are sugar and starch base biomass such as corn, sugarcane and cassava. One of the most crucial problems with increasing bioethanol production is that it competes for agricultural resources with food production and food-related use. According to the latest data available (2012), 15.1% of global corn production, 17.0% of global sugarcane production and 0.6% of global cassava production were used to produce bioethanol. Moreover, current bioethanol production in tropical countries has experienced some problems like high temperature in summer season and high processing costs. To achieve sustainable energy, ethanol production from lignocellulosic biomass which does not undermine the food supply was challenged. Moreover, the application of a thermotolerant, stress-resistant and highly efficient microbe for ethanol fermentation is a crucial point for industrial application.

1.2 Literature Review

1.2.1 Biomass feedstocks

The term biomass (Greek *bio*, life + *maza* or mass) refers to non-fossilized and biodegradable organic materials derived from plants, animals and microorganisms. Biomass includes forest products, agricultural crops such as energy crops, food crops, sugar crops, biorenewable wastes, animal wastes, the organic portion of municipal solid waste, aquatic plants, algae, mosses and lichens (Table 1.1).

Table 1.1 Major categories of biomass feedstocks (Demirbas and Demirbas 2010)

Forest products	Wood; logging residues; trees, shrubs and wood residues; sawdust, bark, etc.
Energy crops	Short-rotation woody crops, herbaceous woody crops, grasses, starch crops, sugar crops, forage crops, oilseed crops, switchgrass, miscanthus
Food crops	Grains, oil crops
Sugar crops	Sugar cane, sugar beets, molasses, sorghum
Biorenewable wastes	Agricultural wastes, crop residues, mill wood wastes, urban wood wastes, urban organic wastes
Organic wastes	Municipal solid waste, industrial organic wastes, municipal sewage and sludges
Animal wastes	Manure from dairy cows, feedlot cattle or pigs
Aquatic plants	Algae, Water weed, water hyacinth, reed and rushes
Algae	Prokaryotic algae, eukaryotic algae, kelps
Mosses	Bryophyta, polytrichales
Lichens	Crustose lichens, foliose lichens, fruticose lichen

Biomass provides a clean, renewable energy source that could dramatically improve our environment, economy and energy security. Biomass energy generates smaller CO_2 emissions than fossil fuels, reduces the amount of waste, decreases oil import and creates job opportunities for rural communities. There are three ways to use biomass. It can be burned to produce heat and electricity, changed to gaslike fuels such as methane and hydrogen, or changed to a liquid fuel such as ethanol and methanol.

1.2.2 Ethanol production from biomass feedstocks

Based on the source of biomass, bioethanol is classified broadly into three major categories.

1.2.2.1 The first generation bioethanol

Ethanol produced by fermentation with sugar and starch based feedstocks which have been used for several decades is referred to as “the first generation” bioethanol. When sugar feedstocks such as sugar cane, molasses, sugar beet and sweet sorghum are used for ethanol fermentation, yeast can directly consume simple sugars and convert to ethanol. However, yeast cannot directly utilize starch feedstocks such as corn, wheat, rice or cassava which usually contains a mixture of linear (amylose) and branched (amylopectin) polyglucans. They have to be converted or depolymerized to glucose prior to yeast fermentation. Depolymerization or hydrolysis of starch can be achieved by the treatment of acid or enzyme or a combination of both (Klanarong et al. 2012). The key enzyme for starch hydrolysis is α-amylase which hydrolyzes α-1,4 but not α-1,6 linkages (in amylopectin), consequently, amylose is broken down to maltose and maltotriose and the latter to be free glucose and maltose, wheres amylopectin is hydrolyzed to a mixture of maltose, glucose and oligosaccharides containing α-1,6-linked glucose residues, thus limiting the amount of fermentable sugars liberated (Fig.1.1) (Davis 2008). Glucose is then subsequentially converted to ethanol by yeast.

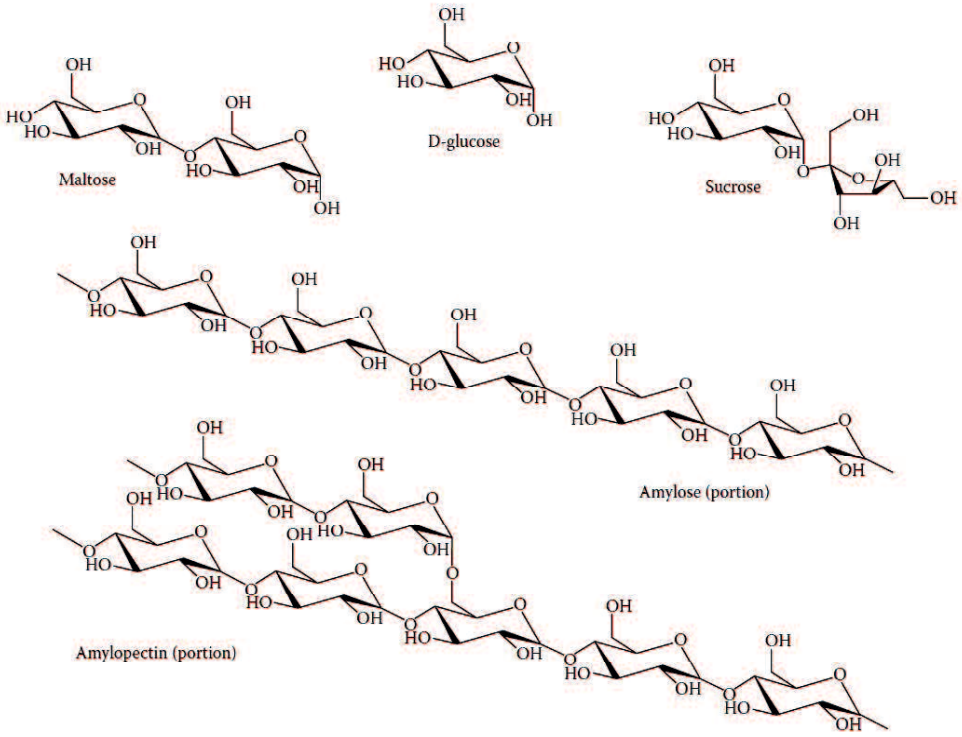

Fig. 1.1 Chemical structures of glucose, disaccharides, and components of starch

1.2.2.2 The second generation bioethanol

The first-generation bioethanol productions from food crops possess certain limitations as it tends to cause food crisis. In order to overcome the problem, the second generation bioethanol which is produced from lignocellulosic materials is a promising alternative and potentially offers greater cost reduction in the longer term. Lignocellulosic biomass is referred to plant biomass which mainly consists of three organic compounds: cellulose, hemicelluloses and lignin. Typically, most of the agricultural lignocellulosic biomass is composed of about 10-25% lignin, 20-30% hemicellulose, and 40-50% cellulose (Malherbe and Cloete 2002; Anwar et al. 2014). Cellulose is an unbranched homopolysaccharide consisting of D-glucopyranosyl units. Hemicelluloses are branched heteropolysaccharides consisting of both hexose and pentose sugar residues, which may also carry acetyl groups (Fig. 1.2). The third main

component, lignin, consists of phenylpropane units linked together by different types of interunit linkages in which ether bonds are the most common.

Compared to starch, the polysaccharides of lignocellulose are more resistant to hydrolysis. There are two major methods to depolymerize lignocellulosic materials, acid hydrolysis and enzymatic hydrolysis. At present, enzymatic hydrolysis is considered as a potent approach for saccharification of complex polymer (Wyman 2007). However, the lignin-hemicellulose matrix, cellulose crystallinity and its low surface area make the lignocellulose very resistant to enzymes (Berlin et al. 2006). Feedstock pretreatment has been recognized as a necessary upstream process to remove lignin and enhance the porosity of the lignocellulosic materials prior to the enzymatic process (Zhu and Pan 2010; Kumar et al. 2009), for which there are three physical, chemical and biological pretreatments. The chemical pretreatment by acidic thermochemical process step usually degrades the hemicellulose leading to the formation of products such as pentose and hexose sugars, sugar acids, aliphatic acids (primarily acetic acid, formic acid and levulinic acid) and furan aldehydes [5-hydroxymethylfurfural (HMF) and furfural]. Sugars derived from hemicelluloses account for a substantial part of the total sugar and it is desirable that they are utilized in the subsequent fermentation step. The monosaccharides obtained through the hydrolysis process are then fermented to ethanol by microbial catalysts (Jönsson et al. 2013).

The pretreatment process is one of the most crucial and expensive steps in the process of converting biomass to fermentable sugars, which is estimated to account for 33% of the total cost. Therefore, the production from lignocellulosic biomass is not cost effective. To overcome this obstacle, conversion technologies including processing of biomass, convenient and cost-effective pretreatment process to detach hemicellulose and cellulose from their complex with lignin are under developing.

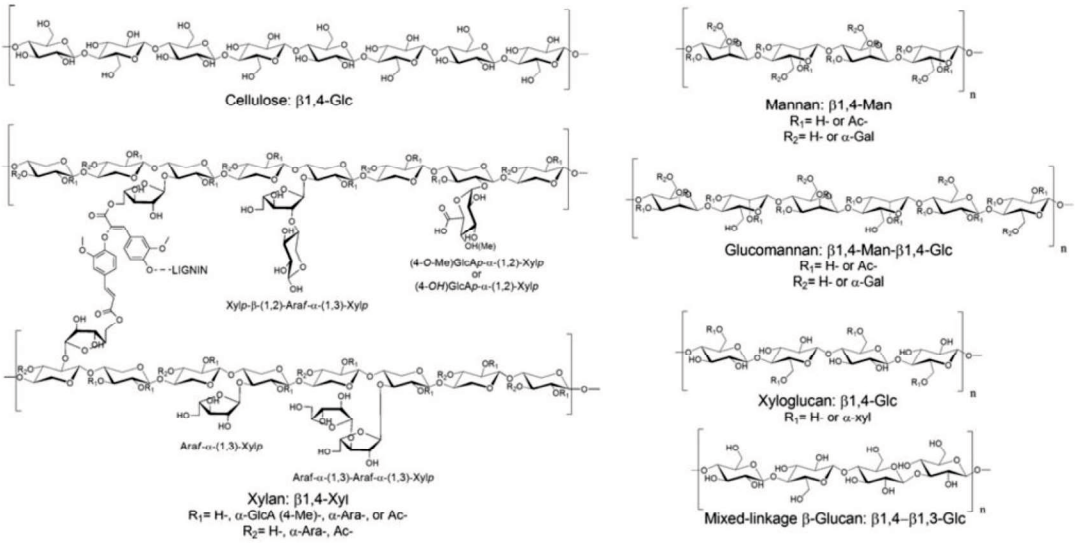

Fig. 1.2 Polymeric structures of cellulose and hemicellulose chains. Cellulose consists of repeating *β*-(1,4)-linked D-glucose residues. Adjacent D-glucose residues are flipped, making cellobiose the fundamental repeating unit. The major hemicelluloses are shown. Xylan consists of repeating *β*-(1,4)-linked D-xylose residues with the potential for L-arabinose or acetyl substitutions at either the 2-O or 3-O positions or both; 2-O substitution with 4-hydroxy- or 4-methyl-glucuronic acid; and added complexity from substitutions (hexose, pentose and/or phenolics) on the L-arabinose side-chain residues. Mannan consists of a *β*-(1,4)-linked D-mannose backbone, whereas the glucomannan backbone has both D-mannose and D-glucose residues with *β*-(1,4) linkages. Xyloglucan has a *β*-(1,4)-linked D-glucose backbone with D-xylose side chains, and the mixed-linkage *β*-glucan backbone has both *β*-(1,4)- and *β*-(1,3)-linked glucose residues (Jordan et al. 2012).

1.2.2.3 The third generation bioethanol

The third generation of bioethanol is algal bioethanol that is still at an early stage of investigation. Algae are photosynthetic, eukaryotic organisms that do not develop multicellular sex organs. Based on their morphology and size, algae are typically divided into two major categories, macroalgae and microalgae. Utilizing of algae as feedstock for bioethanol production does not compete with food production

in either land or water. Moreover, algae grow rapidly and can easily grow in various aquatic environments such as fresh water, saline water or municipal waste water (Gouveia et al. 2009; Harun et al. 2010). The microalgal cells have a very fast productivity and harvesting cycle (1–10 days) compared with other feedstocks that are harvested once or twice a year and thus provides enough supplies to meet ethanol production demands (Schenk et al. 2008). Like the second generation ethanol, bioethanol production from algae requires four major processes including pretreatment, hydrolysis, fermentation and distillation but algae have the advantages of having no lignin and low hemicellulose, which result in an increased hydrolysis efficiency and fermentation yields (Eshaq et al. 2011). Additionally, the pretreatment of the algae is easier than those of lignocellulosic biomasses because algae biomass is soft organization and high moisture content. (Li et. al, 2014)

1.2.3 Process of ethanol fermentation

1.2.3.1 Overall process of bioethanol production

The process of ethanol production depends on the raw materials used. Ethanol production is commonly carried out by the major three steps (Fig. 1.3):

(1) Obtainment of a solution of fermentable sugars: this step is the main difference between the ethanol production processes from sugar-based, starch-based or lignocellulosic feedstocks. Sugar-based feedstocks need only a milling process for the extraction of sugars to fermentation without any step of hydrolysis.

- Pretreatment: carbohydrates from lignocellulosic feedstocks are extracted or made more accessible to the subsequent steps. Pretreatment generally includes a mechanical step to reduce the particle size and a chemical pretreatment strep (diluted acid, alkaline, solvent extraction) to make the biomass more digestible.

- Hydrolysis or saccharification: polysaccharides from starch or hemicellulose and cellulose of the lignocellulosic biomass are broken down into simple sugars by acid or enzymatic hydrolysis.

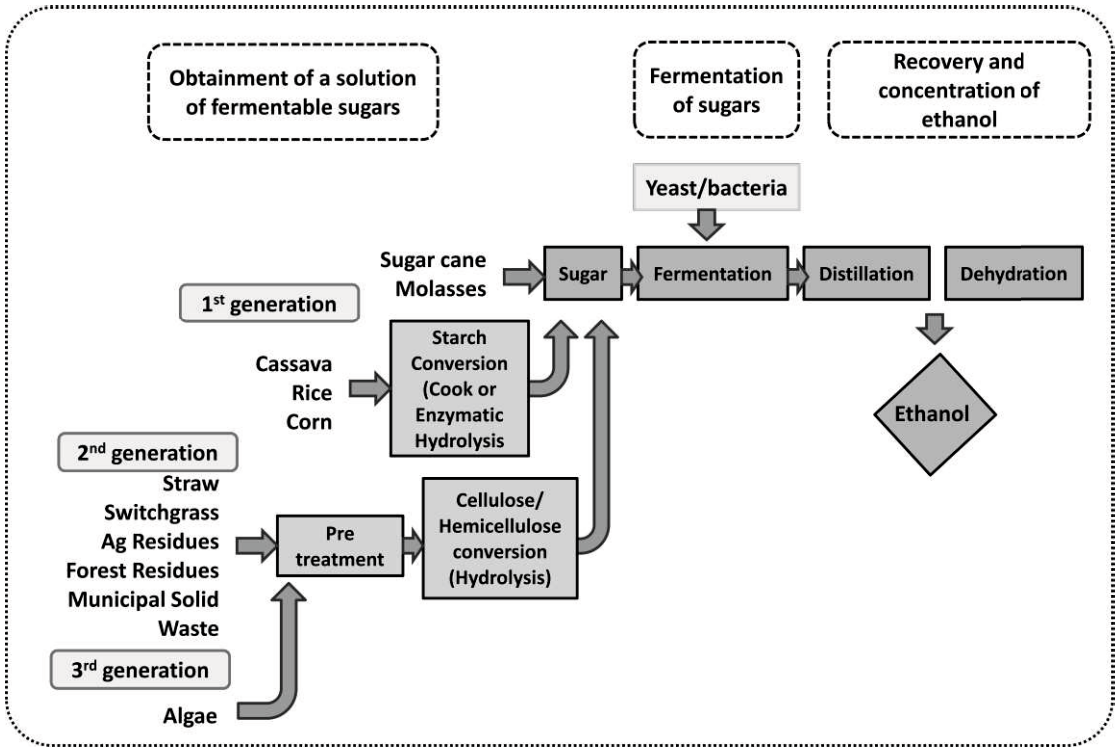

Fig. 1.3 Flowchart with the main raw materials and processes used for ethanol production

(2) Fermentation of sugars: hexoses and pentoses are converted to ethanol by a variety of microorganisms, such as bacteria, yeast or fungi. The conversion reaction for hexoses (C6) and pentoses (C5) is as follows:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO2$$

The best known microorganisms for ethanol production from hexoses are the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* (Claassen et al. 1999). For a better use of materials containing substantial quantities of pentoses, it is interesting to use

microorganisms able to convert also these sugars to ethanol, such as pentose-fermenting yeasts, *Candida chehatae* and *Scheffersomyces stipitis*, or genetically modified *S. cerevisiae* (Agbogbo and Coward-Kelly 2008; Watanabe et al.2007). The fermentation reactions occur at temperatures between 25°C and 30°C and it last between 6 h and 72 h depending on the composition of the hydrolysate, cell density, microbe species and their physiological activity. The cultured broth typically contains 8–14% of ethanol on a volume basis. Above the latter concentration, the growth and their activity of microbes may be inhibited.

(3) Recovery and concentration of ethanol: ethanol is separated from water usually by distillation and dehydration. The distillation step yields an azeotropic mixture made up of 95.5% alcohol that is the ‘‘hydrous’’ or ‘‘hydrated’’ ethanol which is then dehydrated to obtain an ‘‘anhydrous’’ ethanol containing up to 99.6% alcohol. Distillation technologies that allow the economic recovery of diluted volatile products from streams containing a variety of impurities have been developed.

1.2.3.2 Simultaneous saccharification and fermentation (SSF)

In this process, saccharification and fermentation are carried out simultaneously in a single reactor, thus allowing for cost saving and reduction of inhibitors, increasing hydrolysis rate (Foust et al. 2009). Comparison with the separate hydrolysis and fermentation process (SHF), SSF offers an easy operation and a low number of equipments since no hydrolysis reactors are needed. The fermentable sugars are gradually supplied into the reactor, which reduces severe osmotic stress to ethanologenic microorganisms. Moreover, the microorganisms can rapidly convert sugars into ethanol and reach higher yields and concentrations of ethanol (Brethauer and Wyman 2010). Nevertheless, SSF has the disadvantages that the optimal conditions for hydrolysis and fermentation are different, which implies a difficult

control and optimization of process parameters and that a larger amount of exogenous enzymes are required (Taylor et al. 2009). Saccharification with amylase for starch based feedstocks or with cellulolytic enzymes for cellulose based feedstocks is best performance at around 50 °C, whereas most fermenting microbes have an optimum temperature for ethanol fermentation between 28°C and 37°C. Accordingly, high-temperature fermentation is in high demand for simultaneous saccharification and fermentation, and thermotolerant yeast strains have been screened for the ability to ferment ethanol (Hasunuma and Kondo 2012a).

