Chapter 7

Potential of Thermotolerant Ethanologenic Yeasts Isolated from ASEAN Countries and Their Application in High-Temperature Fermentation

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Abstract

Thermotolerant ethanologenic yeasts receive attention as alternative bio-ethanol producers to traditionally used yeast, *Saccharomyces cerevisiae*. Their utilization is expected to provide several benefits for bio-ethanol production due to their characteristics and robustness. They have been isolated from a wide variety of environments in a number of ASEAN countries: Thailand, Vietnam, Laos, and Indonesia. One of these yeasts, Kluyveromyces marxianus has been investigated regarding characteristics. Some strains efficiently utilize xylose, which is a main component of the 2nd generation biomass. In addition, the genetic basis of *K. marxianus* has been revealed by genomic sequencing and is exploited for further improvement of the strains by thermal adaptation or gene engineering techniques. Moreover, the glucose repression of *K. marxianus* and its mechanisms has been investigated. Results suggest that *K. marxianus* is an alternative to *S. cerevisiae* in next-generation bio-ethanol production industry. Indeed, we have succeeded to apply *K. marxianus* for bio-ethanol production in a newly developed process, which combines high-temperature fermentation with simultaneous fermentation and distillation under low pressure. This chapter aims to provide valuable information on thermotolerant ethanologenic yeasts and their application, which may direct the economic bioproduction of ethanol and other useful materials in the future.

Keywords: thermotolerant yeast, high-temperature fermentation, genomic aspects

1. Introduction

Worldwide economic growth with the related increase in CO_2 emissions from fossil fuels causes global warming. Utilization of renewable energy with low CO_2 emission therefore has been getting increased attention. Renewable energy is generated from renewable natural resources, such as sunlight, wind, rain, tides, waves, geothermal heat, as well as biomass. One such important source of renewable energy, *bio-ethanol*, has been highlighted due to the characteristics of its production from biomass, which is generated by plants using sunlight for CO_2 fixation, resulting in carbon neutrality. Bio-ethanol is the name for ethanol produced from biomass by fermentation. This bio-process is thoroughly researched and well-established, and to-date, it produces the most prominent and cost-effective biofuel [1]. Although bio-ethanol production is increasing worldwide and the production of biofuels including ethanol in 2022 is forecasted to be more than 126 billion L [2], biofuels are still more costly than fossil fuels [3]. Therefore, several industrial companies and researchers aim to develop new technologies, enabling the cost-effective production of bio-ethanol from biomass. Since microorganisms are essential for material production through bio-processing, their characteristics and traits are crucial for the production process efficiency. Ethanologenic yeast, *Saccharomyces cerevisiae*, has been traditionally and widely utilized for the production of alcoholic beverages and bio-ethanol [4, 5]. Industrially common problems in bio-ethanol production related to *S. cerevisiae* strains are temperature level (35–45°C) and high ethanol concentration (over 20%) [6]. These two factors inhibit yeast proliferation and fermentation activity if they reach the upper limit. In addition, for cost-effective bio-ethanol production, the production source must be changed from 1st generation biomass (sugarcane, corn, and wheat, which are important food sources) to 2nd generation biomass (lignocellulosic biomass or woody crops, which are agricultural residues or waste) [7]. Lignocellulosic biomass is composed of hemicellulose, cellulose, and lignin, and the first consists of six (e.g., glucose) and five (e.g., xylose) carbon sugars. However, the low efficiency of ethanol production by *S. cerevisiae* from lignocellulosic biomass hydrolyzates is mainly due to its little ethanol productivity from xylose [8]. Although the *S. cerevisiae* genome encodes all components necessary for xylose utilization, most of them are rarely expressed [9]. In addition, *S. cerevisiae* preferably utilizes glucose while repressing the uptake and catabolism of alternate carbon sources by a mechanism such as glucose repression [10]. This results in the reduction of ethanol production rates from several kinds of biomass. For economically feasible bioethanol production from lignocellulosic biomass, the efficient co-fermentation of glucose and other sugars is also necessary. Therefore, genetic engineering of *S. cerevisiae* strains has been extensively performed, and metabolically engineered strains were developed [11], which have showed higher stress tolerance and/or improved xylose utilization [12, 13]. However, the utilization of genetically recombinant strains in industry has been very limited, especially due to the instability of the desirable phenotype and the necessary confinement to a closed system to prevent their leakage into the environment, which can eventually endanger public health or biodiversity. Therefore, the development of new feasible strains for next-generation bio-ethanol production is under way, and new yeast strains have been isolated that may have advantages compared to *S. cerevisiae*.

Recently, thermotolerant microorganisms were found among mesophiles with optimum growth temperatures that are 5–10°C higher than those of the typical mesophilic strains

belonging to the same genus or even to the same species [14]. These thermotolerant mesophiles are mainly and widely distributed in foods, plants, soils, and waters from tropical environments in ASEAN countries [15]. In these environments, relatively high temperature presumably becomes a selective pressure to enrich thermotolerant strains. These thermotolerant strains are expected to provide a benefit for the industries because they are more robust and resistant to many stressors [14]. In addition, some of these thermotolerant microorganisms can produce distinctive enzymes that function under relatively high temperature conditions [16–18]. Thermotolerant yeasts have been found and isolated from a number of countries [19–28]. Of these, *K. marxianus* is a haploid, homothallic, thermotolerant, and hemiascomycetous yeast [29, 30]. One such yeast, *K. marxianus* DMKU 3-1042 isolated in Thailand, shows relatively high ethanol productivity and fermentation ability at high temperatures [31], assimilates various sugars including xylose and/or arabinose [32], and exhibits relatively weak glucose repression on utilization of some sugars including sucrose [33]. Therefore, *K. marxianus* is, in comparison to *S. cerevisiae,* a promising candidate for next-generation bio-ethanol production. In addition, the genomic sequences of *K. marxianus* are available [34, 35], and genetic technology and tools have also been developed [36]. Moreover, *K. marxianus* has been a platform for next-generation protein production for structural and biochemical studies [18, 29]. However, it is possible that unidentified and more beneficial thermotolerant yeasts exist in ASEAN countries, especially, thermotolerant high xylose-utilizing and ethanol-producing yeasts, which are needed for 2nd generation biomass utilization. None of the isolated *K. marxianus* strains, however, are able to more efficiently convert xylose to ethanol than strains of other xylose-utilizing yeasts, such as *Pichia stipitis* (*Scheffersomyces stipitis*) [32, 37].

Thermotolerant strains allow the development of high-temperature fermentation (HTF) technology, which enables fermentation at 5–10°C higher than the traditional fermentative process [38, 39]. HTF is thus expected to reduce cooling costs, running costs at the simultaneous saccharification and fermentation (SSF) stage, and contamination risks [6, 31, 38–40], therefore offering a promising technology for bio-ethanol production. Moreover, thermotolerant yeast can also be applied for temperature-uncontrolled fermentation, hence offering another economical advantage. A combination of efficient bioreactors and robust hosts, such as thermotolerant strains, leads to lowest energy consumption and emission of CO_2 in biofuel production [41].

In this chapter, we outline a number of thermotolerant yeasts including *K. marxianus* species isolated in Thailand and their characteristics, including utilization of various sugars, glucose repression, and genetic information, that are beneficial for high-temperature fermentation. In addition, new strains of thermotolerant yeasts that have been isolated in Indonesia, Vietnam, and Laos are summarized. Subsequently, the trial results of HTF with some of these strains for ethanol production are presented.