1.2.3.3 Simultaneous saccharification and co-fermentation (SSCF)

This kind of integration of fermentation steps is oriented to the complete assimilation by the microorganisms of all the sugars from pretreated lignocellulosic biomass. The use of mixed cultures of a microbe that assimilates hexoses and a microbe that assimilates pentoses has been proposed, but problems related to the facts that hexose-utilizing microorganisms grow faster than pentose-utilizing microorganisms and that the conversion of hexoses to ethanol is consequently predominant, are arisen (Sanchez and Cardona 2008). Other variant of co-fermentation consists of a single microorganism capable of assimilating both hexoses and pentoses in an optimal way, allowing high conversion and ethanol yield (Banerjee et al. 2010). Although in the nature such microorganisms exist, a high efficiency in the conversion of both sugar types to ethanol can be reached through the genetic modification of yeasts or bacteria already adapted to the ethanolic fermentation (Cardona and Sa´nchez 2007).

1.2.3.4 Consolidated bioprocessing (CBP)

CBP is the integration of enzyme production, polysaccharide hydrolysis (saccharification) and multisugar fermentation into one step, ideally achieved by a single organism (Xu et al. 2009). However, co-cultures may also be applied such that one organism is saccharolytic and the other is ethanologenic, or both organisms are

ethanologenic but each provides different key saccharifying enzymes. CBP requires fewer operation units, reducing maintenance and capital costs (Xu et al. 2009; Olson et al. 2012). In contrast to the usage of exogenous saccharifying enzymes, CBP organisms provide their own cellulolytic and hemicellulolytic enzymes for lignocellulose decomposition, offering large cost saving (Olson et al. 2012; Lu et al. 2006). Cell surface engineering has been applied to a thermotolerant strain of the yeast *K. marxianus* for the display of cellulolytic enzymes on its cell surface. A recombinant *K. marxianus* strain codisplaying endoglucanase and β-glucosidase on the cell surface grows well at temperature as high as 48 °C, at which ethanol was produced from the cellulosic material β-glucan with a yield of 0.47 g ethanol per gram of consumed carbohydrate. This study supports the development of CBP yeast for effective bioethanol production (Hasunuma and Kondo 2012b).

1.2.4 Fermenting organisms

1.2.4.1 *Saccharomyces cerevisiae*

S. cerevisiae or baker's yeast has commonly been used for the first generation ethanol production. This yeast species naturally ferment hexoses with a high ethanol yield, and exhibit high tolerance to end products and high level of heterologous gene expression, but cannot metabolize xylose. Despite its tolerance to ethanol, growth and ethanol production are temporarily inhibited by furfural, hydroxymethylfurfurral and vanillin that are derived from substrate degradation during pretreatment steps (Delgenes et al. 1996). Due to their inability to ferment pentoses (i.e., xylose and arabinose), wild-type yeasts have been genetically engineered to express genes for xylose isomerase from filamentous fungi or xylose reductase and xylitol dehydrogenase from *S. stipitis* that allow them to efficiently ferment xylose. In its natural form, *S. cerevisiae* is also unable to ferment cellobiose and other oligosaccharides that are products of cellulose hydrolysis. Even in cases where it has been genetically engineered, *S. cerevisiae* preferentially ferments glucose before other sugars, so certain genes must be disrupted in order for it to utilize

multiple sugars simultaneously (Zyl et al. 2007). Additionally, cells need sufficient transporters that are not suppressed by other sugars to increase xylose uptake. Introduction of transporter genes from *S. stipitis* has improved xylose uptake at low concentrations (Vleet and Jeffries 2009).

The main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden-Meyerhof-Parnas or EMP pathway), by which one molecule of glucose is metabolized and two molecules of pyruvate are produced (Bai et al. 2008), as illustrated in Fig. 1.4. Theoretically, the yield is 0.511 for ethanol and 0.489 for CO_2 on a mass basis of glucose metabolized. Two ATP molecules produced in the glycolysis are used to drive the biosynthesis that includes a variety of energy-requiring reactions in yeast cells. Therefore, ethanol production is tightly coupled with yeast cell growth, which means that cells are produced as a co-product. In addition, various by-products are also produced during ethanol fermentation such as glycerol, organic acids and higher alcohols. These by-products are inevitably generated by the corresponding metabolic pathways in addition to increase in yeast cell mass and thus decreases the ethanol yield to some extent (Bai et al. 2008).

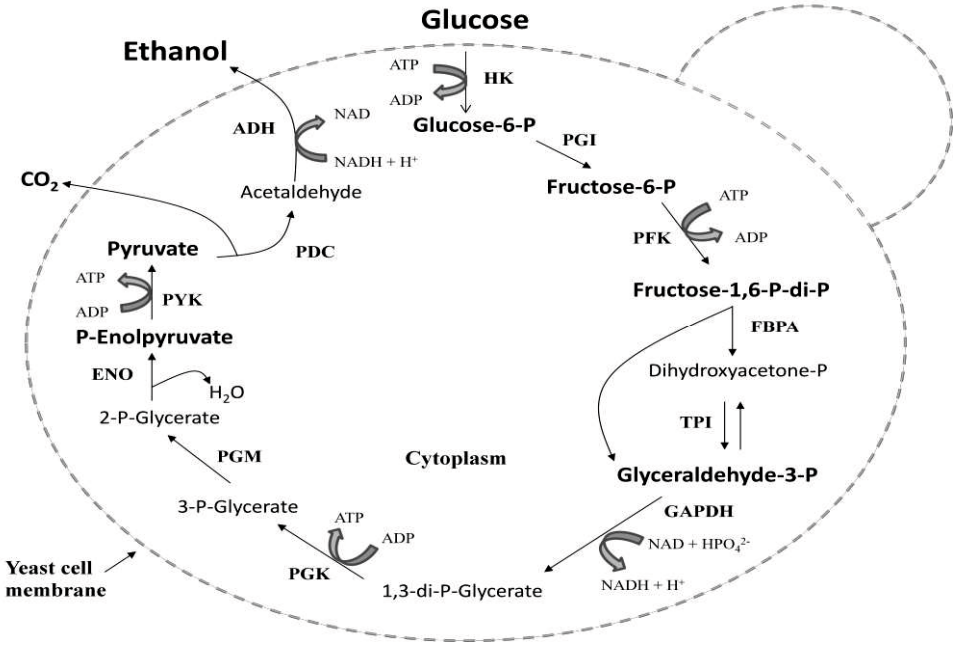

Fig. 1.4 Metabolic pathway of ethanol fermentation in *S. cerevisiae*

1.2.4.2 *Scheffersomyces stipitis*

S. stipitis or *Pichia stipitis* was originally isolated from insect larvae and is closely related to several yeast endosymbionts of passalid beetles (Nardi et al. 2006;) that inhabit and degrade white-rotted hardwood (Suh et al. 2003). It has the high native capacity for fermentation of xylose which *S. cerevisiae* cannot ferment, and thus numerous laboratories have borrowed genes from *S. stipitis* to modify *S. cerevisiae* for xylose fermentation. *S. stipitis* is also able to ferment mannose, glucose, galactose and cellobiose, but cannot ferment arabinose (du Preez et al. 1986). *S. stipitis* is a predominantly haploid, homothallic, hemiascomycetous yeast (Kurtzman 1990; Gupthar 1994; Melake et al. 1996) that forms buds along with pseudomycelia during vegetative growth, and two hat-shaped ascospores from each ascus. The optimal temperature of *S. stipitis* is between 25°C and 33°C and the optimal pH is 4.5–5.5 (du Preez et al. 1986). Some studies have shown that *S. stipitis* produces ethanol under anaerobic conditions (Delgenes et al. 1986), but microaerobic conditions are optimal for ethanol production (Krahulec et al. 2012). It is capable of fermenting sugars from hemicellulosic acid hydrolysates with a yield equivalent to about 80% of the theoretical conversion efficiency (Nigam 2001a, b).

1.2.4.3 *Kluyveromyces marxianus*

K. marxianus that is a homothallic, hemiascomycetous yeast, is phylogenetically related to *S. cerevisiae*, and is a sister species to the well-known *Kluyveromyces lactis* (Lachance 1998; Llorente et al. 2000). *K. marxianus*, like *S. cerevisiae*, is a respiro-fermentative yeast and can generate energy either via the TCA cycle by oxidative phosphorylation or by fermentation. *K. marxianus* strains are more thermotolerant than *Saccharomyces*, which in turn can produce higher ethanol yields at high temperatures. Many strains of *K. marxianus* grow well at temperatures as high as 45–52°C and can efficiently produce ethanol at temperatures between 38°C and 45°C. Moreover, *K. marxianus* offers additional benefits including a high growth rate and the ability to utilize a wide variety of sugar substrates (e.g., arabinose, galactose,

mannose and xylose) at elevated temperatures (Cardona and Sa´nchez 2007; Hasunuma and Kondo 2012a). The underlying interest in *K. marxianus* is undoubtedly driven by applications in the biotechnology industry. The industrial potential of *K. marxianus* has been reviewed by Lane and Morrissey (2010) and some key applications are listed in Table 1.2.

Table 1.2 Overview of key industrial applications of *K. marxianus*

Application	Strains	References
Production of ethanol from various feedstocks		
Whey or lactose	*K. marxianus* NBRC 1963	Oda and Nakamura (2009)
	K. marxianus DSMZ-7239	Ozmihci and Kargi (2007)
	K. marxianus MTCC 1288	Zafar and Owais (2006)
	n/a	Kourkoutas et al. (2002)
	K. marxianus IMB3	Brady et al. (1997)
Sugar cane juice	*K. marxianus* DMKU3-1042	Limtong et al. (2007)
Sugar cane syrup	*K. marxianus* DMKU3-1042	Pimpakan et al. (2012)
Cashew apple bagasse	*K. marxianus* ATCC36907	Rodrigues et al. (2015)
Jerusalem artichokes	*K. marxianus* Y179	Gao et al. (2015)
	K. marxianus DBKKU Y-102	Charoensopharat et al. (2015)
Lignocellulosic biomass	*K. marxianus* DMB1	Goshima et al. (2013)
Production of biomass or single cell protein		
Continuous, batch and fed batch fermentation	*K. marxianus* ZIM 1867	Pas et al. (2007)
Iron and nucleotide rich biomass	*K. marxianus* CBS 6556	Schultz et al. (2006)
Comparable autolysate composition to *S. cerevisiae*	*K. fragilis* NRS 5790	Ghaly et al. (2005)
	K. marxianus FII 510700	Lukondeh et al. (2005)
	K. marxianus CBS 6556	Revillion et al. (2003)
	K. marxianus ATCC10022; CBS 7894	Pinheiro et al. (1998)
Production of endogenous enzymes		
β-xylosidase	n/a	Rajoka (2007)
β-galactosidase	*K. marxianus* CBS 7894	Pinheiro et al. (2003)
β-glucosidase	*K. fragilis* IpF1	Szczodrak (2000)
Inulinase	*K. marxianus* CDBB-L278	Cruzguerrero et al. (1995)

Table 1.2 (Continued)

Application	Strains	References
Heterologous expression of enzymes		
Thermostable cellulases	*K. marxianus* NBRC 0219; NBRC 0541; NBRC 0617; NBRC 1777	Hong et al. (2007)
Lactate dehydrogenase	*K. marxianus* KM1	Pecota et al. (2007)
Endopolygalacturonase	*K. marxianus* BKM Y-719	Siekstele et al. (1999)
α-galactosidase	*K. marxianus* CBS 6556	Bergkamp et al. (1993a) Bergkamp et al. (1993b)
Food industry		
Natural emulsifier – mannoprotein	n/a	Vasallo et al. (2006)
	n/a	Lukondeh et al. (2003)
Aroma compounds	*K. marxianus* ATCC10024	Medeiros et al. (2000)
Bioflavours	*K. marxianus* NRRL YB-155	Güneşer et al. (2015)
Xylitol	*K. marxianus* 36907-FMEL1	Kim et al. (2015)
Bakers yeast	*K. marxianus* NRRL-Y-2415; NRRL-Y-1109	Caballero et al. (1995)
Environmental applications		
Treatment of paper wastes and sludge	*K. marxianus* Y01070	Kadar et al. (2004)
Removal of lactose/other sugars from wastewater	*K. marxianus* NRRL Y-610	Hang et al. (2003)
Biosorption of dyes	*K. marxianus* IMB3	Meehan et al. (2000)
Recovery of heavy metals from wastewater	n/a	Pal et al. (2009)

1.2.4.4 *Zymomonas mobilis*

Z. mobilis is a Gram-negative bacterium, which was first isolated in tropical countries from alcoholic beverages such as the African palm wine. The optimum pH of *Z. mobilis* is 5.5-7.0 and the optimum temperature is 25-31°C (Swings and Deley, 1977). *Z. mobilis* has been of considerable interest in recent years for ethanol production at high temperatures (Pappas et al. 1997, Dung and Huynh, 2013) because it gives a near-theoretical yield of ethanol from glucose and fructose. It is an osmo- and ethanol-tolerant bacterium that has high specific rates of glucose uptake and ethanol production and strong ethanol tolerance of up to 16% (v/v) (Choi et al. 2008). Numerous reports show that this bacterium can ferment a wide spectrum of natural

materials, e.g. pulp juices, Jerusalem artichokes (Thanonkeo et al. 2011), sugarcane molasses (Cazettaa et al. 2007), sugarcane juice, cassava and sago starch and raisin extracts.

Z. mobilis produces ethanol from glucose via the Entner–Doudoroff (ED) pathway in conjunction with enzymes of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Conway 1992). Compared with the EMP pathway of *S. cerevisiae*, which performs the cleavage of fructose-1,6-bisphosphate by fructose bisphosphate aldolase to yield one molecule each of glyceraldehydes-3-phosphate and dihydroxyacetone phosphate, the ED pathway forms glyceraldehyde-3-phosphate and pyruvate by the cleavage of 2-keto-3-deoxy-6-phosphogluconate by 2-keto-3-deoxy-gluconate aldolase and thus yields only one molecule ATP per glucose molecule (Fig. 1.5). As a consequence, *Z. mobilis* produces less biomass than *S. cerevisiae* and more ethanol. It can tolerate to high concentrations of sugar up to 40% (v/v) (Wang 2008) and does not require the controlled addition of oxygen during fermentation. *Z. mobilis* has a homoethanol fermentation pathway. Its specific ethanol productivity is 2.5-fold higher than that of *Saccharomyces* sp. and its ethanol yield approaches 97% of the theoretical yield at an optimal temperature of 30°C (Weber et al. 2010). Although *Z. mobilis* is better than yeast in many aspects, it has not been used commercially, despite of that considerable researches have carried out in a lab or pilot scale. The following factors have been considered as issues that prevent its commercial use. Its substrate range is restricted to glucose, fructose and sucrose. Using biotechnological methods, scientists are currently trying to overcome this limitation. A variant of *Z. mobilis* that is able to use certain pentoses as a carbon source has been developed. By genetic engineering technology, engineered *Z. mobilis* could potentially use all sugars present in most biomass feedstocks. Moreover, specific rates of sugar uptake and ethanol production in *Z. mobilis* are at maximum when glucose is used compared to those when fructose or sucrose is used (Choi et al. 2008). Additionally, using sucrose as substrate for ethanol production, *Z. mobilis* can form other products such as sorbitol, levan polysaccharides and fructose oligosaccharides (FOS), causing a significant decrease in ethanol production (Pinilla et al. 2011). In sucrose-based substrates, ethanol production may be reduced to 70% of that of glucose-based

substrates due to the formation of by-products such as levan and sorbitol (Vignoli et al. 2010). Moreover, *Z. mobilis* cannot tolerate toxic inhibitors present in lignocellulosic hydrolysates such as acetic acid and various phenolic compounds

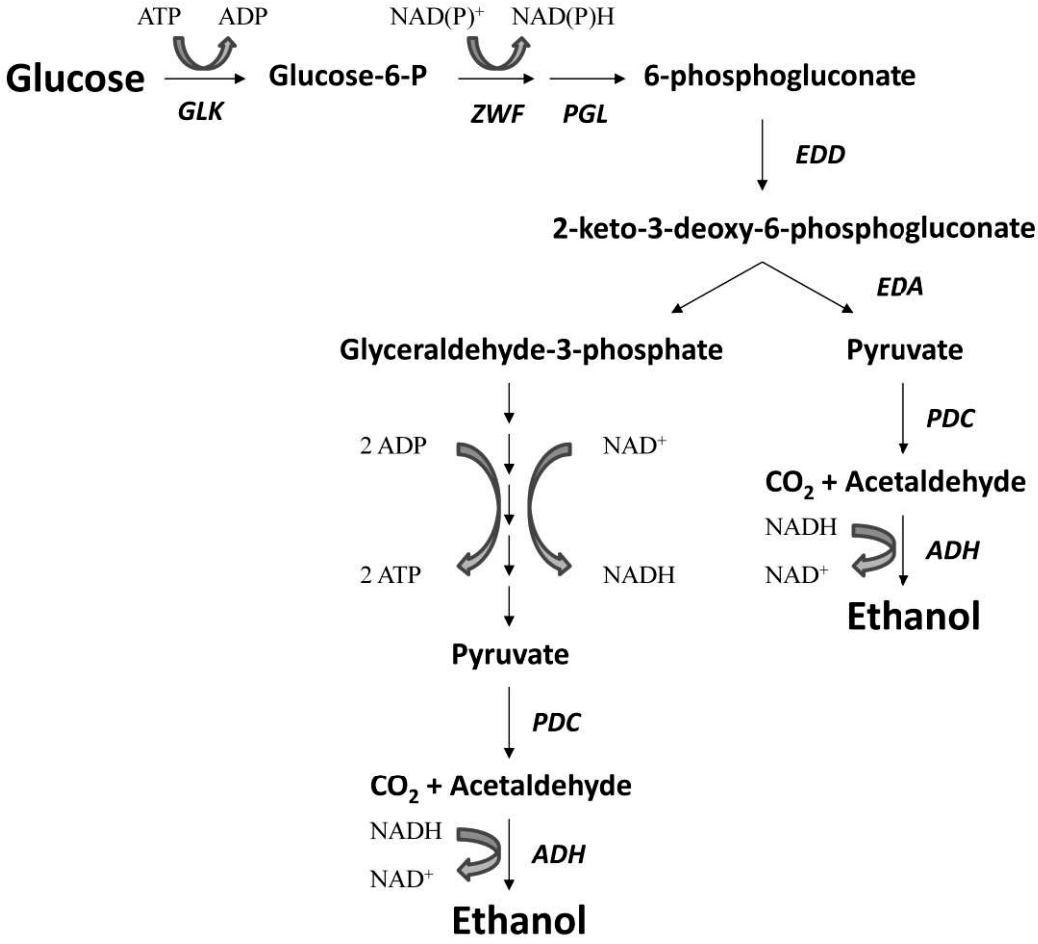

Fig. 1.5 The Entner-Doudoroff (ED) pathway and ethanologenesis. The branch from glyceraldehyde-3-phosphate to pyruvate is identical to the Embden-Meyerhof-Parnas (EMP) pathway. The key enzyme of the ED pathway is pyruvate decarboxylase (PDC), which is only rarely found in bacteria. The ED pathway produces only 1 mole of ATP per mole of consumed glucose (Jungwoo, 2011).