2. Various ethanologenic thermotolerant yeasts and their characteristics

Increasing global energy demand that exceeds the finite supply of fossil fuel has spurred scientific research to deliver alternative fuels. Microbial fermentation and efficient conversion

technologies now allow the extraction of biofuels from biomass, such as wood, crops, and waste materials. Supplies of ethanol have increased tremendously and are expected to continue rising rapidly in both developed and developing countries [41]. A variety of feedstocks from the 1st, 2nd, and 3rd generation have been used in bioethanol production [42]. First-generation bioethanol involves feedstocks rich in sucrose (sugar cane juice, molasses, and sweet sorghum) and starch (corn, wheat, cassava, and potato). Second-generation bioethanol comes from lignocellulosic biomass such as wood, straw, and other agricultural wastes. Third-generation bioethanol is derived from algal biomass including microalgae and macroalgae [43, 44]. The process of ethanol production depends on the types of feedstocks used. Generally, there are three major steps in ethanol production: decomposition of biomass, fermentation, and product recovery. During fermentation, the cooling of fermenters is one of the major energy consuming steps because the metabolism of yeast releases a large amount of heat. Therefore, the application of thermotolerant yeasts can significantly reduce the cooling cost and help prevent contamination [38]. High-temperature ethanol fermentation will also benefit a simultaneous saccharification and fermentation process.

Many thermotolerant yeasts have been isolated from various natural habitats and tested for their capability to produce ethanol at high temperatures (**Table 1**). Many strains of *K. marxianus*, *Pichia kudriavzevii*, and *S. cerevisiae* were often isolated as ethanol-producing yeasts at high temperatures. Of these, *K. marxianus* was found to be the most thermotolerant yeast. Limtong et al. [31] isolated *K. marxianus* DMKU 3-1042 in Thailand and found optimum ethanol production at 40°C. The strain was compared with other *K. marxianus* strains including NCYC587, NCYC1429, and NCYC2791 and found to be the best ethanol producer at 45°C [36]. Kumar et al. [45] isolated *Kluyveromyces* sp. IIPE453 from a soil sample in a sugar mill, which showed high ethanol production rate at 45–50°C. Yanase et al. [46] reported that *K. marxianus* NBRC1777 efficiently produced ethanol corresponding to 92.9% of the theoretical yield. *K. marxianus* DBKKUY-103, that was recently isolated, achieved the maximum ethanol concentration of 83.5 g/L, corresponding to 96.6% of the theoretical yield [47]. Nitiyon et al. [37] reported that *K. marxianus* BUNL-21 is a highly competent yeast for high-temperature ethanol fermentation with lignocellulosic biomass. When compared with the strain DMKU 3-1042, the strain BUNL-21 had stronger ability for conversion of xylose to ethanol and tolerance to various stresses including high temperature and hydrogen peroxide.

Recently, there have been several reports on ethanol production at high temperatures using *P. kudriavzevii* (formerly known as *I. orientalis*). Several *P. kudriavzevii* strains were reported to grow and produce high levels of ethanol at high temperatures. The strain DMKU 3-ET15 was isolated from traditional fermented pork sausage in Thailand by an enrichment technique in a medium supplemented with 4% ethanol at 40°C. The strain produced 78.6 g/L ethanol from 180 g/L glucose at 40°C [20]. The strain KVMP10 that was isolated from soil located beneath apple trees for ethanol production from orange peel achieved 54 g/L ethanol at 42°C [48]. Strain RZ8-1 that was recently isolated from various samples collected from plant orchards in Thailand produced 33.8 g/L ethanol from 160 g/L glucose at 40°C [49].

Yeast strain	Temp. (°C)	P (g/L)	Q*p* (g/L/h)	T.Y (%)	Refs.
Kluyveromyces marxianus					
DMKU 3-1042	40	67.8	1.13	60.4	[31]
IIPE453[a]	50	82.0	nd	nd	[45]
NBRC1777	40	47.4	nd	92.9	[46]
DBKKUY-103	40	83.5	1.39	96.6	[47]
Pichia kudriavzevii					
DMKU 3-ET15	40	78.6	3.28	85.4	[20]
KVMP10	42	54.0	2.25	nd	[48]
RZ8-1	40	33.8	1.41	77.9	[49]
Saccharomyces cerevisiae					
VS3	40	60.0	nd	nd	[50]
C3867	41	38.8	nd	nd	[51]
DBKKUY-53	40	85.0	2.83	—	[52]
KKU-VN8	40	89.3	2.48	96.3	[53]

[a]*Kluyveromyces* sp.
P, ethanol concentration; Q*p*, volumetric ethanol productivity; T.Y, fraction of theoretical yield; nd, no data.

Table 1. Thermotolerant yeasts used in bioethanol production.

Several *S. cerevisiae* strains were also isolated for high-temperature ethanol fermentation. Sree et al. [50] reported a strain VS3 that could grow at 40°C and produced ethanol up to 60 g/L. Auesukaree et al. [51] reported a strain C3867 that produced 38.8 g/L of ethanol at 41°C. Recently, Nuanpeng et al. [52] and Techaparin et al. [53] isolated *S. cerevisiae* DBKKUY-53 and KKU-VN8, respectively, in Thailand. The former strain produced the maximum ethanol concentration and volumetric ethanol productivity of 85.0 g/L and 2.83 g/L h, respectively, at 40°C, and the latter strain produced the maximum ethanol concentration of 89.3 g/L with a productivity of 2.48 g/L h and a theoretical ethanol yield of 96.3% from sweet sorghum juice at 40°C.

Table 1 shows a number of ethanologenic thermotolerant yeasts. A temperature of 40°C was found to be the best condition for most strains to produce ethanol.

3. Utilization of various sugars in thermotolerant yeasts

Bioethanol significantly contributes to the reduction of crude oil consumption and environmental pollution. Thus, it has been identified as the mostly used biofuel worldwide [42]. Feedstocks for biofuel currently seem to be the option for sustainable development in the

context of economical and environmental considerations. There are various types of feedstocks for ethanol production [54], and accordingly, different processes including biomass pretreatment are required. Feedstock rich in sugar that mainly contains sucrose is readily fermented to ethanol. Feedstock rich in starch must first be hydrolyzed to glucose monomers by the action of enzymes [55]. Lignocellulosic and algal biomass needs further pretreatment and hydrolysis before liberating simple sugars, which can be readily converted to ethanol by microorganisms [56–58]. The resulting hydrolysates of these raw materials contain various sugars depending on the type of biomass [59]. In case of algal biomass, the sugar composition varies largely, based not only on algal species but also on their environmental and nutritional conditions [43, 56]. Lignocellulosic biomass is a complex mixture of carbohydrate polymers, and the biomass hydrolysate mainly contains hexoses (D-galactose, L-galactose, and D-mannose) and pentoses (D-xylose and L-arabinose) [60]. Glucose and xylose are the most abundant monosaccharides in this biomass taking up 60–70% and 30–40% of the total hydrolysate, respectively [61, 62]. Predominant pentose sugars derived from the hemicellulose of most feedstocks are xylose and arabinose. Like in higher plants, algae biomass is comprised of rigid cellulose-based cell walls and various complex polysaccharides, which can be hydrolyzed to sugars and subsequently fermented to ethanol [43, 63]. However, algae biomass contains a low percentage of lignin and hemicellulose compared to other lignocellulosic plants [64].

Microorganisms are the key factor in the conversion of sugars to ethanol. One of their several desired characteristics is thermotolerance. Ethanol production at high temperatures by thermotolerant yeasts has earned much interest due to several advantages as described above [38]. There are several ethanologenic yeasts that have been characterized and classified as thermotolerant yeasts such as *K. marxianus* [31, 37, 47], *P. kudriavzevii* (formally known as *I. orientalis*) [20, 48, 49, 65, 66], *Hansenula polymorpha* [67], and some strains of *S. cerevisiae* [21, 52, 68–70]. However, for cost-effective and efficient ethanol production, not only thermotolerance but also a broad spectrum in sugar assimilation and fermentation capability is beneficial for the conversion of a variety of raw materials containing various sugars to ethanol, especially xylose, which is the most common pentose sugar and the second most abundant after glucose in lignocellulosic biomass and algal biomass [71, 72].