1.2.5 Stresses for fermenting microbes

During ethanol fermentation, especially in low-cost bioethanol production systems conducted under high-temperature and high-gravity fermentations, cells of ethanologenic microbes suffer from various stresses including physical stress and chemical stress.

1.2.5.1 High temperature stress

Ethanol production at elevated temperatures has received much attention because of the potential cost savings, which could be obtained by continuous evaporation of ethanol from the broth under reduced pressure (Banat et al. 1998). However, the temperature increase has a negative effect on ethanol yield and also reduces the cell viability (Anderson et al. 1986; Ballesteros et al. 1991). The maximum temperature for growth is relatively constant within a species (van Uden 1984). Most yeast exploited in biotechnology generally grows best between 20-30°C (Walker 1998). Table 1.3 summarizes general adverse influences of heat on yeast cell physiology.

1.2.5.2 Osmotic stress

Osmotic stress can be defined as any situation that there is an imbalance of intracellular and extracellular osmolarities, being sufficient to cause a deleterious change in physiology (Csonka and Hanson 1991). During the ethanol fermentation process, the principal source of osmotic stress is the highly concentrated medium containing high concentrations of sugars, especially, in high-gravity fermentation, and such osmotic stress may reduce cell growth and viability of microbes.

1.2.5.3 Ethanol stress

Ethanol is a major metabolic product of ethanologenic microorganism. However, as fermentation proceeds, the ethanol concentration increases and cells are exposed to increasingly toxic levels of ethanol that initially inhibit the growth of fermenting microbes and latterly cause lethal effect on them. In brewing and ethanol-based biofuel industries, high-gravity fermentation produces 10–15% (v/v) ethanol (Koppram et al. 2014). Table 1.4 summarizes principle inhibitory effects of ethanol on yeast cells.

1.2.5.4 Oxidative stress

Another major chemical stress that cells confront, particularly, during aerobic growth, is reactive oxygen species (ROS). The ROS include the superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$), which can damage cell components, contribute to cellular ageing and ultimately lead to cell death (Beckman and Ames, 1998). During the bioethanol production process, oxidative stress is generated inside the cells by several ways such as increasing of temperature or exposed to some chemicals and cause cell damage and low fermentation ability (Attfield 1997).

1.2.5.5 Other chemical stress

Because of the close associations of cellulose and hemicellulose with lignin in the plant cell wall, pretreatment is necessary to make carbohydrates available for enzymatic hydrolysis and subsequent fermentation. For economic reasons, diluted acid pretreatment is commonly used to prepare lignocelluloses for enzymatic saccharification and fermentation (Balat et al. 2008) and this process generates by-products, including furan derivatives, weak organic acids and phenolic compounds. It has been suggested that many of these components inhibit the growth and fermentation of yeast (Olsson and Hahn-Hagerdal 1996; Saha 2003; Palmqvist and

Hahn-Hagerdal 2000). Furfural and 5-hydroxymethylfurfural (HMF) that are generated by the breakdown of sugars, have been reported to be fermentation inhibitors (Klinke et al. 2004; Shima and Nakamura, 2015). It is also known that furfural and HMF induce oxidative stress in yeast cells (Kim and Hahn 2013), which reduce enzymatic and biological activities as well as cause the breakdown of DNA (Modig et al. 2002; Khan and Hadi 1994).

Table 1.3 General effects of high temperature on yeast cell physiology

(Walker 1998)

Physiological function	Comment
Cell viability	At the highest growth temperature of many yeasts, there is also appreciable cell death. At supramaximal growth temperatures, thermal death rate is exponential.
General cell morphology	Atypical budding, irregular cell wall growth and increased cell size.
Cell division and growth	Growth of non-thermotorant yeasts inhibited at temperature >40°C. Actively-dividing cells in S-phase are more thermosensity compared with resting cells. Heat shock transiently arrests cells in G1 phase of the cell cycle.
Plasma membrane structure/ function	Increased fluidity and reduced permeability to essential nutrients. Ergosterol is known to increase thermotolerance. Decrease in unsaturated membrane fatty acids. Stimulation of ATPase and RAS-adenylate cyclase activity. Decline in intracellular pH.
Cytoskeletal integrity	Extensive disruption of filaments and microtubular network.
Mitochondrial structure/ function	Decrease in respiratory activity and induction of respiratory-deficient petite mutants. Aberrant mitochondrial morphology.
Intermediary metabolism	Inhibition of respiration and fermentation above T_{max}. Immediate increase in cell trehalose an MnSOD following heat shock.
Protein synthesis	Repression of synthesis of many proteins, but specific induction of certain heat-shock proteins. Mitochondrial protein synthesis more thermolabile than cytoplasmic.
Chromosomal structure/ function	Increased frequency of mutation of mitotic cross-over and gene conversion. Increased mitotic chromosomal non-disjunction. Inefficient repair of heat-damaged DNA

Table 1.4 Important effects of ethanol on yeast cell physiology (Walker 1998)

Physiological function	Ethanol influence
Cell viability and growth	- General inhibition of growth, cell division and cell viability - Decrease in cell volume - Induction of morphological transitions (e.g. promotion of germ-tube formation in *Candida albicans*) - Enhancement of thermal death
Intermediary metabolism and macromolecular biosynthesis	- Denaturation of intracellular proteins and glycolytic enzymes - Lowered rate of RNA and protein accumulation - Reduction of Vmax of main glycolytic enzyme - Enhancement of petite mutation - Induction of heat shock-like stress proteins - Increase in oxygen free radical - Induced synthesis of cytochrome P540
Membrane structure and function	- Alteration of fatty acid and sterol composition - Induced lipolysis of cellular phospholipids - Increased ionic permeability - Inhibition of nutrient uptake - Inhibition of H^+-ATPase and dissipation of proton-motive force - Uncoupling of electrogenetic processes by promoting passive re-entry of proton and consequential lowering of cytoplasmic PH - Hyperpolarization of plasma membrane

CHAPTER 2

High-temperature fermentation technology for low-cost bioethanol

2.1 Abstract

Considering its advantages including reduction of cooling cost and saving water during the fermentation process, which consequently cut down the total running cost, high-temperature fermentation with thermotolerant microbes is expected to be one of next-generation fermentation technologies. I focused on the establishment of high-temperature fermentation technology for ethanol production from biomass in Thailand, for which thermotolerant microbes suitable for various types of biomass were selected and advanced fermentation processes including a temperature-uncontrolled fermentation and a simultaneous fermentation and distillation under a low pressure were investigated.

2.2 Introduction

Global temperatures have been increasing due to increases in the amounts of carbon dioxide emission by utilization of fossil fuels including coal, petroleum and natural gas (IPCC, 2007). Reduction of carbon dioxide emission has thus become an urgent issue, and biofuels derived from biomass have attracted attention as an alternative to fossil fuels. Since the worldwide demand for bioethanol has been increasing (OECD, 2011), the development of energy-saving, efficient ethanol production technology is required.

Since the ethanol fermentation process is exothermic, the cooling of fermenter is indispensable for stable fermentation with microbes. The cooling, however, becomes difficult in hot seasons, so that fermentation industries in tropical countries and even in Japan stop their fermentation process for 1-2 months during such seasons. Thus, high temperature fermentation with thermotolerant microbes is

expected to be developed. High-temperature fermentation of ethanol has several advantages including high fermentation rate, decreased risk of contamination, reduction of cooling and operating costs. High-temperature fermentation could thus achieve the running cost reduction of fermentation process as well as a stable fermentation.

For high-temperature fermentation, thermotolerant microbes that are stable under high temperature conditions and efficient in useful material production are essential. In this study, we introduced two ethanologenic microbes, *Kluyveromyces marxianus* and *Zymomonas mobilis*. The former is thermotolerant yeast and is able to efficiently produce ethanol at high temperatures, and it has the potential to assimilate a wide variety of substrates (Rodrussamee et al. 2011; Lertwattanasakul et al. 2011). On the other hand, the latter as a bacterium has a unique metabolic pathway that enables an approximately 3-times higher rate of ethanol production than that of *Saccharomyces cerevisiae* (Rogers et al. 1980; Buchholz et al. 1987). *Z. mobilis* with thermotolerance is thus expected to perform high-speed and high-temperature fermentation. We screened thermotolerant microbes suitable for various types of biomass and bred more thermotolerant and/or stress-resistant microbes that are useful for high-temperature fermentation of ethanol. One of such thermotolerant yeasts was applied for ethanol fermentation under temperature-uncontrolled conditions. I also developed the new process of simultaneous fermentation and distillation under a low pressure. This process may have some benefits, for example, in ethanol recovery from a low concentration of ethanol, which occurs when raw garbage or cellulosic materials are used as raw materials for ethanol fermentation.

2.3 Materials and methods

2.3.1 Strains

K. marxianus DMKU3-1042 was obtained from soil and water samples from sugar cane plantations and sugar factories in four provinces, namely Phra Nakhon Si Ayutthaya, Ratchaburi, Suphanburi and Uthaithani, Thailand (Limtong et al. 2007).

Isolation was carried out at 35°C by an enrichment technique using sugar juice medium to be incubated for 3 days under shaking condition, and then streaked on agar plates containing the same medium and incubated at 35°C. *K. marxianus* DMKU3-1042 derivatives (which will be reported elsewhere) were isolated under various stress conditions from DMKU3-1042 as a parental strain, and of those, one strain that was isolated as an iron-resistant on KMYPD plates (see below) containing 17 mM $FeSO_4$ at 37°C, was used in this study. *Z. mobilis* TISTR548 was obtained from the TISTR culture collection (Bangkok MIRCEN), and ZM4 (NRRL B-14023) was provided by E. Yanase. Thermo-adapted strains (which will be reported elsewhere) were isolated from TISTR548 as a parental strain by repeated-cultivation more than 80 times at high temperatures (from 37°C to 40°C) in ZMYPD medium (see below) under a static condition, and one of the most thermotolerant strains was used in this study.

2.3.2 Media and cell growth condition

K. marxianus cells were grown in KMYPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 3% (w/v) glucose at temperatures indicated under a shaking condition at 160 rpm, and *Z. mobilis* cells were grown in ZMYPD medium containing 0.3% (w/v) yeast extract, 0.5% (w/v) peptone and 3% (w/v) glucose at temperatures indicated under a static condition. Cell growth of *K. marxianus* and *Z. mobilis* was determined by measuring an optical density at OD660 and OD550, respectively. The media for the process of simultaneous fermentation and distillation were KMYP-20% rice medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 20% (w/v) rice hydrolysate (equivalent to 13% glucose) for *K. marxianus* cells and ZMYP-20% rice medium containing 0.3% (w/v) yeast extract, 0.5% (w/v) peptone and 20% (w/v) rice hydrolysate (equivalent to 13% glucose) for *Z. mobilis* cells. Minimum-20% rice medium for fermentation contained 20% (w/v) rice hydrolysate (equivalent to 13% glucose), 0.2% urea, 0.05% potassium dihydrogen phosphate and 0.1% magnesium sulfate heptahydrate. Rice was hydrolyzed into glucose by Yuniase S containing α-amylase and glucoamylase (2,000 U/ g rice;

Yakult Pharmaceutical Industry Co, Tokyo) at 55°C for 24 h. Cell growth in rice medium was not analyzed due to the strong turbidity of the medium.

2.3.3 Fermentation with rice hydrolysate

K. marxianus was pre-cultivated in 50 ml of KMYP-5% rice medium at 30°C for 18 h under a shaking condition at 100 rpm. The pre-culture was inoculated at 10% into KMYP-20% rice medium. Fermentation was carried at 100 rpm for 72 h at 45°C. At the times indicated, the culture medium was collected for analysis of cell growth, ethanol concentration and sugar concentration.

2.3.4 Simultaneous fermentation and distillation

After rice hydrolysis, the hydrolysate was transferred into a fermentation and distillation tank, and fermentation was performed at 41°C by *K. marxianus* DMKU3-1042 under a shaking condition at 160 rpm or by a thermo-adapted strain of *Z. mobilis* under a static condition. After fermentation, the vapor pressure of the fermentation and distillation unit was decreased to 70 mbar and ethanol was collected as the first recovery ethanol into a primary ethanol recovery bottle for about 12 h. After the first distillation under the low air pressure, ethanol from the primary ethanol recovery bottle was collected as the secondary recovery ethanol at 70 mbar. Some leaking ethanol was trapped in a drain tank.

2.3.5 Measurement of glucose and ethanol concentrations in culture medium

Glucose and ethanol concentrations in culture medium were determined at 60°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 GLC610-S Gelpack® column (Hitachi Chemical, Tokyo, Japan) using distilled water from an RFD240NA Water Distillation

Apparatus (Aquarius, ADVANTEC®, Japan) as a mobile phase at a flow rate of 0.3 ml/min.

2.4 Results and discussion

2.4.1 *K. marxianus* as a thermotolerant and ethanologenic yeast

2.4.1.1 Yeasts suitable for biomass

K. marxianus is able to utilize various sugars including glucose, mannose, galactose, xylose and arabinose (Rodrussamee et al. 2011; Lertwattanasakul et al. 2011; Yuangsaard et al. 1998) and possesses many sugar transporters encoded by its genome (Lertwattanasakul et al. 2015). I performed screening of yeasts for biomass of molasses, sugar cane juice, cassava starch and lignocelluloses hydrolysate at high temperatures, and I found highly efficient ethanol fermenting species suitable for each biomass (Table 2.1). These yeasts listed in Table 2.1, however, exhibited not always good efficiencies when different biomasses were tested. I thus utilized *K. marxianus* DMKU3-1042 in the following experiments because it showed a good performance with all biomasses tested.

Table 2.1 Yeast strains suitable for various biomasses at high temperatures

Biomass	Microorganism	Growth Temperature
Molasses	*Saccharomyces cerevisiae* DMKU3-S087	40°C
Sugar cane	*Kluyveromyces marxianus* 3-KS07	40°C
Cassava starch*	*Pichia kudriavzevii* KU-ET1	40°C
Lignocellulose*	*Candida tropicalis* DMKU3-K22(6)	40°C

* The biomass was used as hydrolysate

2.4.1.2 Improvement of stress-tolerance of thermotolerant *K. marxianus*

I also developed several derivatives under various stress conditions from *K. marxianus* DMKU3-1042 as a parent, which was isolated in Thailand as the most thermotolerant and efficiently ethanol-producing yeast (Rodrussamee et al. 2011; Lertwattanasakul et al. 2011). Fermentation abilities of an iron-resistant DMKU3-1042 derivative, DMKU3-1042 as a parental strain and *Saccharomyces cerevisiae* as a yeast generally utilized in the fermentation industry were examined in minimum-20% rice medium under a shaking condition at 100 rpm and under a static condition at 45°C (Fig. 2.1). The derivative showed high levels of glucose utilization and ethanol production compared to those of the other two strains under both conditions. On the other hand, *S. cerevisiae* could not utilize glucose in the hydrolysate under conditions. These experiments indicate the possibility that thermotolerant strains isolated from tropical environments can be improved to be more thermotolerant or efficient ethanol-producing strains.

2.4.1.3 Ethanol fermentation under a temperature uncontrolled condition

Due to the availability of thermotolerant strains, one may expect that a temperature-uncontrolled fermentation can be performed and is economical under high-temperature circumstance like tropical countries or hot seasons. Ethanol fermentation with *K. marxianus* DMKU3-1042 was thus compared under temperature-uncontrolled and –controlled (40°C) conditions in YPD medium containing 9% glucose by using a 5-L fermenter. As a result, cell growth and ethanol production at 7 h under temperature-controlled condition were better than those under temperature-uncontrolled, and the ethanol concentration reached a maximum at 12 h under both tested conditions (Fig. 2.2). Under the temperature uncontrolled condition, temperature was approximately 25°C at the beginning of fermentation and increased gradually to 35°C at 11 h. The final temperature at 48 h was 33°C. Optimal temperature for this yeast strain was around 37°C which is the average temperature

during summer time in Thailand. Furthermore, such a temperature-uncontrolled ethanol fermentation with sugar cane juice was successfully performed even in 3,000 L scale in Thailand, where the initial and maximun temperatures were 30°C and 39°C, respectively (data not shown). These findings and facts suggest the possibility of temperature-uncontrolled ethanol fermentation in industry, which could be an economical process under high-temperature circumstances because it may cut down the cost of cooling units and for cooling fermenter.

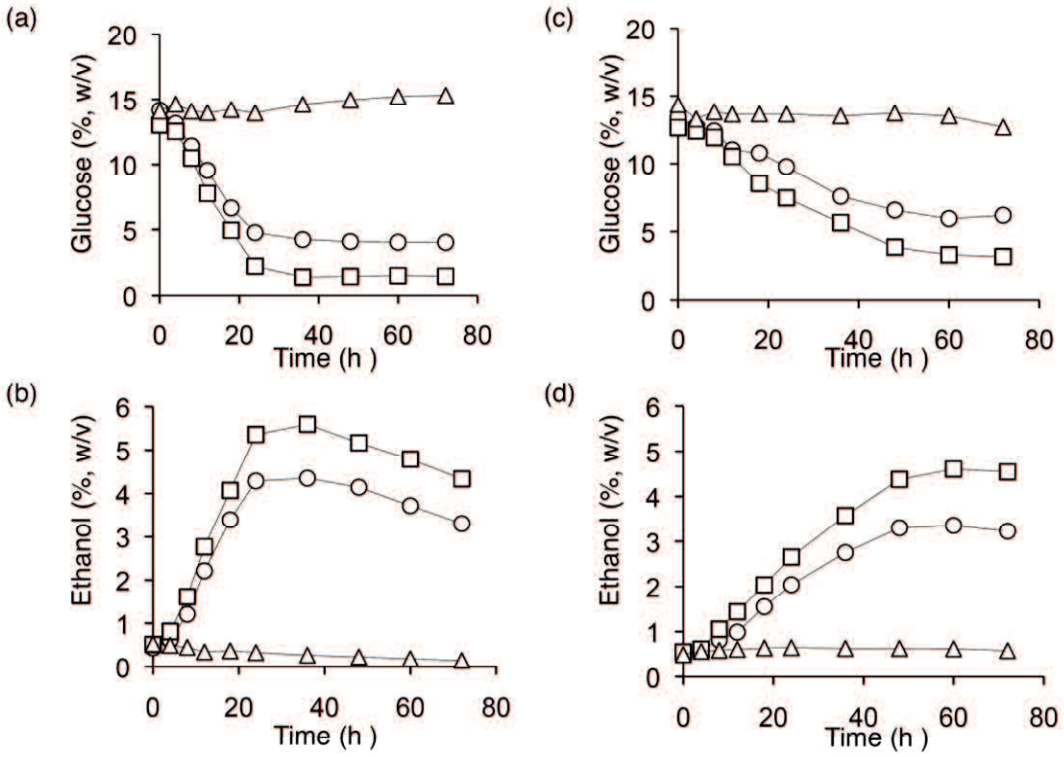

Fig. 2.1 Comparison of ethanol fermentation ability. The *K. marxianus* DMKU3-1042 derivative, *K. marxianus* DMKU3-1042 and *S. cerevisiae* were grown in 50 ml of minimum-20 % rice medium at 45ºC under a shaking condition at 100 rpm (a, b) and a static condition (c, d). Concentrations of glucose (a, c) and ethanol (b, d) in the culture medium were determined by HPLC. Open squares, open circles and open triangles represent the *K. marxianus* DMKU3-1042 derivative, *K. marxianus* DMKU3-1042 and *S. cerevisiae*, respectively.

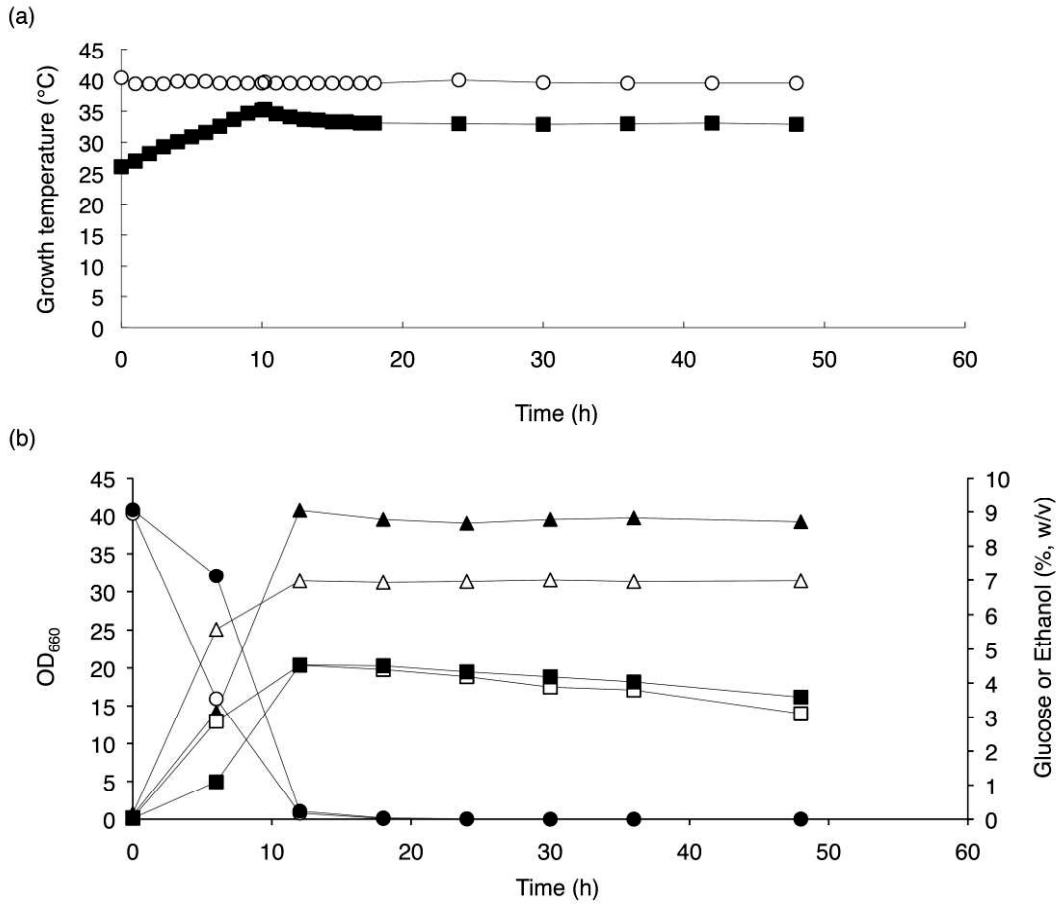

Fig. 2.2 Temperature-uncontrolled fermentation. Time course of ethanol production from 2-L of YPD medium containing 9% glucose by *K. marxianus* DMKU3-1042 in a 5-L fermenter. Cell growth was determined by measuring cell density at OD660 and temperature of the culture was measured automatically. Concentrations of glucose and ethanol in the culture were determined by HPLC. (a) Closed and open circles represent temperatures during fermentation under temperature-uncontrolled and -controlled (40°C) conditions, respectively. (b) Cell growth of temperature-uncontrolled and -controlled conditions is represented by closed and open triangles, respectively. Closed and open circles represent glucose concentrations under temperature-uncontrolled and -controlled conditions, respectively. Ethanol concentration under temperature-uncontrolled and -controlled conditions is represented by closed and open squares, respectively.

2.4.1.4 Application of distillation under a low pressure at 40°C

Since the saturation vapor pressure of ethanol is 177.8 mbar at 40°C as a theoretical value, ethanol is recoverable from the culture tank by reducing the vapor pressure below that value. DMKU3-1042, which is able to efficiently produce ethanol even at a relatively high temperature, was thus used for the simultaneous fermentation and distillation test at 40°C in KMYP-20% rice medium. For this test, a system consisting of a fermentation and distillation tank and a distillation apparatus, primary and secondary ethanol recovery units, a vacuum pump and a drain unit was constructed (Fig. 2.3). Due to the constitution of this system, the air in the tank was discharged outside during vacuum distillation, some of which was trapped in the drain unit. The operation procedure of this system is described in Experimental. Simultaneous fermentation and distillation under a low pressure was repeated three times, and glucose consumption and ethanol production were monitored (Fig. 2.4a). The reproducible results suggest that *K. marxianus* can survive under a low pressure. The maximum concentration of ethanol in the medium was about 5% (Fig. 2.4a). The ethanol concentrations in primary and secondary bottles were about 35% and 60%, respectively (Fig. 2.4b). Considering the concentration of ethanol, the technology of the simultaneous fermentation and distillation under a low pressure could be connected to the technology of membrane separation of ethanol and water.

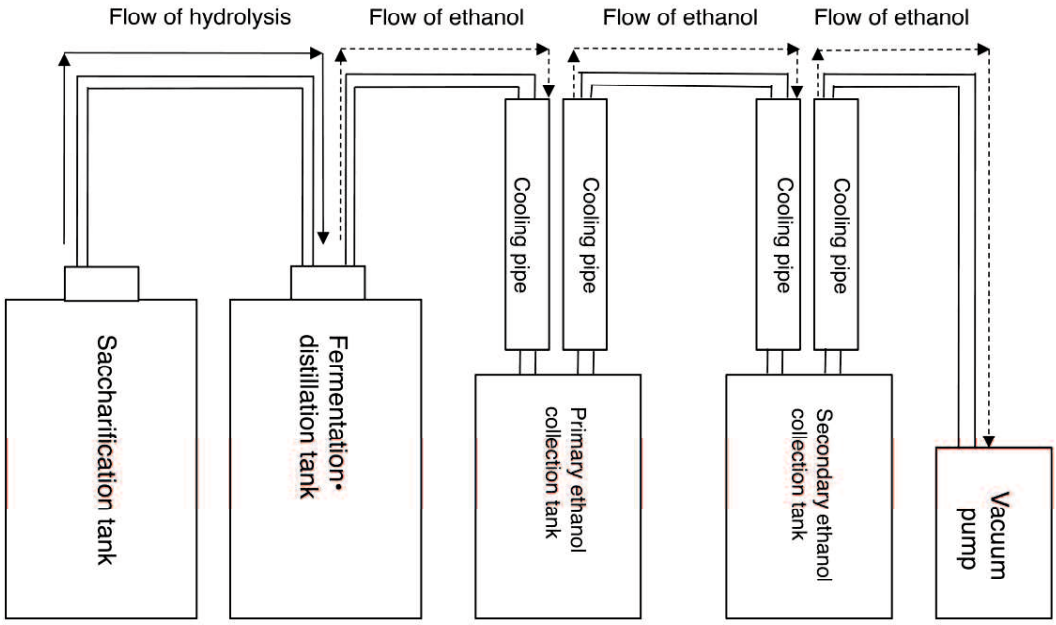

Fig. 2.3 Apparatus for saccharification, fermentation and distillation. This apparatus consists of a saccharification tank, a fermentation and distillation tank, primary and secondary recovery units, a drain unit and a vacuum pump.

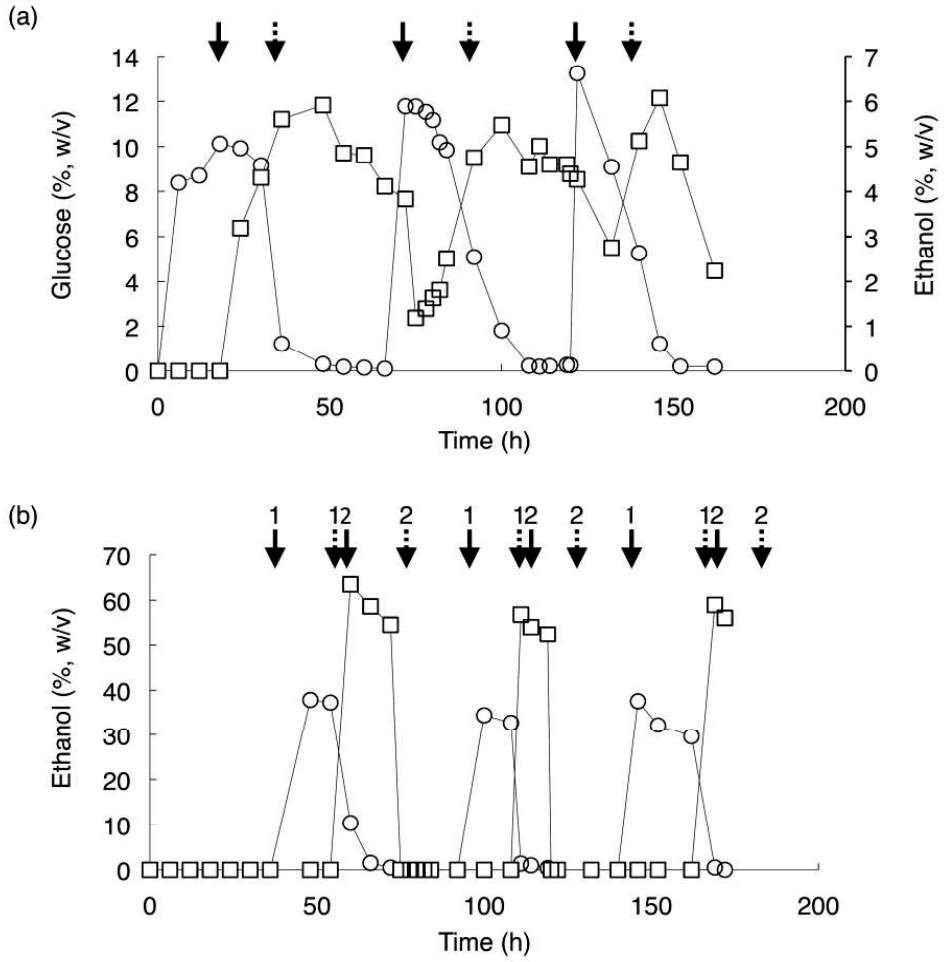

Fig. 2.4 Ethanol production by DMKU3-1042. After saccharification in KMYP-20% rice medium, fermentation with *K. marxianus* DMKU3-1042 was initiated. The initiation times (straight arrows) of the 1st, 2nd and 3rd fermentation were 18 h, 72 h and 132 h, respectively, and the finishing times (dotted lines) were 36 h, 98 h and 148 h, respectively. The initiation times (straight arrows with 1) of the first recovery of ethanol under a low pressure were 36 h, 98 h and 148 h, respectively, and finishing times (dotted lines with 1) were 52 h, 110 h and 160 h, respectively. The initiation times (straight arrows with 2) of the second recovery of ethanol were 52 h, 110 h and 160 h, respectively. Other experimental conditions are described in Experimental. (a) Open circles and open squares indicate concentrations of glucose and ethanol, respectively, in the culture. (b) Open circles and open squares indicate ethanol concentrations in the primary and secondary recovery bottles, respectively. Concentrations of glucose and ethanol were measured by HPLC at the times indicated

2.4.2 Thermotolerant ethanologenic *Z. mobilis*

2.4.2.1 Potential of *Z. mobilis* as an ethanol producer

Z. mobilis as a Gram-negative and facultative anaerobe is known to be an efficient ethanol producer, achieving an approximately 3-times higher rate of ethanol production than that of yeast by its strong Entner-Doudoroff pathway (Rogers et al. 1980; Buchholz et al. 1987), and it is generally recognized as having a safe (GRAS) status. The bacterium, however, can assimilate only three sugars, glucose, fructose and sucrose. Thermotolerant *Z. mobilis* can thus be used for high-speed and high temperature fermentation and is suitable for biomass such as sugar cane juice. I thus compared the cell growth and ethanol productivity among six *Z. mobilis* strains from the TISTR culture collection in ZMYPD medium at 30°C and 39°C under a static condition, and it was found that TISTR548 showed highest optical density and the most abundant ethanol in the medium at 39°C, suggesting that TISTR548 is the most thermotolerant strain.

2.4.2.2 Ethanol production from acid hydrolysate of Jerusalem artichoke roots

The selection and characterization of *Z. mobilis* for ethanol production from acid hydrolysate of Jerusalem artichoke (*Helianthus tuberosus* L.) roots were first investigated (Thanonkeo et al. 2011). Growth and ethanol production of four *Z. mobilis* strains isolated in Thailand, TISTR405, TISTR548, TISTR550 and TISTR551, were compared with those of the type strain *Z. mobilis* ZM4 (NRRL B-14023) at different temperatures. Among the strains tested, TISTR548 gave the highest optical density and ethanol concentration at 39°C. Therefore, this strain was chosen for ethanol production from acid hydrolysate of Jerusalem artichoke roots, called Jerusalem artichoke juice. To optimize the medium for ethanol production from Jerusalem artichoke juice, effects of several factors were examined and the results showed that the maximum ethanol concentration (95.9 g/L) with 98% of the theoretical ethanol yield was obtained when the fermentation was carried out in a

medium containing 250 g/L total sugars, pH 5.0, inoculation size at 10% and using 0.5 g/L diammonium phosphate as a nitrogen source (Thanonkeo et al. 2011). The maximum ethanol yield obtained in this study was higher than yields previously reported.

2.4.2.3 Improvement of the thermotolerance of thermotolerant *Z. mobilis*

In order to elevate thermotolerance of thermotolerant *Z. mobilis*, I conducted repeated cultivation at high temperatures and isolated a thermo-adapted strain as a TISTR548 derivative as described in Experimental. The derivative showed about 4-times higher optical density than that of the parent at 40°C in ZMYPD medium. The growth and ethanol production capacity of the derivative were then compared with those of *S. cerevisiae* and *K. marxianus* DMKU3-1042 (Fig. 2.5). The *Z. mobilis* TISTR548 derivative, *K. marxianus* DMKU3-1042 and *S. cerevisiae* were grown in KMYPD medium at 30°C and at 41°C. As a result, cell growth, glucose consumption and ethanol accumulation of the derivative were shown to be faster than those of the others at both 30°C and 41°C. Therefore, it is likely that the TISTR548 derivative is the most efficient ethanol producer among the three strains even at 41°C under conditions tested.

2.4.2.4 Application of distillation under a low pressure at 40 °C

To evaluate fermentation ability from rice, the TISTR548 derivative was subjected to fermentation experiments in ZMYP-10% rice medium at 40 °C (Fig. 2.6). In three repetitions of the fermentation and distillation process, patterns of glucose consumption and ethanol production showed periodic changes (Fig. 2.6a), suggesting that the derivative can grow under a reduced pressure. Although the maximum concentration of ethanol in the medium was almost the same as that in the experiments with yeast, an average time for fermentation was about 3 h shorter than that with *K. marxianus* DMKU3-1042 (Fig. 2.4a and Fig. 2.6a). Therefore, it is likely that the simultaneous fermentation and distillation process with the TISTR548

derivative is more efficient than that with the yeast. Ethanol concentrations recovered by primary and secondary recovery processes were 25-35% and about 60%, respectively (Fig. 2.6b).