S. cerevisiae is commonly employed in ethanol production due to its high ethanol productivity and high ethanol tolerance [73]. It is capable of converting different types of sugars, such as glucose, mannose, galactose, fructose, sucrose, and maltose to ethanol via the glycolysis pathway under anaerobic conditions [55]. Unfortunately, it is not able to ferment other carbon sources from plant or algal hydrolysates such as D-xylose, L-arabinose, and L-rhamnose [59]. A few types of yeasts can ferment both glucose and xylose but their performance regarding the rate of ethanol production from xylose, and the yield is lower than those from the main hexose sugars (for example, *S.* (*Pichia*) *stipitis* [74], *Scheffersomyces* (*Candida*) *shehatae* [75], *Pachysolen tannophilus* [76], *H. polymorpha* [67], and *K. marxianus* [32, 37]). Among these xylose-fermenting yeasts, it seems that *K. marxianus* has the potential for practical application in high-temperature ethanol fermentation because of its thermotolerance and ability to utilize a variety of sugars.

Feedstock	Substrate	Organism	Temp. (°C)	P (g/L)	T.Y (%)	Refs.
Sugar containing materials	Sugar cane juice	*K. marxianus* DMKU 3-1042	40	67.8	60.4	[31]
	Jerusalem artichoke	*K. marxianus* DBKKU-Y102	40	97.5	92	[77]
	Sweet sorghum juice	*K. marxianus* DBKKUY-103	40	83.5	100	[47]
	Palm sap	*K. marxianus* TISTR 5925	40	45.4	92.2	[39]
	Jerusalem artichoke	*K. marxianus* PT-1	40	73.6	90	[21]
Starchy materials	Taro waste	*K. marxianus* K21	40	43.8	94.2	[78]
Lignocellulosic biomass	Kanlow switchgrass	*K. marxianus* IMB3	45	22.5	86	[79]
	Switchgrass	*K. marxianus* IMB4	45	16.6	78	[80]
	Solka-floc	*K. marxianus* L. G.	42	37.6	98	[81]
	Rice straw	*K. marxianus* NRRLY-6860	45	21.5	86	[82]

P, ethanol concentration; T.Y, fraction of theoretical yield.

Table 2. Ethanol production of *K. marxianus* from various substrates at high temperatures.

K. marxianus's most important characteristics in this respect are thermotolerance to temperatures between 45 and 52°C, efficient ethanol production at temperatures between 38°C and 45°C, and a rapid growth rate that is twice as high as that of *S. cerevisiae* in rich media. Moreover, it has a broad spectrum of sugar assimilation, which includes glucose, mannose, galactose, fructose, arabinose, xylose, xylitol, sucrose, raffinose, cellobiose, lactose, and inulin [32, 36]. However, there has been little ethanol production from xylose and none from arabinose [32]. This strain can utilize a wide variety of industrially relevant substrates and efficiently converts substrates to ethanol. Especially, with lignocellulosic raw materials, it resulted in 78–98% of the theoretical ethanol yield (**Table 2**).

4. Complete genome sequence of thermotolerant yeast *K. marxianus* DMKU 3-1042 and transcriptomic analysis

High-temperature fermentation technology with thermotolerant microbes has been expected to reduce the cost of bioconversion of biomass to fuels or chemicals. *K. marxianus* was included in GRAS (FDA) and QPS (EU) lists of safe microorganisms for use in foods [83, 84]. The capacity of *K. marxianus* to utilize a wide variety of sugars reflects its potential for biotechnological applications [29, 84], which has been indicated by many studies with diverse substrates such as whey permeate, crop plants, and lignocellulosic biomass [32, 33, 78, 85, 86]. *K. marxianus* is also distinguished by its thermotolerance [36, 87] and the highest growth rate

in eukaryotes [88]. In recent years, interest also increased in several new applications such as production of biomolecules [89, 90], biocatalysts [91, 92], and heterologous protein expression [93, 94].

Genomic and transcriptomic studies have started to shed light on *K. marxianus*, and a growing number of genome sequences of *K. marxianus* strains are now available. Those include KCTC 17555 [34], DMB1 [95], CCT 7735 [96], NBRC1777 [97], DMKU 3-1042 [35], B0399 [98], UFS-Y2791 [99], and other nine strains: L01, L02, L03, L04, L05, CBS397, NBRC0272, NBRC0288, and NBRC0617 [100].

4.1. Genomic information and comparative genomics

The genome sequence of *K. marxianus* DMKU 3-1042 as one of the most efficient thermotolerant strains was determined, and the complete genome sequence of 11.0 Mb including all centromeric regions and boundary regions containing up to one to several sequence repeats (GGTGTACGGATTTGATTAGTTATGT) of telomeres was obtained [35]. The genome was composed of eight chromosomes in total, including mitochondrial DNA. Annotation of the genome of DMKU 3-1042 revealed a total of 4952 genes. UniProt and KAAS assignments led to the assignment of homologous genes of about 86.4% of predicted genes and KEGG Orthology numbers of 50.5% respectively.

A total of 202 tRNAs and 8 rDNAs were identified. According to the optical mapping experiment, 140 rDNA copies were observed on chromosome 5 instead of 6 rDNA copies found in the genome sequence in the database. The rDNA copy number and the thermotolerance were expected to positively relate. However, there was no such correlation among 10 *K. marxianus* strains, which exhibited different growth at different temperatures, and at least 31 copies of rDNA are sufficient to support its thermotolerance [35].

The yeast shares 1552 genes with other hemiascomycetous yeasts, including *K. lactis, Ashbya gossypii, Candida glabrata, S. cerevisiae, Ogataea parapolymorpha, Debaryomyces hansenii, S. stipitis, Clavispora lusitaniae, Yarrowia lipolytica,* and *Schizosaccharomyces pombe* [101–105]. *K. marxianus* was found to be phylogenetically closest to *K. lactis*. There are 193 genes specific to *K. marxianus*, which may be responsible for its species-specific characteristics [35]. The 422 genes shared between *K. marxianus* and *K. lactis* may be related to their genus-specific characteristics, such as production of β-galactosidase [106], assimilation of a wide variety of inexpensive substrates [84], efficient productivity of heterologous proteins [107–109], and synthesis of a killer toxin against certain ascomycetous yeasts [110, 111].

The two attractive traits of *K. marxianus* for fermentation applications were the thermotolerance and pentose assimilation capability. The thermotolerant ability was also found in *O. parapolymorpha*, and 30 genes were found to be shared between the two thermotolerant yeasts, including genes for three siderophore-iron transporters and three vacuolar proteins. For pentose assimilation capability, there are 27 putative genes for sugar transporters in the *K. marxianus* genome, and some of them (*KLMA_60073, KLMA_70145 and KLMA_80101*)

were induced by xylose. The initial xylose catabolism after its uptake in *K. marxianus* is accomplished by three reactions catalyzed by enzymes, xylose reductase (*XYL1*), xylitol dehydrogenase (*XYL2*), and xylulokinase (*XKS1*), which are involved in the conversion of xylose to xylulose-5-phosphate as an intermediate in the pentose phosphate pathway (PPP). Genes for utilization of various other sugars and alcohol dehydrogenases were also found [35, 112, 113].