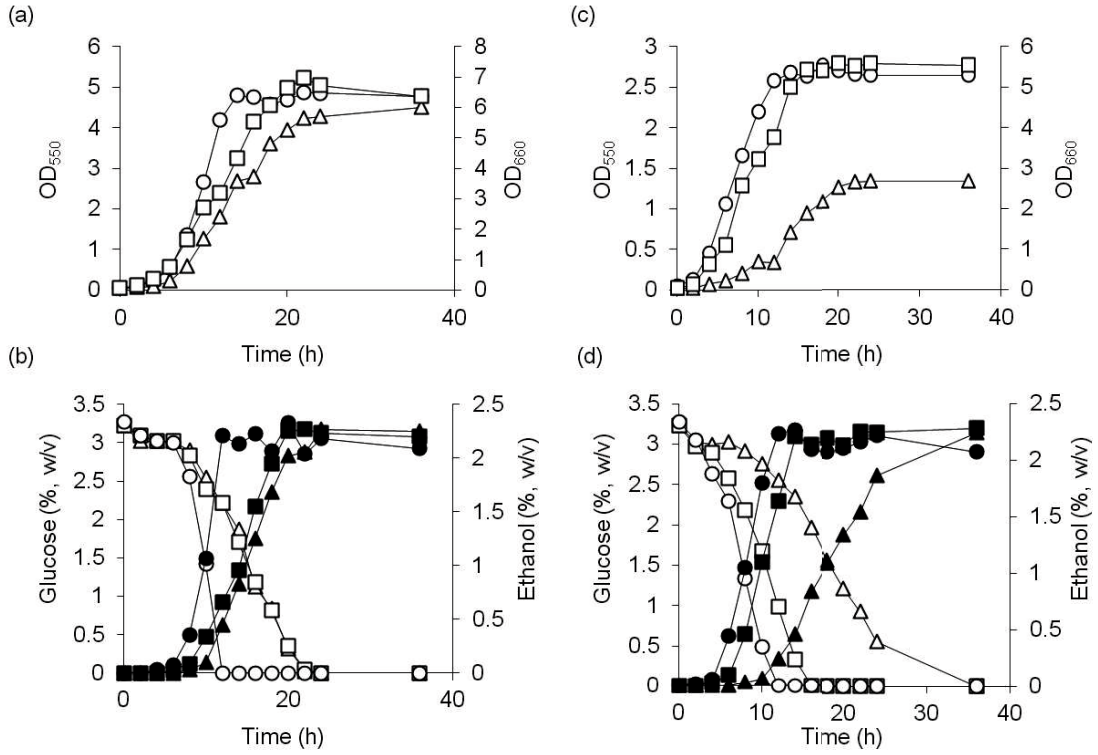

Fig. 2.5 Fermentation activities of a TISTR548 derivative and DMKU3-1042. The *Z. mobilis* TISTR548 derivative, *K. marxianus* DMKU3-1042 and *S. cerevisiae* were grown in KMYPD medium at 30ºC (a) and at 41ºC (c) and their growth was monitored by measuring OD660. Concentrations of glucose and ethanol in the culture at 30ºC (b) and at 41ºC (d) were measured by HPLC. Open circles, open squares and open triangles represent the *Z. mobilis* TISTR548 derivative, *K. marxianus* DMKU3-1042 and *S. cerevisiae*, respectively. Open symbols and closed symbols in b and d represent concentrations of glucose and ethanol in the culture, respectively.

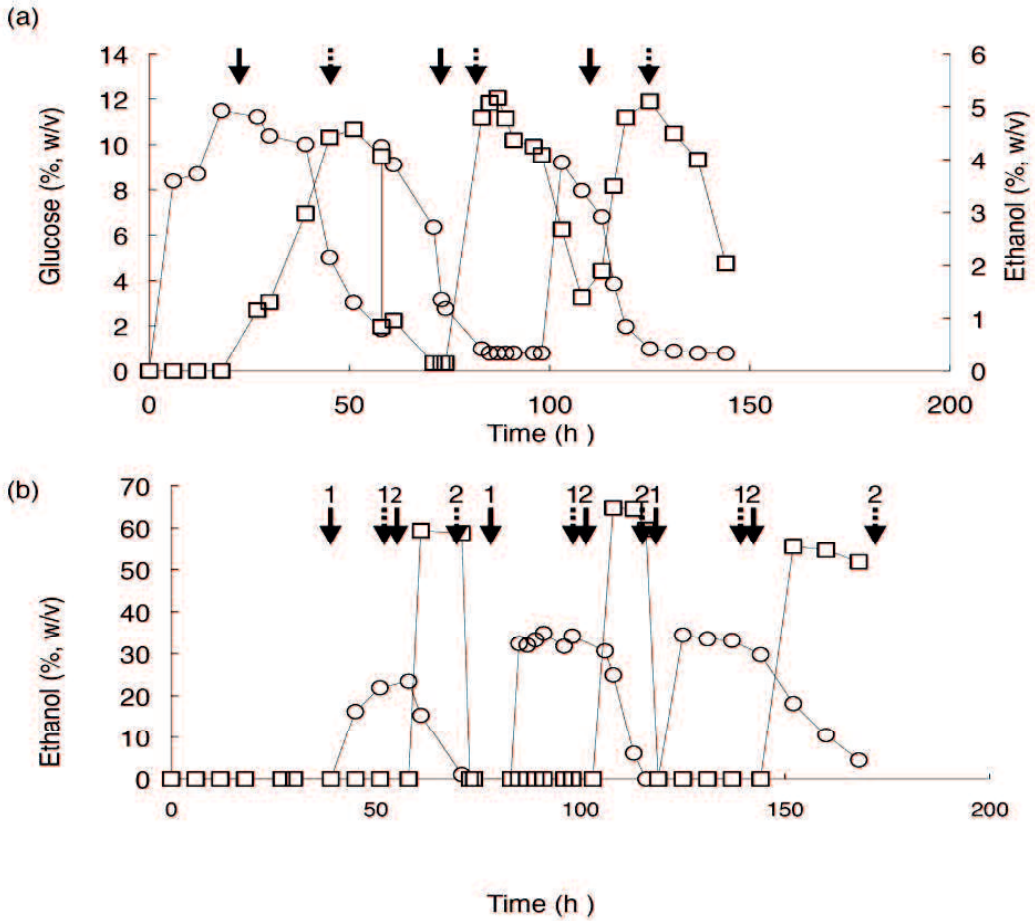

Fig. 2.6 Ethanol production by a TISTR548 derivative. After saccharification in ZMYP-20% rice medium, fermentation with *Z. mobilis* TISTR548 derivative was initiated. The initiation times (straight lines) of the 1st, 2nd and 3rd fermentation were 18 h, 72 h, and 112 h, respectively, and the finishing times (dotted lines) were 42 h, 84 h and 130 h, respectively. The initiation time (straight lines with 1) of the first recovery of ethanol under a low pressure were 42 h, 84 h and 130 h, respectively, and finishing times (dotted lines with 1) were 60 h, 110 h and 142 h, respectively. The initiation times (straight lines with 2) of the second recovery of ethanol were 60 h, 110 h and 142 h, respectively. Other experimental conditions are described in Experimental. (a) Open circles and open squares indicate concentrations of glucose and ethanol, respectively, in the culture medium. The arrows and dotted arrows indicate the start and finish time of fermentation, respectively. (b) Open circles and open squares indicate ethanol concentrations in the primary and secondary bottles,

respectively. The arrows and dotted arrows indicate the start time of first and second recovery of ethanol, respectively. Concentrations of glucose and ethanol were measured at the times indicated by HPLC.

2.5 Conclusion

In this study, I selected thermotolerant yeasts suitable for various biomasses (Table 2.1). I also showed the possibility that the thermotolerance of naturally isolated thermotolerant microbes can be further improved and the possibility of temperature-uncontrolled fermentation with *K. marxianus* DMKU3-1042 (Fig. 2.2). *Z. mobilis* is able to perform high-speed fermentation compared to yeast (Buchholz et al. 1987; Limtong et al. 2007), and thus high-speed and high-temperature fermentation is expected with thermotolerant *Z. mobilis*. A thermo-adapted derivative from thermotolerant *Z. mobilis* TISTR548 showed significantly high levels of performance in growth and ethanol fermentation at a high temperature compared to those of the parent and also to *K. marxianus* DMKU3-1042 and *S. cerevisiae* (Fig. 2.5). Further improvement for stable high-temperature fermentation might be possible by the addition of sorbitol because its supplementation promotes cell growth and increases the ethanol fermentation capability of *Z. mobilis* under heat, ethanol, and osmotic stress conditions (Sootsuwan et al. 2013). The thermotolerance of *K. marxianus* DMKU3-1042 and the thermo-adapted derivative of *Z. mobilis* TISTR548 were applied for a new technology of simultaneous fermentation and distillation under a low pressure at a high temperature. Both microbes were shown to be applicable to the new technology. Notably, the *Z. mobilis* TISTR548 derivative showed much better performance than that of *K. marxianus* DMKU3-1042 in the simultaneous fermentation and distillation. It is expected that high temperature fermentation or simultaneous fermentation and distillation at high temperatures will become widely used in the fermentation industry in the near future, and thermotolerant microbes isolated from tropical countries or further thermo-adapted or stress-adapted mutants may be key factors for such application.

CHAPTER 3

Efficient conversion of xylose to ethanol by stress-tolerant *Kluyveromyces marxianus* BUNL-21

3.1 Abstract

The fermentation ability of thermotolerant *Kluyveromyces marxianus* BUNL-21 isolated in Laos was investigated. Comparison with thermotolerant *K. marxianus* DMKU3-1042 as one of the most thermotolerant yeasts isolated previously revealed that the strain possesses stronger ability for conversion of xylose to ethanol, resistance to 2-deoxyglucose in the case of pentose, and tolerance to various stresses including high temperature and hydrogen peroxide. *K. marxianus* BUNL-21 was found to have ethanol fermentation activity from xylose that is slightly lower and much higher than that of *Scheffersomyces stipitis* (*Pichia stipitis*) at 30°C and at higher temperatures, respectively. The lower ethanol production seems to be due to large accumulation of acetic acid. The possible mechanism of acetic acid accumulation is discussed. In addition, it was found that both *K. marxianus* strains produced ethanol in the presence of 10 mM hydroxymethylfurfural or furfural, at a level almost equivalent to that in their absence. Therefore, *K. marxianus* BUNL-21 is a highly competent yeast for high-temperature ethanol fermentation with lignocellulosic biomass.

3.2 Introduction

Compared with *Saccharomyces cerevisiae,* which is widely used in ethanol fermentation industries, *Kluyveromyces marxianus* has advantageous potentials for application in ethanol production. First, *K. marxianus* is thermotolerant and is able to efficiently produce ethanol at high temperatures (Limtong et al. 2007), allowing us to develop high-temperature fermentation technology that will reduce cooling cost,

enable efficient simultaneous saccharification and fermentation, reduce the risk of contamination and enable stable fermentation even in tropical countries (Anderson et al. 1986; Banat et al. 1998; Limtong et al. 2007). Second, the yeast can assimilate various sugars including xylose, arabinose, sucrose, raffinose and inulin in addition to several hexoses (Lertwattanasakul et al. 2011; Rodrussamee et al. 2011). This broad spectrum in sugar assimilation capability is very beneficial for conversion of biomass consisting of various sugars to ethanol. Third, the yeast exhibits a relatively weak glucose repression on utilization of some sugars including sucrose (Lertwattanasakul et al. 2011) and is thus highly suitable for biomass such as sugar cane juice including glucose, fructose and sucrose as main sugars.

For application of such beneficial characteristics, however, some crucial points regarding *K. marxianus* should be improved. One point is a relatively strong glucose repression on utilization of other sugars including xylose (Rodrussamee et al. 2011). Another point is its low conversion ability of xylose to ethanol. The ethanol fermentation potential and sugar utilization profile of thermotolerant *K. marxianus* DMKU3-1042 have been extensively studied (Limtong et al. 2007; Lertwattanasakul et al. 2011; Rodrussamee et al. 2011; Pimpakan et al. 2012). The strain is capable of producing ethanol from all sugar components in lignocelluloses except arabinose. Lignocellulose, which is a second-generation biomass for biofuels, contains glucose, xylose and arabinose as abundant sugars. Tremendous exploration (Toivola et al. 1984; Barnett et al. 2000; Kurtzman et al. 2011) has revealed that only a few yeast species can efficiently ferment D-xylose to ethanol. Xylose fermentation ability of *K. marxianus* strains isolated previously, however, is much lower than those of *Scheffersomyces stipitis* (*Pichia stipitis*) and *Spathaspora passalidarum* (Nguyen et al. 2006; Krahulec et al. 2012; Su et al. 2015), and the yield of ethanol production varies in different strains (Margaritis and Bajpai 1982; Banat et al. 1996; Wilkins et al. 2008; Rodrussamee et al. 2011). Gene engineering has thus been attempted to improve the ethanol productivity from xylose in *K. marxianus* (Wang et al. 2013; Zhang et al. 2013). In addition, it is known that toxic compounds, including hydroxymethylfurfural (HMF) and furfural, that are generated in the process of hydrolysis of lignocellulosic materials prevent the growth or fermentation efficiency

of microbes (Mussatto and Roberto 2004; Behera et al. 2014), and strains that are resistant to them should thus be developed. It is notable that some ethanol-fermenting microbes such as *S. stipitis* are resistant to furfural at low concentrations (Nigam 2001c).

In this study, I characterized a newly isolated strain of *K. marxianus,* BUNL-21 strain, which is one of the most thermotolerant yeasts screened in Laos. The BUNL-21 strain was shown to be superior to the DMKU3-1042 strain, which is one of the most thermotolerant and efficient *K. marxianus* strains isolated previously, in terms of the conversion activity of xylose to ethanol, resistance to 2-deoxyglucose in the case of pentose and tolerance to various stresses. In addition, I found efficient conversion of xylose to ethanol in the presence of HMF or furfural in *K. marxianus* strains tested. I also noticed a large accumulation of acetic acid on the xylose medium, which was more than that of ethanol, and I discuss its accumulation in comparison with *S. stipitis.*

3.3 Materials and Methods

3.3.1 Yeast strains

The yeast strains used in this study were thermotolerant *K. marxianus* BUNL-21 (NITE P-01739) and DMKU3-1042 (NITE AP-283), which were isolated in Lao PDR (Results for its screening and identification will be published elsewhere.) and Thailand (Limtong et al. 2007), respectively, and *S. stipitis* CBS 5773. They were stored in YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) supplemented with 10% (v/v) glycerol at -80°C.

3.3.2 Analysis of ethanol fermentation ability

To investigate the ethanol fermentation ability of *K. marxianus* strains, YP medium (10 g/l yeast extract and 20 g/l peptone) supplemented with 20 g/l of glucose (Glc) or xylose (Xyl), designated as YPD or YPXyl, respectively, was used. When

mixed sugars were used, YP media supplemented with 2 g/l or 20 g/l Glc and 20 g/l of Xyl, designated as YPXyl + 0.2% Glc and YPXyl + 2% Glc, respectively, were used. Cells were precultured in 30 ml of YPXyl or YPD medium in a 100-ml Erlenmeyer flask sealed with aluminium foil at 30°C under a rotary shaking condition at 160 rpm for 18 h. The preculture was inoculated to 30 ml fresh medium of YPXyl or YPD in a 100-ml Erlenmeyer flask sealed with aluminium foil at the initial OD_{660} value of 0.1 and incubated at 30°C or 37°C under a shaking condition at 160 rpm. The closure with aluminum foil may reduce oxygen transfer to flasks (Chain and Gualandi 1954) and cause an oxygen-limited condition. Yeast growth was determined by measuring optical density at 660 nm on a UVmini-1240 spectrophotometer (Shimadzu, Japan) and the values were converted to dry cell weight (DCW, g/l) by using conversion factors in equations in Fig. 3.1. To determine sugar and ethanol concentrations in culture media, cultures were sampled and centrifuged at 14,000 rpm for 5 min. The supernatant was then subjected to quantitative analysis of sugars on a high-performance liquid chromatography apparatus (Hitachi, Japan) with a GLC610-S Gel pack column (Hitachi) connected to a refractive index detector Model L-2490 (Hitachi) in the mode of 0.5 ml/min eluent of deionized water at 60°C. Acetic acid was analyzed on a high-performance liquid chromatography apparatus (Hitachi) with a GLC610-H Gel pack column (Hitachi) connected to a UV detector Model L-2400 (Hitachi) in the mode of 1 ml/min eluent of 0.1% phosphoric acid at 60°C. Ethanol concentration was analyzed by a gas chromatography GC-2014 apparatus (Shimadzu) with a glass column packed with PEG-20M (Shimadzu) connected to a flame ionization detector.

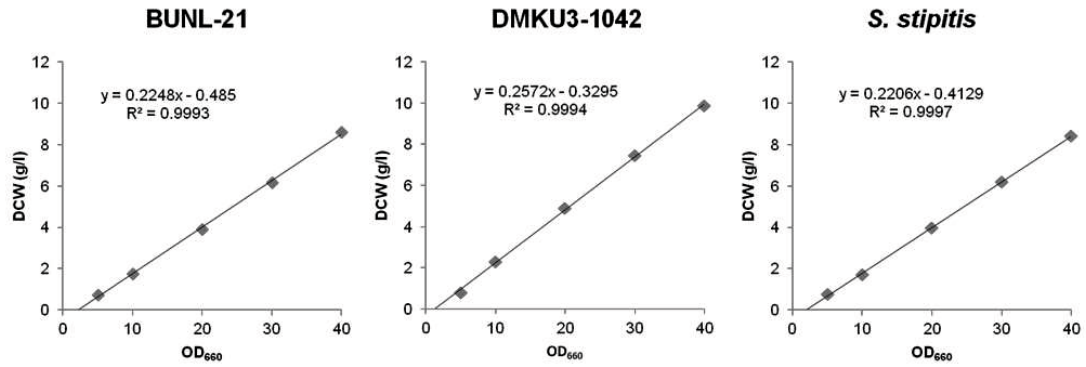

Fig. 3.1 Relation of dry cell weight to OD_{660} of *K. marxianus* BUNL-21 and DMKU3-1042 and *S. stipitis*. Cells were precultured in 3 ml of YPD medium. The precultures were inoculated to 100 ml fresh medium of YPD in a 200-ml Erlenmeyer flask at 30ºC under a shaking condition at 160 rpm. The cells were harvested and washed 2 times with sterilized dH_2O. Samples were set to 1 ml of cell solution of 0, 5, 10, 20, 30 and 40 of OD_{660} in 1.5-ml test tubes, dried up at 80ºC, and kept in a desiccator. All of the experiments were carried out in triplicate, and the values in this figure are mean values.

3.3.3 Analysis of stress resistance and glucose repression

Cells were grown at 30°C for 18 h in YPD medium, harvested, washed with distilled water, and resuspended in distilled water (1×10^7cells/ml). The cells were then 10-fold sequentially diluted and spotted onto YPD agar plates supplemented with 5 mM hydrogen peroxide (H_2O_2), 8% (w/v) ethanol, 35% (w/v) Glc, 10 mM furfural or 10 mM HMF. The plates were incubated at 30°C for 48 h. To examine glucose repression on the assimilation of other sugars, 2-deoxyglucose (2-DOG) as a glucose analog was used. Cells were spotted onto YP agar plates with or without 0.01% 2-DOG in the presence of different carbon sources: 20 g/l mannose (YPMan), 20 g/l galactose (YPGal), 20 g/l xylose (YPXyl) and 20 g/l arabinose (YPAra). These plates were incubated at 30°C or 37°C for 48 h. I performed each experiment at least three times and obtained similar results.

3.4 Results

3.4.1 Comparison of ethanol fermentation ability on Xyl in *K. marxianus* BUNL-21 with those in *K. marxianus* DMKU3-1042 and *S. stipitis* at different temperatures.

Comparison of several *K. marxianus* strains allowed us to select BUNL-21 as the highest ethanol fermenting strain on Xyl. Its growth and ethanol fermentation ability were thus compared with those of the DMKU3-1042 strain, which is one of the most efficient strains that has been analyzed in detail (Rodrussamee et al. 2011; Limtong et al. 2007). In the case of Xyl as a carbon source, its assimilation and ethanol fermentation of DMKU3-1042 have been shown to be greatly reduced at high temperatures (Rodrussamee et al. 2011). Ethanol fermentation of DMKU3-1042 under a temperature-uncontrolled condition was performed, and the maximum temperature for the fermentation process was found to be 35°C (Murata et al. 2015). I thus compared the effects of temperatures (30°C and 37°C) on ethanol fermentation from Xyl in BUNL-21, DMKU3-1042 and *S. stipitis* (Fig. 3.2, Table 3.1). At 30°C, biomass yields of BUNL-21 and *S. stipitis* were found to be lower than that of DMKU3-1042 in YPXyl medium. *S. stipitis* completely consumed Xyl within 48 h, but a trace amount of Xyl remained in both *K. marxianus* strains. The maximum ethanol yields of BUNL-21, DMKU3-1042 and *S. stipitis* were 0.15, 0.09 and 0.31 g/g, respectively. At 37°C, *S. stipitis* could neither grow nor produce ethanol, but both *K. marxianus* strains grew well at this temperature. The time required to reach the maximum ethanol level was shorter, but the maximum ethanol yields were slightly lower than those at 30°C. BUNL-21 and DMKU3-1042 showed maximum ethanol yields of 0.14 and 0.07g/g, respectively, and they completely consumed Xyl within 48 h.

During xylose utilization, xylitol was accumulated in both *K. marxianus* strains but not in *S. stipitis*. Interestingly, there was little accumulation of xylitol in BUNL-21 compared to that in DMKU3-1042 at 30°C, which seems to be consistent with a relatively small amount of ethanol in the latter, but the xylitol concentration

significantly increased in both *K. marxianus* strains at 37°C. In addition, a large amount of acetic acid accumulated in both strains compared to that in *S. stipitis*, suggesting that the relatively low level of ethanol production in *K. marxianus* strains may be due to a high level of acetic acid accumulation. Glycerol accumulation, however, was not detected in any of the strains tested (data not shown). These findings clearly indicate that the capability of conversion of Xyl to ethanol at a high temperature in *K. marxianus* is greater than that in *S. stipitis* and suggest that BUNL-21 is preferable to DMKU3-1042 for conversion at low and high temperatures.

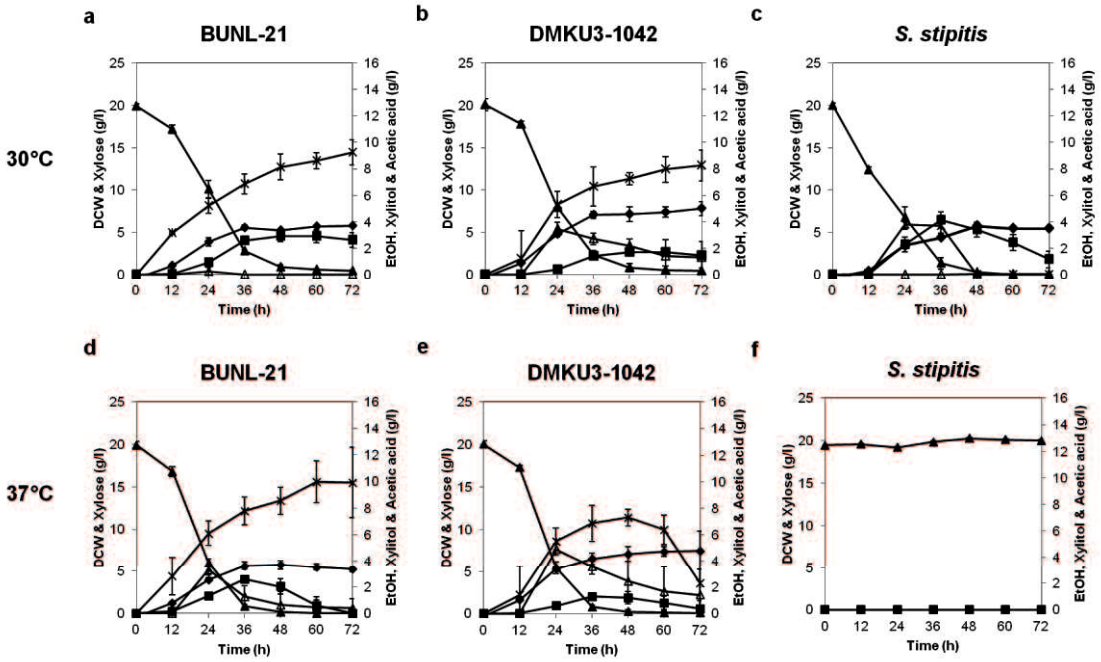

Fig. 3.2 Growth and metabolite profiles of *K. marxianus* BUNL-21 and DMKU3-1042 and *S. stipitis* in YPXyl medium at 30°C and 37°C. *K. marxianus* BUNL-21 (a, d) and DMKU3-1042 (b, e) and *S. stipitis* (c, f) were grown in YPXyl medium at 30ºC (a, b, c) or 37ºC (d, e, f) under a shaking condition at 160 rpm, and samples were taken every 12 h until 72 h of incubation. The dry cell weight (filled diamonds) and concentrations of Xyl (filled triangles), ethanol (filled squares), xylitol (open triangles) and acetic acid (crosses) are shown. Bars represent the ±SD of values from experiments performed in triplicate.

3.4.2 Stress resistance of *K. marxianus* BUNL-21 and DMKU3-1042 and *S. stipitis*

During the fermentation process, yeast cells are exposed to several stresses including osmotic stress, ethanol stress and heat stress, all of which have severe effects on cell viability and ethanol production (Gibson et al. 2007; Puligundla et al. 2011). In addition, high temperature fermentation tends to generate oxidative stress inside cells (Zhang et al. 2015). Therefore, characteristics of not only efficient ethanol producibility but also tolerance to these stresses are potentially required for candidate yeasts for the ethanol fermentation industry.

The degree of stress tolerance of BUNL-21 was thus compared with those of DMKU3-1042 and *S. stipitis*. Cells were spotted onto YPD agar plates supplemented with 5 mM H_2O_2 (oxidative stress), 35% Glc (osmotic stress) or 8% ethanol (ethanol stress), and effects of the supplements on cell growth were evaluated after incubation at 30°C for 48 h (Fig. 3.3a). The three supplements were found to more strongly inhibit the growth of DMKU3-1042 and *S. stipitis* than that of BUNL-21.

The effects of furfural and HMF were also examined. The inhibitory effects of both compounds on the growth of BUNL-21 were nearly the same as those on the growth of DMKU3-1042. *S. stipitis* showed significantly weaker resistance than the two *K. marxianus* strains to furfural. BUNL-21 was also more resistant than DMKU3-1042 at 47°C (Fig. 3.3b). Taken together, the results clearly show that BUNL-21 is much more robust than DMKU3-1042 under the stress conditions tested.

3.4.3 Effects of furfural and HMF on growth and ethanol fermentation on Xyl

The effects of furfural and HMF on growth and ethanol fermentation on Xyl in *K. marxianus* BUNL-21, DMKU3-1042 and *S. stipitis* were further examined in liquid media at 30°C and 37°C (Figs. 3.4 and 3.5, Table 3.1). Study of *S. stipitis* at 37°C was omitted due to it being a non-permissive temperature for this yeast (Fig. 3.2). Furfural and HMF showed strong or slightly negative effects, respectively, on growth and Xyl utilization of *S. stipitis* but hardly any or very slight negative effects

on BUNL-21 and DMKU3-1042. The timing of ethanol accumulation in the medium was retarded in the presence of the two compounds. At 30°C, the maximum ethanol yields of BUNL-21, DMKU3-1042 and *S. stipitis* were 0.10, 0.10 and 0.20 g/g, respectively, in YPXyl medium supplemented with 10 mM HMF, and they were 0.14, 0.10 and 0.12 g/g, respectively, in the same medium supplemented with 10 mM furfural. At 37°C, maximum ethanol yields of BUNL-21and DMKU3-1042 were 0.13 and 0.07 g/g, respectively, in YPXyl medium supplemented with 10 mM HMF, and they were 0.14 and 0.10 g/g, respectively, in the same medium supplemented with 10 mM furfural. These findings and data shown in Fig. 2a suggest that both strains, especially BUNL-21, are relatively resistant to the typical toxic compounds derived from lingocellulosic biomass.

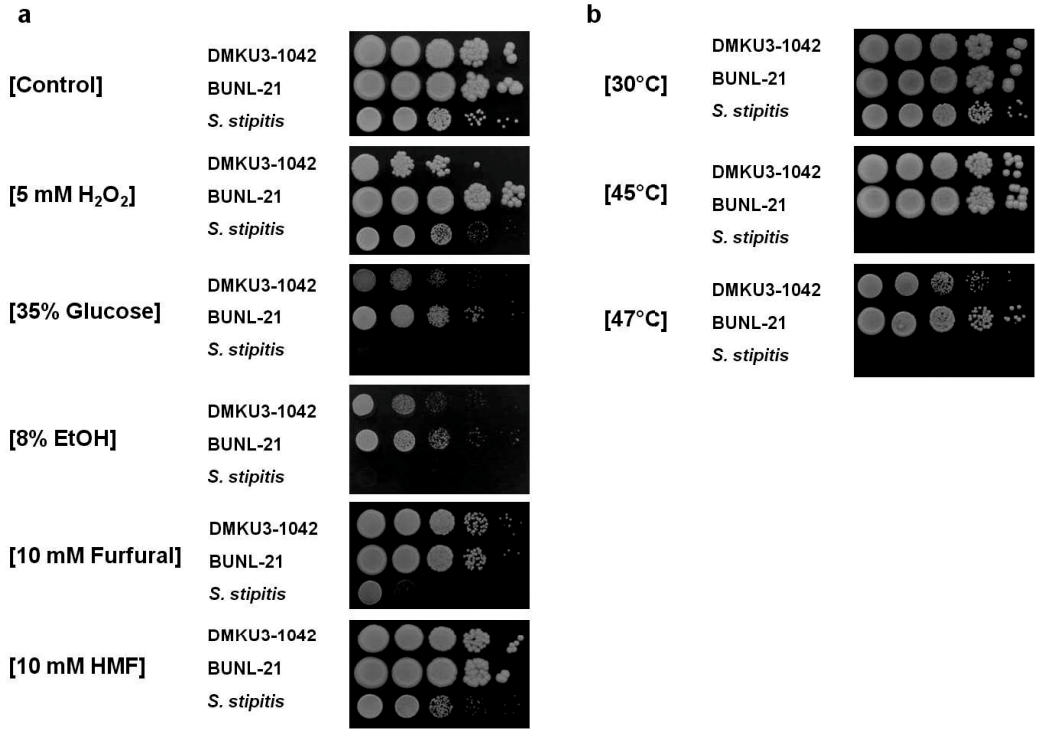

Fig. 3.3 Stress resistance of *K. marxianus* BUNL-21 and DMKU3-1042 and *S. stipitis*. Analysis of stress resistance was performed as described in Materials and Methods. Diluted cells were spotted onto (a) YPD agar plates supplemented with 5 mM H_2O_2, 8% ethanol, 35% Glc, 10 mM furfural or 10 mM HMF. The plates were incubated at 30°C for 48 h. (b) YPD agar medium was incubated at 30°C, 45°C or 47°C for 48 h.

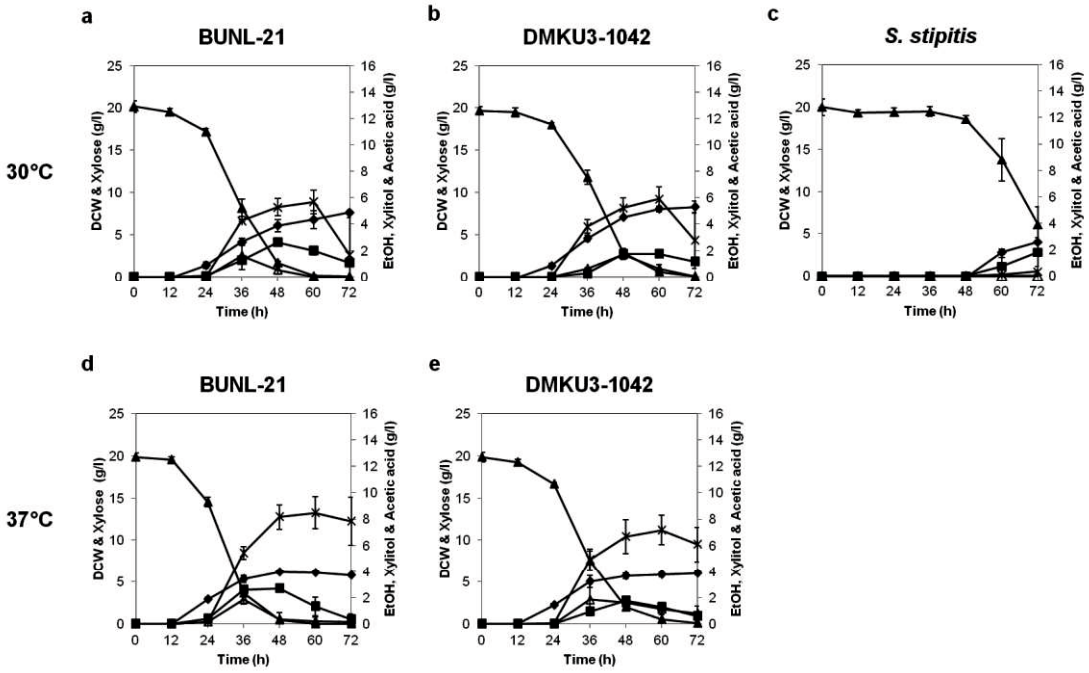

Fig. 3.4 Effects of furfural on growth and ethanol fermentation on Xyl in *K. marxianus* BUNL-21 and DMKU3-1042 and *S. stipitis*. *K. marxianus* BUNL-21 (a, d) and DMKU3-1042 (b, e) and *S. stipitis* (c) were grown in YPXyl medium supplemented with 10 mM furfural at 30ºC (a, b, c) or 37ºC (d, e) under a shaking condition at 160 rpm, and samples were taken every 12 h until 72 h of incubation. The dry cell weight (filled diamonds) and concentrations of Xyl (filled triangles), ethanol (filled squares), xylitol (open triangles) and acetic acid (crosses) are indicated. Bars represent the ±SD of values from experiments performed in triplicate.

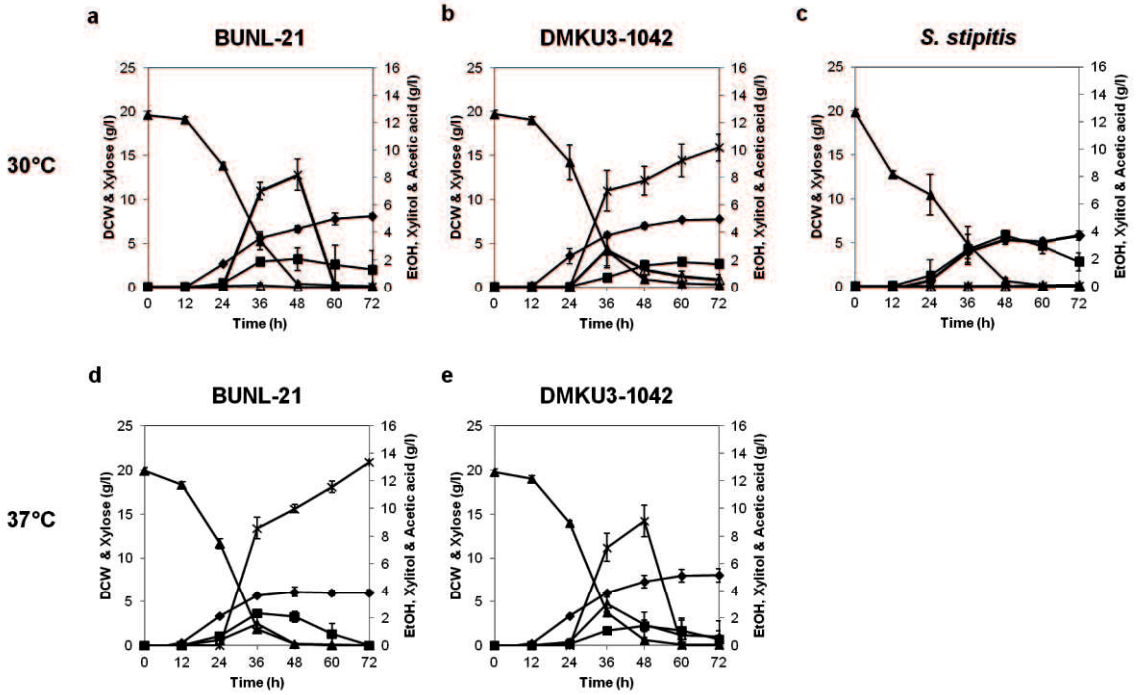

Fig. 3.5 Effects of HMF on growth and ethanol fermentation on Xyl in *K. marxianus* BUNL-21 and DMKU3-1042 and *S. stipitis*. *K. marxianus* BUNL-21 (a, d) and DMKU3-1042 (b, e) and *S. stipitis* (c) were grown in YPXyl medium supplemented with 10 mM HMF at 30ºC (a, b, c) or 37ºC (d, e) under a shaking condition at 160 rpm, and samples were taken every 12 h until 72 h of incubation. The dry cell weight (filled diamonds) and concentrations of Xyl (filled triangles), ethanol (filled squares), xylitol (open triangles) and acetic acid (crosses) are shown. Bars represent the ±SD of values from experiments performed in triplicate.