4.2. Ploidy variation in *K. marxianus*

K. marxianus showed a high level of phenotypic variation. Recently, the single nucleotide polymorphisms (SNIPs) in 14 strains of *K. marxianus* were analyzed [100]. On the basis of SNIP analysis and flow cytometry, it was found that the isolates included haploid, diploid, and triploid strains. All isolates from dairy environments were diploid or triploid, whereas most isolates (6 out 7 isolates) from nondairy environments were haploid.

4.3. Transcriptomic analysis

A major potential future application of *K. marxianus* may be ethanol production from lignocellulosic biomass, which is an anaerobic or oxygen-limited process where both glucose and xylose may be present. Detailed transcription start site sequencing (TSS Seq) to explore the response of *K. marxianus* DMKU 3-1042 was reported for four different conditions: shaking condition in rich medium at 30°C (30D) or 45°C (45D), static condition in rich medium at 30°C (30DS), and shaking condition in xylose-containing rich medium at 30°C (30X) [35].

Under the 30DS condition, there were 159 and 154 significantly upregulated and downregulated genes, respectively. In brief, *K. marxianus* may increase the turnover of RNAs and proteins in addition to suppression of transporters that depend on mitochondrial respiratory activity. Most genes for several oxygen-dependent biosynthetic pathways (**Figure 1**), such as those for heme, sterols, unsaturated fatty acids, pyrimidine, and deoxyribonucleotides [114], are crucial for the cellular metabolism under the static condition.

Under the 45D condition, there were 199 and 508 significantly upregulated and downregulated genes, respectively. *K. marxianus* seems to drastically change metabolic pathways under the 45D condition, that is, the enhancement of PPP and the attenuation of TCA cycle after the fumarate-producing step (**Figure 2**). Several genes for homologous recombination and nonhomologous end joining, which function in the repair of DNA-double stranded breaks, were also upregulated. As expected, heat shock proteins and chaperones, such as Hsp26, Hsp60, Hsp78, Hsp82, Ssa3, and Cpr6, are crucial for survival at high temperatures. The thermotolerance of *K. marxianus* is likely achieved by systematic mechanisms consisting of various strategies. The yeast prevents reactive oxygen species (ROS) generation by minimizing mitochondrial activity and mainly acquires ATP from glycolysis rather than from TCA cycle at high temperatures.

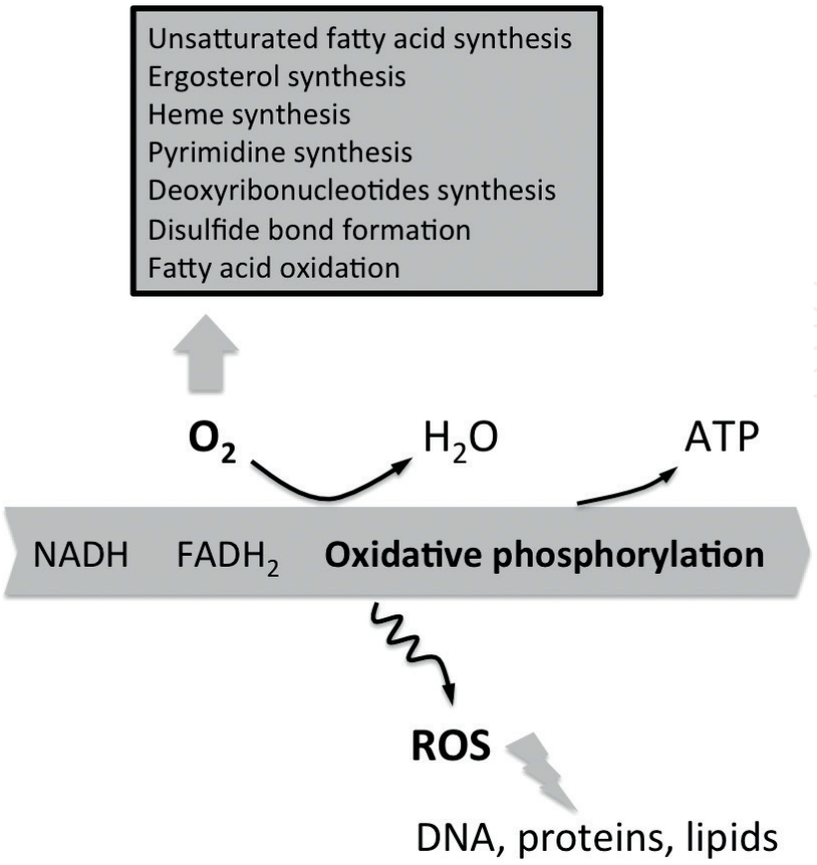

Figure 1. Oxygen-related metabolism in budding yeast. Oxygen is used for the biosynthesis of unsaturated fatty acids, ergosterol, heme, pyrimidine, and deoxyribonucleotides, as well as during disulfide bond formation and fatty acid oxidation. Oxygen is also the final electron acceptor for the electron transport chain, which oxidizes reduced equivalents of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) for the synthesis of ATP. However, ROS are produced as a by-product during some of these processes. The ROS can cause damage to DNA, proteins, and lipids.

Under the 30X condition, there were 89 and 79 significantly upregulated and downregulated genes, respectively. This condition may stimulate the degradation of lipids in the peroxisome and keep a low level of amino acid synthesis, indicating the possibility that fatty acids could be a subsidiary intracellular carbon source in xylose medium (**Figure 3**). Similarly, Schabort et al. [99] also reported that peroxisomal fatty acid catabolism was dramatically upregulated in a defined xylose mineral medium without fatty acids, along with mechanisms to activate fatty acids and transfer products of β-oxidation to the mitochondria. It is known that *K. marxianus* tends to suffer from cofactor imbalance in xylose medium [115, 116]. Redox balancing mechanisms between the cytoplasm and mitochondria are probably used to resolve the NADH/NADPH imbalance owing to lack of transhydrogenases [117]. In *S. cerevisiae*, five cytosolic-mitochondrial redox shuttles have been proposed [118]. Of these, genes for enzymes related to ethanol-acetaldehyde, citrate-oxoglutarate, and oxaloacetate-malate shuttles were relatively upregulated under the 30X condition, which were different from those found in *S. cerevisiae* and *S. stipitis* [103, 119].

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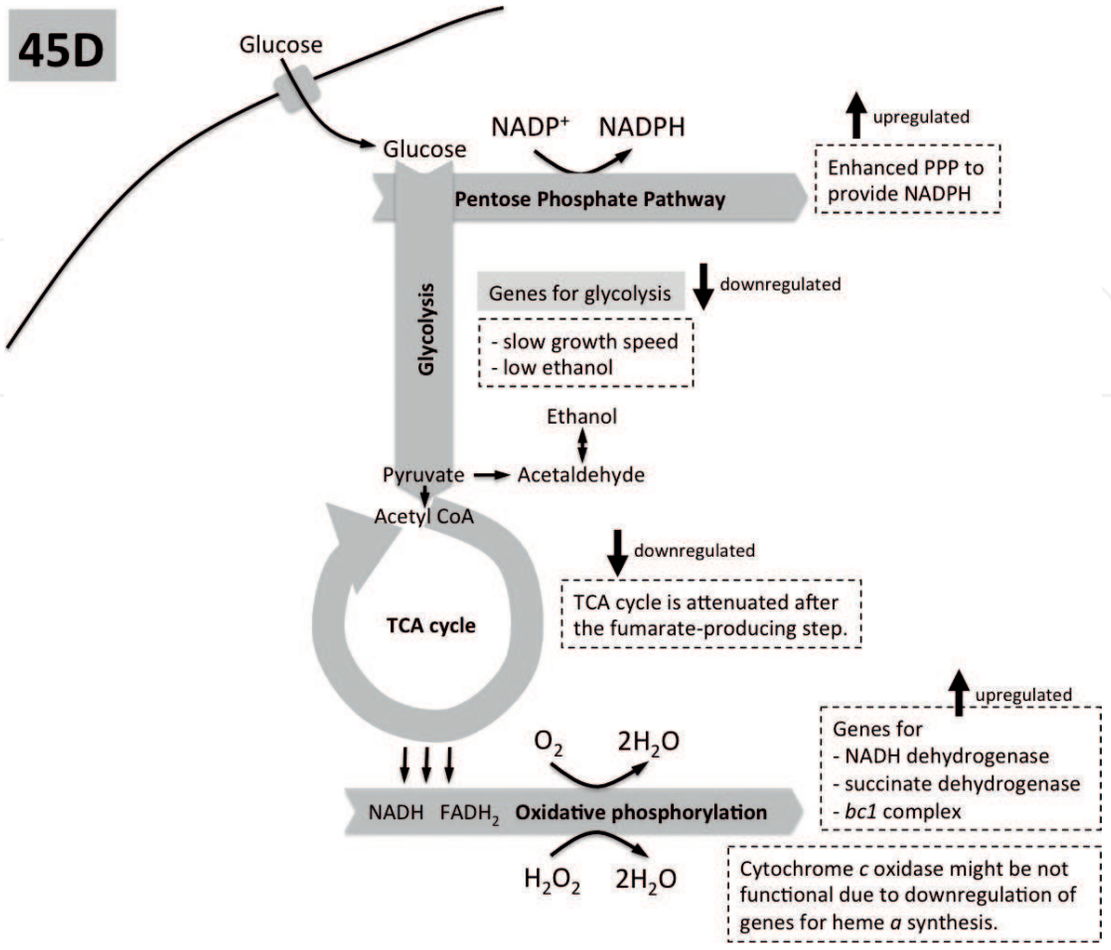

Figure 2. Difference of metabolism under the 45D condition from that under the 30D condition in *K. marxianus* DMKU 3-1042 (see more detail in Ref. [35]).

TSS seq analysis revealed that the oxidative stress-response genes were highly induced under the three conditions tested, indicating that ROS is accumulated in the cytoplasm, mitochondria, and peroxisome under the 30DS and 30X conditions and in the cytoplasm and mitochondria under the 45D condition.

Moreover, *K. marxianus* has been exploited as a cell factory to produce valuable enzymes, showing retention of the activity in a broad temperature range [120]. The 30X condition showed high expression of *INU1* for inulinase, which is useful for the production of recombinant proteins [108, 109, 121]. These useful characteristics may allow simultaneous production of ethanol and valuable proteins, thus generating additional revenue from ethanol production.

In conclusion, the transcriptome analyses clarified distinctive metabolic pathways under three different growth conditions, static culture, high temperature, and xylose medium, in comparison to the control condition of a glucose medium under a shaking condition at 30°C. Interestingly, the yeast appears to overcome the issue of ROS, which tend to accumulate under all three conditions. Nicotinamide adenine dinucleotide phosphate (NADPH) synthesis from several reactions is the key for cells to cope with ROS (**Figure 4**).

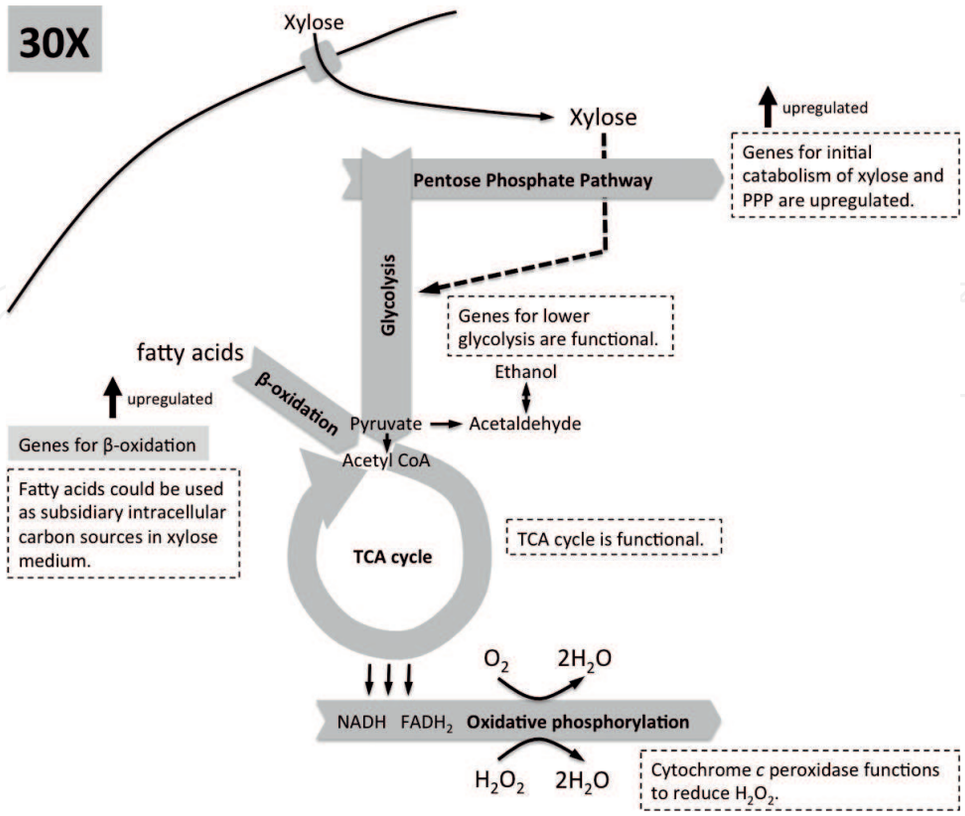

Figure 3. Difference of metabolism under the 30X condition from that under the 30D condition in *K. marxianus* DMKU 3-1042 (see more detail in Ref. [35]).

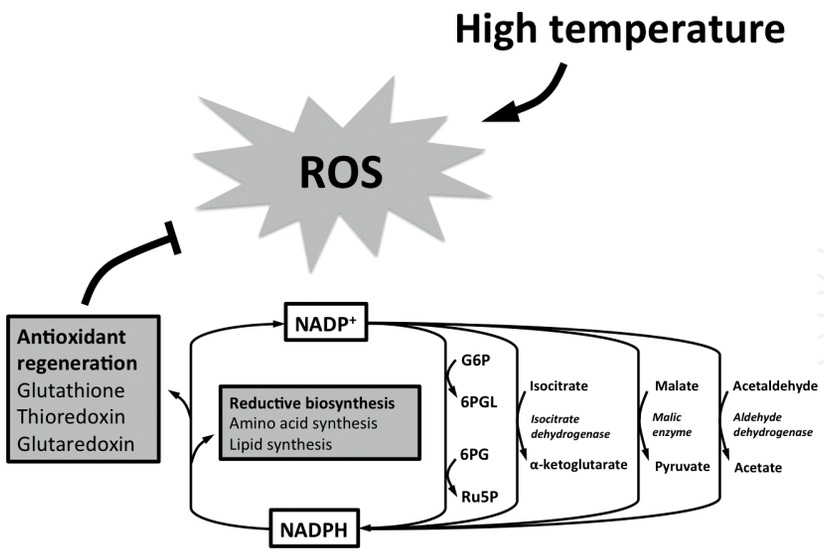

Figure 4. Generation and utilization of NADPH in budding yeast. A major source of cellular-reduced NADPH is thought to be produced via the oxidative branch of the pentose phosphate pathway. Oxidation of isocitrate, malate, and acetaldehyde generates NADPH. NADPH is consumed during the synthesis of amino acids and lipids. The reducing power of NADPH is also used to regenerate a variety of antioxidants and antioxidant enzymes, which protect the cell from ROS and engage in deoxyribonucleotide triphosphate (dNTP) synthesis. Abbreviations: G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate.