3.4.4 Effect of 2-DOG on sugar utilization

Glucose repression of the utilization of other sugars is one of the disadvantages for ethanol fermentation when biomass containing several sugars in addition to Glc is used. Thus, the effect of 2-DOG as an analog of Glc on sugar utilization was examined. Cells were spotted onto agar plates of YPGal, YPMan, YPXyl and YPAra with and without 0.01% 2-DOG and were incubated at 30°C and 37°C (Fig. 3.6). In the absence of 2-DOG, *K. marxianus* BUNL-21 and DMKU3-1042 grew better on YPMan and YPGal than on YPXyl and YPAra at both temperatures. In the medium supplemented with 0.01% 2-DOG, the growth on YPGal, YPXyl and YPAra, but not that on YPMan, significantly decreased. The inhibitory effect of 2-DOG on DMKU3-1042, but not that on BUNL-21, was slightly enhanced with an increase in temperature. In addition, BUNL-21 showed better growth than that of DMKU3-1042 on YPXyl and YPAra plates supplemented with 2-DOG. These results suggest that *K. marxianus* BUNL-21 exhibits a relatively weak glucose repression, especially of Xyl or Ara utilization, and is thus more suitable than DMKU3-1042 for application of lignocellulosic biomass.

3.4.5 Effect of Glc on Xyl utilization

To further analyze the glucose repression of *K. marxianus* BUNL-21 and DMKU3-1042, effects of Glc on their Xyl utilization were examined (Fig. 3.7). Consumption patterns of Glc in the presence of Xyl were similar to those in the absence of Xyl (data not shown), and Glc in 0.2% and 2% Glc YP media was completely used within 12 h. On the other hand, when Xyl consumption with and that without 2% Glc in YPXyl medium were compared, the utilization of Xyl was greatly delayed, especially in DMKU3-1042, in the presence of Glc (Figs. 1 and 6). Consistently, in the YP medium of 2% Xyl + 0.2% Glc, a relatively strong effect on Xyl consumption was observed in DMKU3-1042, but the effect was relatively weak in BUNL-21, indicating that BUNL-21 consumed Xyl faster than did DMKU3-1042 both at low and high Glc concentrations. These results seem to agree with the finding

that BUNL-21 exhibited weaker glucose repression than that of DMKU3-1042 in Xyl plates as shown in Fig. 5. Moreover, the maximum ethanol concentration of BUNL-21 in the mixed sugar medium was higher than that of DMKU3-1042.

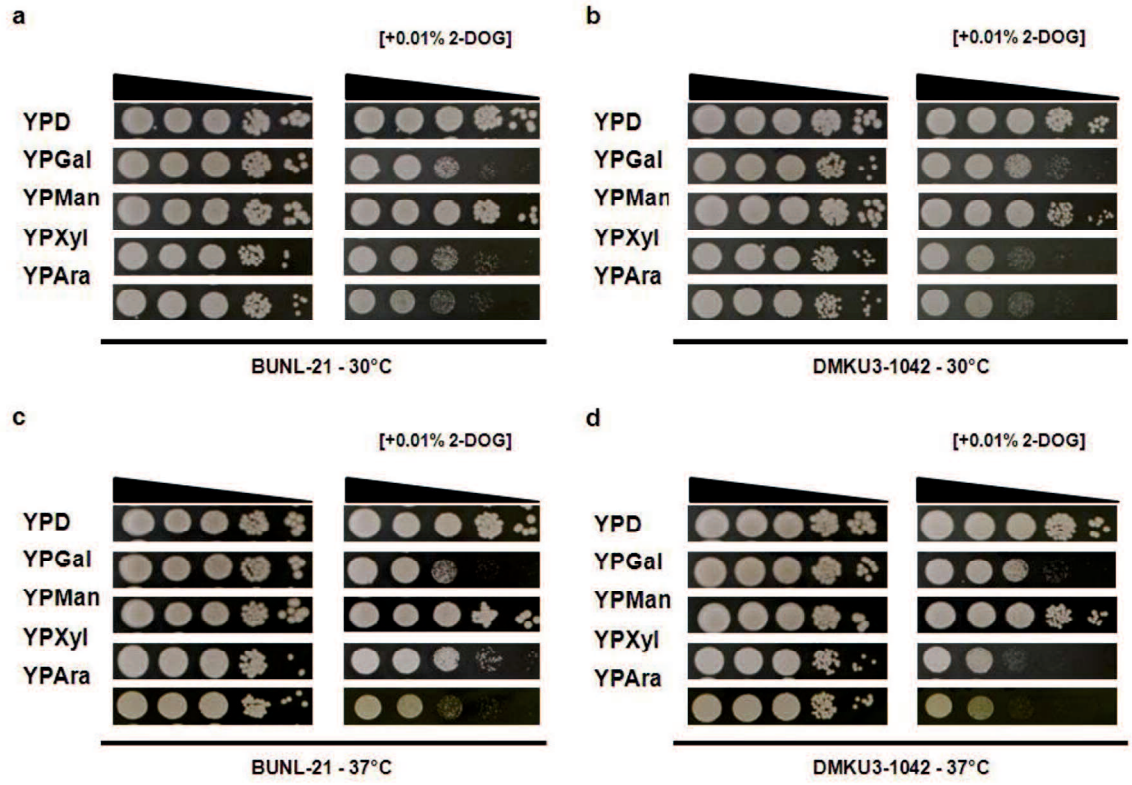

Fig. 3.6 Effect of 2-DOG on utilization of other sugars at different temperatures in *K. marxianus* BUNL-21 and DMKU3-1042. Analysis of the effect of 2-DOG on utilization of other sugars was performed as described in Materials and Methods. Diluted cells were spotted onto YPD, YPGal, YPMan, YPXyl, and YPAra agar plates supplemented with or without 0.01% 2-DOG. The plates were incubated at 30°C (a, b) or 37°C (c, d) for 48 h.

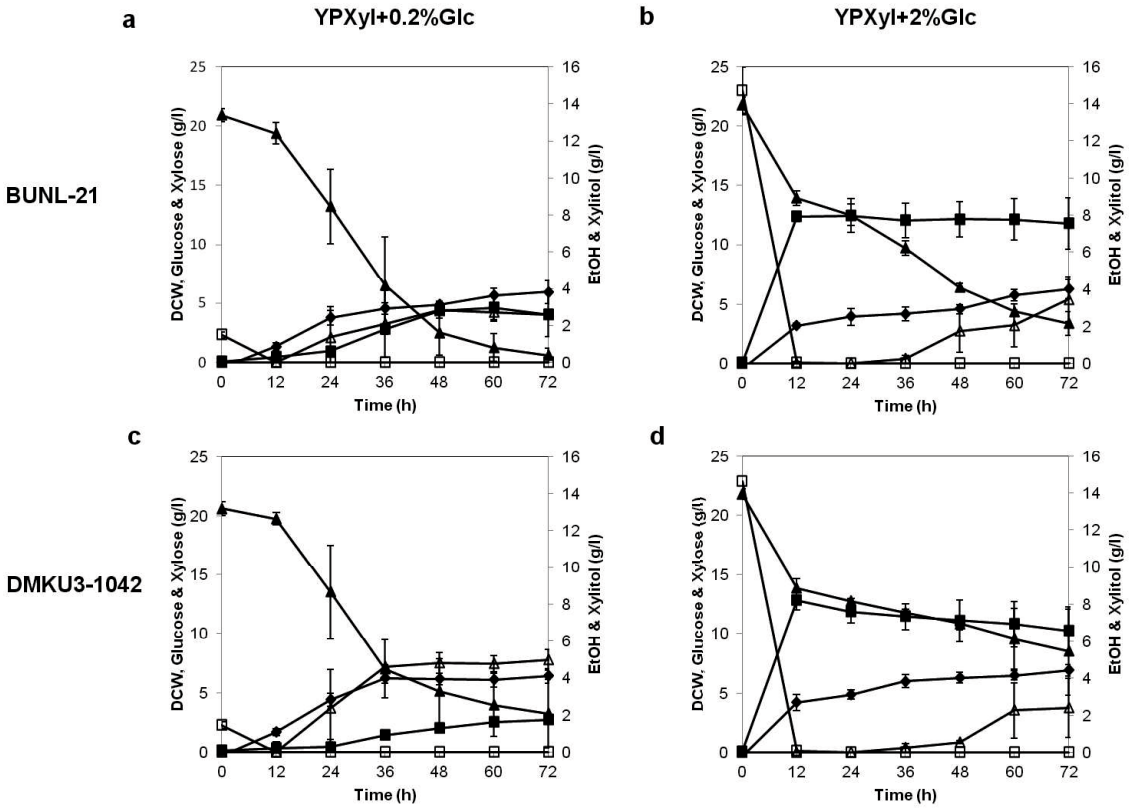

Fig. 3.7 Growth and metabolite profiles of *K. marxianus* BUNL-21 and DMKU3-1042 in a mixed sugar medium. *K. marxianus* BUNL-21 (a, b) and DMKU3-1042 (c, d) were grown in 30 ml of mixed sugar YP medium of 2% Xyl + 0.2% Glc (a, c) or 2% Xyl + 2% Glc (b, d) at 30ºC under a shaking condition at 160 rpm, and samples were taken every 12 h until 72 h of incubation. The dry cell weight (filled diamonds) and concentrations of Xyl (filled triangles), Glc (open squares), ethanol (filled squares) and xylitol (open triangles) are shown. Bars represent the ±SD of values from experiments performed in triplicate.

Table 3.1 Comparison of xylose consumption, ethanol formation, and xylitol and acetic acid accumulation among various strains of *K. marxianus*

Strains	Inhibitor	Temp. (°C)	Xylose Conc. (g/l)	Xylose Consumption (g/l)	Time (h)	Dry cell weight (g/l)	Ethanol (g/l)	Xylitol (g/l)	Acetic acid (g/l)	Dry cell yield (g/g)	Ethanol yield (g/g)	Xylitol yield (g/g)	Acetic acid yield (g/g)	Reference
S.stipitis														
CBS 5573	-	30	20	15.0± 0.69 [a]	36	4.40±0.26	4.12±0.61	0.00±0.00	3.72±0.57	0.24±0.01	0.31±0.15	0.00±0.00	0.20±0.03	This study
CBS 5573	HMF	30	20	18.7±0.49	48	5.32±0.52	3.73±0.38	0.01±0.00	0.00±0.00	0.28±0.01	0.20±0.02	0.00±0.00	0.00±0.00	This study
CBS 5573	Furfural	30	20	13.91±2.99	72	4.06±0.21	1.82±1.09	0.00±0.00	0.37±0.16	0.30±0.05	0.12±0.05	0.00±0.00	0.03±0.02	This study
K. marxianus														
BUNL-21	-	30	20	18.9±0.46	48	5.29±0.19	2.91±0.40	0.00±0.00	8.15±0.98	0.28±0.01	0.15±0.02	0.00±0.00	0.43±0.04	This study
BUNL-21	-	37	20	19.1±0.23	36	5.67±0.48	2.58±0.05	1.29±0.81	7.80±1.05	0.30±0.03	0.14±0.00	0.07±0.04	0.41±0.05	This study
BUNL-21	HMF	30	20	19.2±0.45	48	6.65±0.37	2.05±0.85	0.00±0.00	8.19±1.14	0.35±0.03	0.10±0.04	0.00±0.00	0.43±0.05	This study
BUNL-21	HMF	37	20	18.1±0.27	36	5.68±0.18	2.33±0.02	1.57±0.44	8.57±0.78	0.31±0.01	0.13±0.00	0.09±0.02	0.47±0.05	This study
BUNL-21	Furfural	30	20	18.5±0.49	48	6.06±0.70	2.61±0.10	0.52±0.31	5.28±0.65	0.33±0.05	0.14±0.01	0.03±0.02	0.29±0.03	This study
BUNL-21	Furfural	37	20	19.4±0.35	48	6.17±0.24	2.70±0.13	0.35±0.53	8.16±0.92	0.32±0.05	0.14±0.00	0.02±0.02	0.42±0.05	This study
DMKU3-1042	-	30	20	19.2±1.09	48	7.12±0.87	1.71±0.45	2.22±0.47	7.25±0.44	0.37±0.02	0.09±0.03	0.12±0.45	0.38±0.01	This study
DMKU3-1042	-	37	20	19.3±0.22	36	6.53±0.69	1.29±0.23	3.57±0.60	6.86±1.38	0.34±0.04	0.07±0.01	0.19±0.03	0.36±0.06	This study
DMKU3-1042	HMF	30	20	19.3±0.42	60	7.69±0.18	1.84±0.24	0.78±0.42	9.23±1.20	0.40±0.05	0.10±0.01	0.04±0.02	0.48±0.05	This study
DMKU3-1042	HMF	37	20	19.2±0.32	48	7.26±0.73	1.42±0.43	1.50±0.94	9.10±1.14	0.38±0.03	0.07±0.02	0.08±0.05	0.47±0.05	This study
DMKU3-1042	Furfural	30	20	16.8±0.80	48	7.04±0.11	1.76±0.07	1.69±0.35	5.23±0.75	0.42±0.03	0.10±0.00	0.10±0.02	0.31±0.03	This study
DMKU3-1042	Furfural	37	20	17.9±0.69	48	5.68±0.34	1.78±0.14	1.63±0.36	6.67±1.27	0.32±0.02	0.10±0.01	0.10±0.02	0.38±0.06	This study

Table 3.1 (Continued)

Strains	Inhibitor	Temp. (°C)	Xylose Conc. (g/l)	Xylose Consumption (g/l)	Time (h)	Dry cell weight (g/l)	Ethanol (g/l)	Xylitol (g/l)	Acetic acid (g/l)	Dry cell yield (g/g)	Ethanol yield (g/g)	Xylitol yield (g/g)	Acetic acid yield (g/g)	Reference
K. marxianus														
SUB-80-S	-	35	20	20	48	NR	5.6	NR	NR	NR	0.28	NR	NR	Margaritis and Bajpai
80-SM-16-10	-	35	20	20	48	NR	0.26	NR	NR	NR	0.11	NR	NR	Margaritis and Bajpai
IMB3	-	45	10	10	22	NR	0.80–1.20	NR	NR	NR	0.08–0.12	NR	NR	Banat et al. 1996
IMB4	-	45	10	~10	48	2.6	1.2	NR	NR	NR	0.12	NR	NR	Banat and Marchant
IMB4	Anaerobic	40	10	5.59	48	NR	0.53	0.40	NR	NR	0.09	0.07	NR	Wilkins et al.
IMB4	Anaerobic	45	10	3.39	48	NR	0.00	0.85	NR	NR	0.00	0.25	NR	Wilkins et al.
DMKU3-1042	-	30	20	20	72	NR	~2.60	~4.50	NR	NR	0.13	~0.23	NR	Rodrussamee et al.
DMKU3-1042	-	40	20	20	72	NR	~2.20	~6.50	NR	NR	0.11	~0.33	NR	Rodrussamee et al.
DMKU3-1042	-	45	20	~16.0	48	NR	~0.96	~3.00	NR	NR	0.06	~0.19	NR	Rodrussamee et al.

Temp. = Temperature, NR = not reported

[a] ± Standard deviation of values from experiments in triplicate

3.5 Discussion

In this study, I compared the capability for conversion of Xyl to ethanol of the thermotolerant *K. marxianus* strain BUNL-21 from Laos with that of the efficient strain DMKU3-1042 from Thailand and *S. stipitis*. Comparison of the ethanol productivity from Xyl or/and Glc (Figs. 1 and 6), degrees of tolerance to various stresses (Figs. 2, 3 and 4) and degree of 2-DOG tolerance indicating susceptibility to glucose repression (Figs. 2 and 5) suggests that BUNL-21 has a high potential that is superior to that of DMKU3-1042 for application in ethanol production from lignocellulosic biomass. In addition, I noticed accumulation of xylitol and large accumulation of acetic acid over ethanol in *K. marxianus* but not in *S. stipitis* when Xyl was used as a carbon source. The accumulation of acetic acid seems to result in a low level of ethanol production in *K. marxianus*.

K. marxianus and *S. stipitis* are Crabtree-negative yeasts. Several studies have revealed that *K. marxianus* is superior to *S. stipitis* in thermotolerance and stress resistance but inferior in terms of ethanol productivity from Xyl and oxygen dependence (Jeffries and Van Vleet 2009; Lertwattanasakul et al. 2015; Signori et al. 2014). *S. stipitis* CBS5773 and CBS 6054 achieved ethanol yields of ~0.41 g/g under both anaerobic and microaerobic conditions (Krahulec et al. 2012). Under the conditions used in this study, *S. stipitis* CBS 5773 achieved ethanol yield of 0.31 g/g at 30°C. On the other hand, *K. marxianus* DMKU3-1042 achieved ethanol yields of 0.13, 0.11 and 0.06 g/g at 30°C, 40°C and 45°C, respectively, under a shaking condition, but its ethanol yield was negligible under a static condition (Rodrussamee et al. 2011). *K. marxianus* IMB4 showed ethanol yields of ~0.12 g/g at 45 °C (Banat and Marchant 1995) under an aerobic condition, but its ethanol yield decreased to 0.09 g/g at 40°C and 0.00 g/g at 45°C under an anaerobic condition (Banat and Marchant 1995; Wilkins et al. 2008). These results suggest that *K. marxianus* has a higher oxygen dependency than that of *S. stipitis*.

As described above, the thermotolerant characteristics of *K. marxianus* are beneficial for high-temperature fermentation or robust fermentation. The two *K. marxianus* strains tested in this study were shown to be able to ferment Xyl at 37°C, a

temperature at which *S. stipitis* cannot grow. The yield of ethanol production from Xyl of *K. marxianus* varies in different strains from 0.1 to 0.28 g/g (Margaritis and Bajpai 1982; Banat et al. 1996; Wilkins et al. 2008; Rodrussamee et al. 2011) (Table 1). However, BUNL-21 achieved ethanol yields of 0.15 and 0.14 g/g on Xyl at 30°C and 37°C, respectively, which were about 1.7- and 2.0-times higher, respectively, than those of DMKU3-1042 (Table 1). Regarding ethanol yield, although the fermentation conditions were not the same, BUNL-21 showed ethanol yields higher than those of strains 80-SM-16-10, IBM3 and IBM4, the ethanol yields of which were 0.11, 0.12 and 0.12 g/g, respectively, but lower than that of strain SUB-80-S, the ethanol yield of which was 0.28 g/g at 35°C (Banat and Marchant 1995; Banat et al. 1996; Margaritis and Bajpai 1982). Moreover, it is notable that the productivity of both strains was hardly inhibited by HMF or furfural, and the ethanol yield of BUNL-21 was 0.14 g/g in the presence of furfural (Figs 3 and 4, Table 1).