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5. Glucose repression in thermotolerant yeast *K. marxianus*

Glucose repression is a general phenomenon in organisms including yeasts, by which glucose prevents the assimilation of other sugars [122, 123]. This process will disturb the fermentation of mixed sugars like hydrolysate of cellulosic biomass. As mentioned in the previous sections, *K. marxianus* is a well-known budding yeast, which has potential for production of bioethanol, hydrolytic enzymes, food biomass, and food additives [29, 31, 124]. *K. marxianus* DMKU 3-1042 is a thermotolerant yeast from Thailand and efficiently produces ethanol at high temperatures [31]. Although the strain can utilize various sugars including xylose [32, 35, 125], it has an intrinsic system of glucose repression like other microbes. In this section, we describe glucose repression in thermotolerant yeast, *K. marxianus*, and in conventional yeast, *S. cerevisiae*.

5.1. Mechanism of glucose repression in *S. cerevisiae*

Glucose repression in *S. cerevisiae* has been well studied. Mig1 and Hxk2 play as the main regulator of glucose repression in this species [126]. The former is a C_2H_2 zinc finger protein [127], and the latter is a bi-functional protein acting as a hexokinase and transcriptional regulator, which is localized in both the cytoplasm and the nucleus [128, 129]. Hxk2 activity in glucose repression mechanism is influenced by the concentration of glucose. Under high concentrations of glucose, Hxk2 in the cytoplasm moves to the nucleus and, as a complex with dephosphorylated Mig1, Cyc8, and Tup1 [126], represses the transcription of several genes including respiratory and gluconeogenic genes. As a result of Hxk2 binding to Mig1, serine 311 in Mig1 is dephosphorylated, resulting in maintenance of repressive conditions [130]. On the other hand, in the presence of a low concentration or absence of glucose, Hxk2 and Mig1 remain in the cytoplasm, where neither Mig1 nor Hxk2 can repress Mig1-regulated genes [126]. In this situation, Hxk2 does not interact with Mig1 but still interacts with Snf1. No interaction between Hxk2 and Mig1 facilitates phosphorylation of serine 311 in Mig1 by the Snf1 kinase. Snf1 is phosphorylated by Sak1 and forms a complex with Snf4 and Gal8 to become activated. The Snf1 complex inhibits formation of a complex of Mig1-Hxk2-Cyc8-Tup1. In this situation, since Mig1 is also phosphorylated or inactive and absent in the nucleus, Mig1-regulated genes are de-repressed [130].

5.2. Mechanism of glucose repression in *K. marxianus*

K. marxianus DMKU 3-1042 exhibits almost no glucose repression on sucrose assimilation unlike *S. cerevisiae* [33]. To acquire glucose repression-defective strains in *K. marxianus*, some researchers performed spontaneous isolation on 2-deoxyglucose (2-DOG) plates or random insertion of *kan*MX4 [131, 132]. According to the characteristics of sugar consumption abilities, cell growth and ethanol accumulation along with cultivation time, only one of 33 isolates of 2-DOG-resistant mutants showed enhanced utilization of xylose in the presence of glucose. Further analysis revealed that this isolate had a single nucleotide mutation to cause amino acid substitution (G270S) in *RAG5* encoding hexokinase and exhibited very low activity of the enzyme [132]. Another technique for obtaining glucose repression-defective strains showed

one group of 2-DOG-resitant mutants with intragenical insertion of *Kan*MX4. This group also exhibits enhanced utilization of xylose in the presence of glucose, presumably due to a defect in the glucose-repression mechanism [131].

On the other hand, Zhou et al. focused on the function of Mig1 in *K. marxianus* and showed that the *MIG1* mutation increased hydrolysis of lactose [133] and production of inulinase [134]. Nevertheless, information on the function of Rag5 as a transcriptional regulator is hardly available, and thus construction of the complete disrupted mutation of *RAG5* and its analysis become a challenge. Thus, disrupted mutants of genes for Mig1 and Rag5 were constructed, and their characteristics were compared with those of the corresponding mutants of *S. cerevisiae*. *MIG1* and *RAG5* mutants exhibited more resistance to 2-DOG in YP plates containing sucrose. *RAG5* and *HXK2* mutants showed more resistant to 2-DOG than the corresponding *MIG1* mutants [135].

Several attractive characteristics of *MIG1* and *RAG5* mutants of *K. marxianus* DMKU 3-1042 were uncovered. *MIG1* mutants consumed almost two times faster xylose and accumulated glycerol and xylitol much more than those of the parental strain and the *RAG5* mutant in the liquid media YPX (containing 20 g/L of xylose) and YPDX (containing 20 g/L of glucose and 20 g/L of xylose) at 30°C. The accumulation of glycerol and xylitol may be due to accumulation of NADH. *RAG5* mutants exhibited very slow utilization of glucose in the liquid media of both YPD (containing 20 g/L of glucose) and YPDS (containing 20 g/L of glucose and 20 g/L of sucrose). However, with this mutant, high amounts of fructose (about 11.9 g/L in YPDS at 30°C for 96 h) were accumulated. *MIG1* and *HXK2* mutants of *S. cerevisiae* also accumulated high amounts of fructose in the same medium, but after 12 h, fructose was consumed.

The fructose accumulation in *RAG5* mutants is probably due to the inability of this mutant to uptake fructose or the lack of kinase activity. To further analyze this phenomenon, Enzyme activities[a] and gene expression levels of inulinase and kinase in *MIG1*- and *RAG5*-disrupted mutants and the parental strain were measured (**Table 3**) [135]. *RAG5* mutants showed very high activities of inulinase, about 77 times higher than those of the parental strain, but almost no activities of hexokinase and glucokinase that are encoded by *RAG5* and *GLK1*, respectively. The inulinase activity in *RAG5* mutant was consistent with the gene expression level of *INU1*, being about 22 times higher than that of the parental strain. However, the expression level of *GLK1* in this mutant was higher, which was inconsistent with glucokinase activity. It is thus likely that there is a post-transcriptional regulation for glucokinase. *MIG1* mutants showed no significant increase in inulinase activity, but *INU1* transcriptional expression was eight times higher than that of the parental strain. This inconsistence may also be due to post-transcriptional regulation for inulinase. These results suggest that Mig1 and Rag5 are related to the glucose repression mechanism in *K. marxianus* and share some functions with Mig1 and Hxk2, respectively, in *S. cerevisiae*.

In conclusion, Mig1 and Rag5 in *K. marxianus* share some functions with Mig1 and Hxk2, respectively, in *S. cerevisiae*. Mig1 and Rag5 in *K. marxianus* may form a complex similar to that consisting of Mig1 and Hxk2 in *S. cerevisiae*.

Strains	Enzyme activities[a]			Gene expression levels		
	Inulinase (U/mg DCW)	Gluco-hexokinase (U/mg)	Hexokinase (U/mg)	*INU1/ACT1*	*GLK1/ACT1*	*RAG1/ACT1*
DMKU 3-1042	127.38	1.107	0.662	0.087	0.136	0.916
MIG1 mutant	160.1	1.466	0.774	0.696	0.141	0.266
RAG5 mutant	9838.16	0.007	0.005	1.927	1.495	0.051
RAG1 mutant	4229.23	0.203	0.027	1.234	0.606	0.091

[a]The data are from Ref. [135].

Table 3. Comparison of enzyme activities and gene expression levels in *MIG1*- and *RAG5*-disrupted mutants of *K. marxianus* in YPD liquid medium.

6. Thermotolerant and ethanologenic yeasts in Vietnam

In Vietnam, ethanol is a compound in many different products from fermentation technology including alcoholic drinks and biofuel. In the national strategy with a vision to 2025 designed by the government, the technology of biofuel production in Vietnam using the various raw material resources that are abundantly available, e.g., pineapple, cassava, sugarcane, etc., will reach the advanced worldwide level. For the scheme on the development of Vietnam's alcoholic beverages with a vision to 2025, the Mekong Delta is one of the top national areas for the improvement of such products. In addition, nowadays due to global warming, the exploration of thermotolerant yeasts for ethanol fermentation at high temperature also falls in the potential priorities in Vietnam.

6.1. Characteristics of thermotolerant and ethanologenic yeasts

Recent research studies under international programs, such as the Asian Core Program (2008–2012) and the Core-to-Core Program (2014–2018), have addressed the exploration of useful thermotolerant ethanologenic yeasts isolated from Vietnam and their applications for fermentation technology at high temperature. The diversity of yeast isolates with high capacities and stability for the controlled processing of alcoholic winemaking and ethanol production from cheap and available raw materials in the region has been studied.

A total of 712 yeast isolates were purified from many different kinds of raw material sources in the Mekong Delta, Vietnam, such as ripe fruits, flowers of fruit-tree, cocoa, fermented products, alcoholic fermentation starters, sugarcane, molasses, sawdust, agricultural by-products, and soil samples. All of these yeast isolates could grow well at 37°C and about 80, 45 and 10% of these yeasts could grow at 40, 43 and 45°C, respectively. More than 80% of yeasts were able to grow in a medium containing 9% (v/v) of ethanol, this number decreased to about 40% of yeasts growing in a medium supplemented with 12% (v/v) of ethanol. For conservation, all pure yeast isolates have been stored at −20 and −80°C in stock culture of glycerol freezing broth.

A bank collection of genetically diverse yeasts with thermotolerant ethanologenic capacity at high temperatures was developed and systemized. The full data of morphological,

physiological, and biochemical characteristics, as well as the nucleotide sequencing analyses of the 88 selected yeasts, have been established. Some predominantly abundant identified species include *Candida tropicalis*, *S. cerevisiae*, *P. kudriavzevii*, and *C. glabrata* (**Table 4**). Besides, a number of other species was also characterized, such as *Torulaspora globosa*, *Candida nivariensis*, *Pichia manshurica*, *C. lusitaniae*, *Hanseniaspora opuntiae*, and *Meyerozyma caribbica*.

With the aim to pave the way for the application of useful thermotolerant ethanologenic yeasts toward industrial fermentation technology, ethanol production, and winemaking by using the selected thermotolerant yeasts, investigations at laboratory-scale and pilot-scale were performed. The optimum fermentation conditions at different temperatures (37, 40, and 43°C) were also tested in a factorial design with three factors including yeast inoculum, initial sugar concentration, and fermentation time. For wine manufacture, different kinds of fruits were employed as raw materials such as: pineapple, watermelon, dragon fruit, guava, jackfruit, rambutan, tangerine, and three-leaved wild vine. The highest ethanol concentration of the final wine product reached about 12% (v/v) and up to 7% (v/v) during the fermentation at 37 and 40°C, respectively. For ethanol production, a number of raw materials were tested including molasses, sugarcane juice, sugarcane waste, and pineapple waste hydrolysate. The highest ethanol concentration could be found at about 7% (v/v) and up to 4% (v/v) during the fermentation at 37 and 40°C, respectively.

No	Isolated yeast species	Vietnam	Laos	Indonesia
1	*Blastobotrys adeninivorans*		2	
2	*Candida glabrata*	7	2	
3	*Candida manshurica*		2	
4	*Candida nivariensis*	4		
5	*Candida stellimalicola*		1	
6	*Candida tropicalis*	16	26	16
7	*Clavispora lusitaniae*	1		
8	*Cyberlindnera rhodanensis*		2	
9	*Hanseniaspora opuntiae*	1		
10	*Issatchenkia orientalis*			1
11	*Kluyveromyces marxianus*		6	3
12	*Meyerozyma caribbica*	1		
13	*Meyerozyma guilliermondii*			2
14	*Pichia kudriavzevii*	35	47	1
15	*Pichia manshurica*	2		
16	*Saccharomyces cerevisiae*	19	1	
17	*Torulaspora globosa*	2		
	Not identified	624	70	56
	Total	712	159	79

Table 4. Isolated yeast strains from Vietnam, Laos, and Indonesia.

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The research findings on the diversified collection of thermotolerant ethanologenic yeasts isolated from Vietnam and the high ethanol yields as well as and fermentation efficiencies by using the selected yeast isolates indicate the promising application of such newly isolated functional thermotolerant yeasts for the controlled ethanol production at high temperatures from agricultural by-products and the winemaking manufacture from different available fruit resources in the region. Further advanced research on the expression levels of the selected genes and the metabolic pathways will be performed to explore the regulation of these genes to get maximum benefits of the superior thermotolerant yeasts for high-temperature ethanol production.

7. Thermotolerant and ethanologenic yeasts in Laos

Ethanol production in Lao PDR is generally used for human consumption and household use, rather than for small or large-scale industries. Until now, no ethanol as a substitute of energy in Lao PDR is produced in the industry. The raw material used to make ethanol for drinking is mostly sticky rice and the starter culture used for fermentation contains sticky rice and many other herbs. Drinking alcohol in Lao PDR is available in all provinces, mainly for consumers in their own province. Currently, alcoholic beverages are still very productive and the most popular products to customers are produced in the Saravan province in Meuangkhong district. High quality ethanol used for medicine, hospitals or laboratories are imported from neighboring countries.

The National Economic Research Institute under the Ministry of Planning and Investment reported that production of ethanol in 2010–2011 was increased 3.2 times compared to 2001. Lao government plans to develop other sources of renewable energy, which have been investigated by the private sector. Demonstration projects including a bio-diesel oil from Jatropha plant and biofuel (bio-gasoline and bioethanol) from Palm and Carmelina plants have been developed. In 2011, the Savannakhet sugar factory has been established by a Thai company to produce biogas and biomass energy. In 2013, a Vietnam company started a biomass power and ethanol production plant in Phouwong District, Attapeu Province.

7.1. Characteristics of thermotolerant and ethanologenic yeasts

Isolation of yeasts was first attempted from fruits, vegetables, leaves and soils in four provinces, Louang Phrabang, Xayaburi, Xiengkhouang, and Vientiane of Lao PDR. The attempt was carried out at 37°C by an enrichment culture. Samples (5–10 g) of fruits pressed in small pieces, leaves cut in small portions, and mashed soil were transferred into 100-mL Erlenmeyer flasks containing 10 mL of YPD (1% yeast extract, 2% peptone and 2% glucose) medium and incubated at 37°C for 3 days with occasional shaking. The cultures were then streaked on YPD agar plates and incubated at 37°C for 24–48 h. As a result, 43 strains were isolated, and their ethanol fermentation ability was characterized under various conditions including different sugars and different temperatures. A second isolation was attempted from similar kinds of samples described above in four provinces, Bolikhamxay, Champasak, Louang Phrabang, and Oudomxay, and 116 strains were obtained after enrichment culture as described above except that 4% ethanol was added in YPD medium. Of a total of 159 strains, 89 were identified by nucleotide sequencing of D1/D2 domains and analysis on MALDI-TOF/MS [28]. Fermentation experiments allowed to classify them into two groups: the first bears

an ethanol-fermenting ability at high temperature (116 strains) and the second the converting ability of xylose to ethanol at 37°C or more (43 strains). In fermentation of ethanol, the first group can use glucose, sucrose, sugar cane juice, and molasses as carbon sources, producing a maximum of ethanol concentrations of 7.9% (w/v), 6.7% (w/v), 7.3% (w/v), and 4.0% (w/v) from 16% sugar concentration, respectively. The second group produced 1.2–1.7% (w/v) ethanol from 4% xylose at 37°C. Species identification revealed that isolates include nine species including *C. tropicalis*, *P. kudriavzevii*, and *K. marxianus* (**Table 4**).