It was found that both *K. marxianus* strains accumulated larger amounts of xylitol than that accumulated by *S. stipitis.* The accumulation of xylitol seems to reduce the yield of ethanol. I have limited evidence regarding the superiority of BUNL-21 to DMKU3-1042. BUNL-21 accumulated a smaller amount of xylitol both at 30°C and 37°C (Fig. 1). Consistently, when xylitol was used as a carbon source, BUNL-21 assimilated xylitol and grew better than DMKU3-1042 did (Fig. 3.8). Notably, the expression level of *ADH2*, which is a gene for the major alcohol dehydrogenase in ethanol fermentation on Glc (Lertwattanasakul et al. 2007) and is down-regulated on Xyl (Lertwattanasakul et al. 2015), in BUNL-21 was found to be about 3-times higher than that in DMKU3-1042 on Xyl (Fig. 3.9). Its relatively high expression level of *ADH2* may provide more NAD^+ to xylitol dehydrogenase to prevent xylitol accumulation and produce more ethanol. Moreover, it was found that a higher temperature caused more accumulation of xylitol in both *K. marxianus* strains (Fig. 1). The accumulation might be due to the limitation of NAD^+ as a result of the so-called cofactor imbalance (Jeffries and Jin 2004; Zhang et al. 2013; Hou et al. 2014). If so, it is assumed that some enzyme activity coupled to NADH oxidation is weakened at a high temperature. Alternatively, the

pathway from Xyl uptake to xylitol might be enhanced or the pathway downstream from xylitol might be weakened as temperature increased.

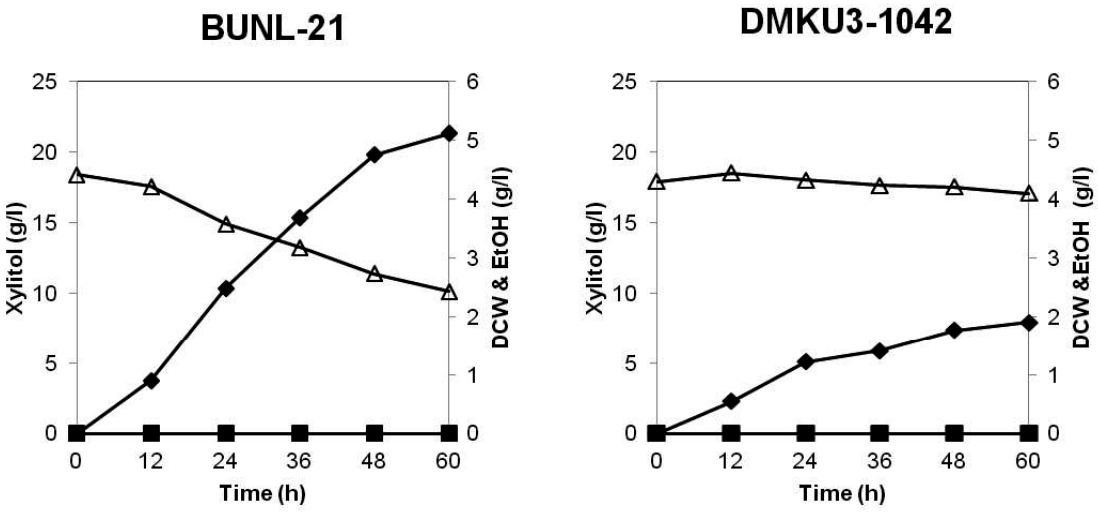

Fig. 3.8 Growth and metabolite profiles of *K. marxianus* BUNL-21 and DMKU3-1042 in YP medium containing 2% xylitol. Cells were precultured in 30 ml of YPXyl medium in a 100-ml Erlenmeyer flask at 30°C under a rotary shaking condition at 160 rpm for 18 h. The preculture was inoculated into 30-ml fresh medium of YP medium containing 2% xylitol in a 100-ml Erlenmeyer flask, at the initial OD_{660} value of 0.1, and incubated at 30°C under a shaking condition at 160 rpm. Samples were taken every 12 h until 72 h of incubation. The dry cell weight (filled diamonds) and concentrations of xylitol (open triangles) and ethanol (filled squares) are shown.

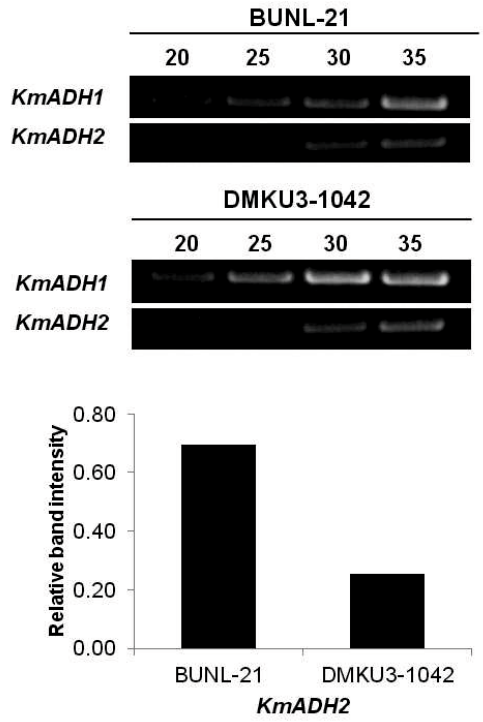

Fig. 3.9 Expression of *KmADH1* and *KmADH2* genes of *K. marxianus* BUNL-21 and DMKU3-1042 in YPXyl medium. Cells were precultured in 30 ml of YPXyl medium in a 100-ml Erlenmeyer flask at 30°C under a rotary shaking condition at 100 rpm for 18 h. The preculture was inoculated to 30-ml fresh medium of YPXyl in a 100-ml Erlenmeyer flask at 30°C under a rotary shaking condition at 100 rpm for 16 h. Total RNA was then isolated and subjected to RT-PCR. RT-PCR was performed with primers specific to *KmADH1* and *KmADH2* as previously performed (Lertwattanasakul et al. 2007). After reverse transcriptase reaction, PCR products of 20, 25, 30, and 35 cycles were subjected to agarose gel electrophoresis and stained with ethidium bromide (a). Band intensity was analyzed by using the UNSCAN-IT gel™ automated digitizing system (Silk Scientific). Ratio of intensity of each band for *KmADH2* to that for *KmADH1*, a constitutive gene, at the 30th cycle (in a liner range) is shown in (b).

Large accumulation of acetic acid (0.36-0.43 g/g) was found in both *K. marxianus* strains at 30° and 37°C, which may be responsible for the low level of ethanol production. One speculative reason for the acetic acid accumulation is the requirement of NADPH for xylose reductase in xylose catabolism (Signori et al., 2014). The accumulation is consistent with the up-regulation of *ALD4* for acetaldehyde dehydrogenase on Xyl (Fig. 3.10) (Lertwattanasakul et al. 2015). At the same time, *RKI1* for ribose-5-phosphate isomerase in the pentose phosphate pathway (PPP) is largely down-regulated (Fig. 3.10). I thus speculated that the NADPH supply for xylose reductase activity by PPP is limited and compensatorily the cofactor is provided by the acetaldehyde dehydrogenase-mediated reaction. On the other hand, no such down-regulation of any of the genes including *RKI1* for PPP occurs in *S. stipitis* (Jeffries and Van Vleet 2009). However, when xylulose was used as a carbon source, the level of acetic acid was hardly changed (Fig. 3.11). Another possible reason is the supply of NADPH for removal of reactive oxygen species (ROS) via antioxidants including glutathione. Evidence that genes for the oxidative stress defense mechanism were up-regulated in Xyl medium (Lertwattanasakul et al. 2015) indicates the possibility that *K. marxianus* accumulates ROS under the condition with Xyl.

Notably, in Glc medium, large accumulation of acetic acid was observed at 45°C, but not at 30°C, and the accumulation of acetic acid was prevented by the addition of the reduced form of glutathione (unpublished data). These findings allow us to speculate that cells accumulate ROS at a high temperature and, via the acetic acid production pathway, supply NADPH for detoxification of ROS. In Xyl medium, however, the addition of reduced glutathione showed no effect on the accumulation of acetic acid. This failure might be due to the lower expression levels of genes for the putative glutathione transporters (*OPT1* and *OPT2*) in Xyl medium (Lertwattanasakul et al. 2015). Another possible reason for the failure is that cells require a much larger amount of NADPH for other cellular activities under the condition with Xyl as a carbon source than those with Glc at 45°C.

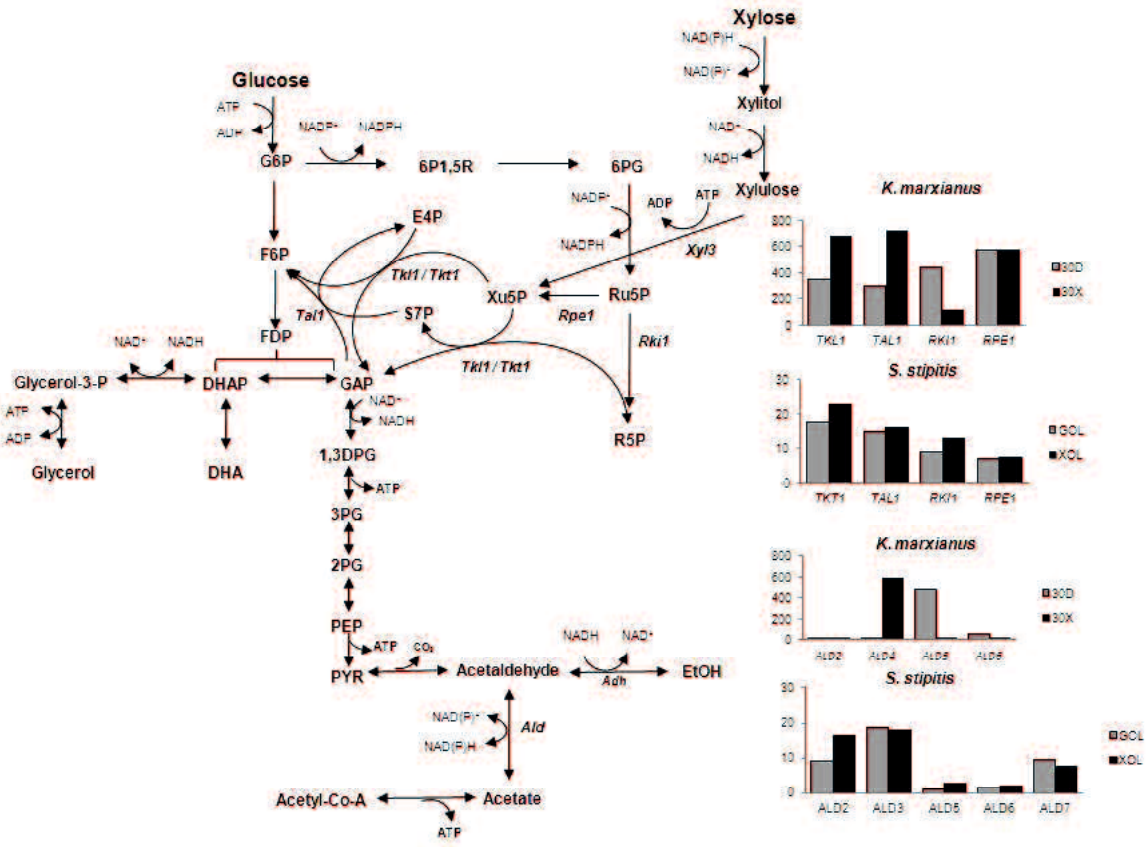

Fig. 3.10 Expressional difference in genes for enzymes in the pentose phosphate pathway and acetaldehyde dehydrogenase between *K. marxianus* and *S. stipitis*. The data for *K. marxianus* and *S. stipitis* were reproduced from Lertwattanasakul et al. (2015) and Jeffries and Van Vleet (2009), respectively. Abbreviations are 30D: in Glc medium at 30°C under a shaking condition; 30X: in Xyl medium at 30°C under a shaking condition; GOL: in Glc medium at 30°C under an oxygen-limited condition; XOL: in Xyl medium at 30°C under an oxygen-limited condition.

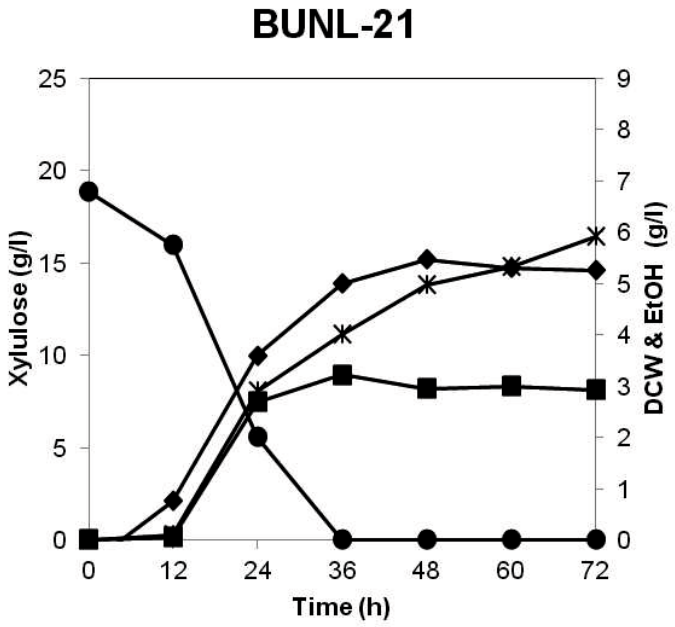

Fig. 3.11 Growth and metabolite profiles of *K. marxianus* BUNL-21 in YP medium containing 2% xylulose. Cells were precultured in 30 ml of YPXyl medium in a 100-ml Erlenmeyer flask at 30°C under a rotary shaking condition at 160 rpm for 18 h. The preculture was inoculated to 30-ml fresh medium of YP medium containing 2% xylulose in a 100-ml Erlenmeyer flask, at the initial OD_{660} value of 0.1, and incubated at 30°C under a shaking condition at 160 rpm. Xylulose of more than 98% purity was prepared by purification after conversion of xylose (Tokyo Chemical Industry) by using xylose isomerase (Novozymes). Samples were taken every 12 h until 72 h of incubation. The dry cell weight (filled diamonds) and concentrations of xylulose (filled circles), ethanol (filled squares) and acetic acid (crosses) are shown.

3.6 Conclusion

Application of a stress-resistant and highly efficient microbe for ethanol fermentation is a crucial point for industrial application. This study provided evidence that the newly isolated strain of *K. marxianus* BUNL-21 bears a high potential for conversion of Xyl to ethanol, has strong resistance to high temperature and to toxic materials, and exhibits relatively weak glucose repression. These beneficial characteristics will allow us to develop a more efficient Xyl-to-ethanol converter by gene engineering on the basis of BUNL-21. The first findings of a large accumulation of acetic acid and its relation to specific gene expression on Xyl in *K. marxianus* motivate us to do gene engineering to improve NADPH production and reduce acetic acid accumulation.

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SUMMARY

Characterization and improvement of thermotolerant ethanologenic microbes for low-cost bioethanol production from various feedstocks

Rising demand of an alternative energy is fueled by the growing of world population and increasing of *petroleum* price. Bioethanol is one of alternative and renewable fuels derived from various plant biomasses by ethanologenic microorganisms. At present, bioethanol production is mainly conducted with sugar or starch base biomass such as corn, sugarcane and cassava. However, to achieve sustainable energy, the production of ethanol with low-cost lignocellulosic biomasses, which does not undermine the food supply, has been challenged. Moreover, the application of thermotolerant, stress-tolerant and highly efficient microbes for high-temperature ethanol fermentation is expected to be a crucial point for industrial application.

In this study, I focused on the development of low-cost ethanol production system. Ethanologenic microbes were screened from natural sources in tropical countries for the utilization of various biomasses and were further improved by using several procedures. The high-temperature fermentation with rice hydrolysate by two microbes, *Kluyveromyces marxianus*, *Zymomonas mobilis* and their derivatives were investigated, and their fermentation capabilities were compared with that of *Saccharomyces cerevisiae,* which is widely used in ethanol fermentation industries. The results showed that naturally isolated microbes can be further improved and increased the ability of ethanol fermentation at high temperature. Moreover, the possibility of temperature-uncontrolled fermentation has been shown, which could be an economical process under high-temperature circumstances because it may cut down the cost of cooling units and their running when *applied* for industries. In addition, thermotolerant *K. marxianus* DMKU3-1042 and the thermo-adapted derivative of *Z. mobilis* TISTR548 were applied for a new technology of simultaneous fermentation and distillation under a low pressure at a high

temperature. Both microbes were shown to be applicable to this new technology. Considering the concentration of ethanol, application of this technology could achieve the final ethanol concentration up to 60 % (w/v) which may reduce the cost of energy used for distillation process.

On the other hand, the capability for conversion of xylose, which is the second most abundant sugar component in lignocellulosic biomass, to ethanol in *K. marxianus* was extensively examined. *K. marxianus* BUNL-21 that was isolated in Laos was characterized and compared with that of the efficient strain DMKU3-1042 previously isolated in Thailand. The results revealed that the strain BUNL-21 possesses greater ability for conversion of xylose to ethanol, stronger resistance to 2-deoxyglucose in the case of pentose, and higher tolerance to various stresses than those of DMKU3-1042. In addition, it was found that both *K. marxianus* strains produced ethanol in the presence of 10 mM hydroxymethylfurfural or furfural, at a level almost equivalent to that in their absence. Comparison with *Scheffersomyces stipitis*, which is a native xylose-fermenting yeast, revealed that *K. marxianus* BUNL-21 was found to have ethanol fermentation activity from xylose that is slightly lower and much higher than that of *S. stipitis* at 30°C and at higher temperatures, respectively. Large accumulation of acetic acid was noticed during the conversion of xylose to ethanol of both *K. marxianus* strains which may be the reason of the lower ethanol production of *K. marxianus* than that of *S. stipits*. These findings motivate us to do gene engineering to improve ethanol production and reduce acetic acid accumulation. However, the beneficial characteristics clearly indicate that *K. marxianus* BUNL-21 is a highly competent yeast for high-temperature ethanol fermentation with lignocellulosic biomass.

This study provides valuable information and technology for application of thermotolerant *K. marxianus* and *Z. mobilis* for low-cost bioethanol production from various feedstocks.

LIST OF PUBLICATIONS

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

1. High-temperature fermentation technology for low-cost bioethanol

Masayuki Murata, Sukanya Nitiyon, Noppon Lertwattanasakul, Kaewta Sootsuwan, Tomoyuki Kosaka, Pornthap Thanonkeo, Savitree Limtong, Mamoru Yamada

Journal of the Japan Institute of Energy (2015), 94: 1154-1162

(CHAPTER 2)

2. Efficient conversion of xylose to ethanol by stress-tolerant *Kluyveromyces marxianus* BUNL-21

Sukanya Nitiyon, Chansom Keo-oudone, Masayuki Murata, Noppon Lertwattanasakul Savitree Limtong, Tomoyuki Kosaka, Mamoru Yamada

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(CHAPTER 3)