7.2. Characteristics of newly isolated *K. marxianus* strains

Out of six isolated *K. marxianus* strains, BUNL-17 was found to be the most efficient ethanol producer at high temperature [28]. Comparison with DMKU 3-1042, which is one of most thermotolerant *K. marxianus* strain isolates from Thailand, revealed that BUNL-17 possesses an efficient conversion activity of xylose to ethanol, resistance to 2-deoxyglucose and tolerance to various stresses including temperature, high sugar concentration, and hydrogen peroxide [37]. Compared to *S. stipitis* the fermentation activity toward xylose of BUNL-21 is slightly lower at around 30°C and much higher at higher temperatures. BUNL-21 is thus a highly competent yeast for high-temperature ethanol fermentation with lignocellulosic biomass. Interestingly, the fermentation activity was shown to be significantly enhanced by over-expression of *KmADH2* for alcohol dehydrogenase 2 [37].

8. Thermotolerant and ethanologenic yeasts in Indonesia

Ethanol production in Indonesia is generally performed for medical, industrial processes, and beverages. Several potential biomass resources for bioethanol production in Indonesia are (1) sugar-based materials including sugar cane (molasses), (2) starch-based including root (cassava and sweet potato) and grain (corn and sorghum), and (3) lignocellulosic-based including bagasse, straw, stalk, wood waste, corn cob, and sap of several plants or trees. The main biomass used for bioethanol production in Indonesia is molasses [136] probably because Indonesia is one of the largest sugarcane producers in the world. Annual cane production in Indonesia is about 32–35 million tons with an average cane productivity of 70–85 ton/ha. Sugar production is about 2.2–2.7 million tons, including molasses with about 1.3–1.5 million tons. Molasses are mainly used for monosodium glutamate production in the ethanol industry and for export to other countries [137].

Bioethanol development for fuel in Indonesia was started from 2006. Its road map until 2010 showed production of 99.5% ethanol as a fuel grade ethanol (FGE), which can be mixed with petroleum for gasohol E10 (10% ethanol and 90% petroleum). For the first period, biomass used for bioethanol production was molasses and cassava and bioethanol supply was about 1.48 mil kL (million kiloliters) or equal to 10% of total gasoline consumption. In the period 2011–2015, bioethanol supply was estimated to increase to 2.78 mil kL or equal to 15% of total gasoline consumption. Until 2025, bioethanol supply is predicted to be 6.28 mil kL or 20% of total gasoline consumption [138]. The application of bioethanol for fuel in Indonesia is E5, and only two bioethanol filling stations are operating in two cities, Malang and Semarang [139]. However, because of

some obstacles such as limitation of fuel grade ethanol market, inconsistency supply, insufficient demand, and price volatility, there is almost no fuel ethanol production since 2010 [136].

8.1. Characteristics of thermotolerant and ethanologenic yeasts

In international programs including the e-ASIA Joint Research Program, yeast strains were isolated from various samples such as soils, waters, flowers, fruits, vegetables, and fermented foods. The isolation method for thermotolerant and ethanol-producing yeast was similar to that applied in Lao PDR. The enrichment culture was carried out in YPD medium without the addition of ethanol. Most of the isolates can grow at relatively high temperatures ranging from 37 to 48°C. Of those, 52 yeast isolates grow well at 37°C on agar plates containing different types of sugar, such as glucose, xylose, and sucrose. Some can produce around 6% ethanol in a rich medium containing 16% (w/v) glucose at 40°C. These prominent characteristics are important for the development of bioethanol production in Indonesia.

Most yeast strains isolated from Indonesia are able to grow at relatively high temperatures not only in glucose medium but also in xylose and sucrose. However, their growth gradually decreases as temperature increases and is very weak at more than 45°C. Indonesian yeast isolates from fruits and fermented foods seem to be more thermotolerant than those from soils and waters. Most of the isolates grow very well at 40°C. These isolates include *C. tropicalis, K. marxianus* and *P. kudriavzevii* (**Table 4**).

9. High-temperature fermentation technologies with thermotolerant yeast

Currently, biofuel-aimed ethanol fermentation in industry is performed at around 30°C because the most frequently applied yeast is nonthermotolerant *S. cerevisiae*. In the fermentation process, the temperature in the fermenter increases close to a nonpermissible level for the yeast by metabolic and mechanical heat sources. A cooling system with a large amount of water and/or by a cooling unit is equipped for effective fermentation. The cooling cost tends to be higher in tropical countries or increases in summer time in other many countries, and the electricity problem largely affects productivity of ethanol. The HTF using a thermotolerant microbe is expected to provide several advantages. First, it can reduce the cooling cost. Second, the amount of enzyme used for saccharification can be reduced in the simultaneous saccharification and fermentation at higher temperature. Third, higher temperature causes lower contamination by various germs. Fourth, when the distillation under reduced pressure is applied at around 40°C, fermentation and distillation can be performed by one tank, which reduces the manufacturing time and the cost of equipment. Here, we introduce a fundamental research for an energy-saving fermentation technology using thermotolerant yeast.

9.1. Temperature-noncontrolled fermentation with thermotolerant yeast

For development of the fermentation technology, *K. marxianus* DMKU 3-1042 was used, which efficiently produces ethanol at high temperatures as mentioned above [32, 33]. The utilization

of the thermotolerant yeast is favorable to fermentation in a tropical country because it can be performed under temperature-noncontrolled conditions. When a bench-scale fermentation, 2 L of 9% glucose medium, was tested, DMKU 3-1042 produced ethanol equivalent to that under the temperature-controlled condition at 30°C [39]. In a fermenter-scale fermentation with 4000 L of 18% sugarcane, 7% ethanol production was achieved [39].

9.2. Distillation-connected fermentation with thermotolerant yeast

As an additional challenge, distillation-connected fermentation was attempted. Because the saturated vapor pressure of ethanol is 177.8 mbar at 41°C, where a thermotolerant microbe can grow well, ethanol can be collected from the fermenting culture when pressure is reduced to less than the saturated vapor pressure. The system shown in **Figure 5** was constructed and tested, which consists of a fermentation and a distillation tank, the primary and secondary ethanol recovery units, a vacuum pump, and a drain unit. In this system, ethanol is concentrated as the process proceeds from the primary to secondary ethanol recovery units. Due to the set-up of this system, the air in the tank was discharged outside during the vacuum distillation, and some ethanol was trapped in the drain unit. When fermentation with *K. marxianus* DMKU 3-1042 and distillation at 70 mbar and 41°C was applied, about 35 and 60% were recovered in the primary and secondary bottles [39]. The process of the simultaneous fermentation and distillation under a low pressure was continuously repeated three times, with 12% rice-hydrolysate [39]. Similar performance was achieved with a thermo-adopted strain of *Zymomonas mobilis* TISTR548, an ethanologenic bacterium [39].

That system provides some benefits: (i) microbes avoid exposure to high concentrations of ethanol or acetic acid or strong oxidative stress and (ii) fermentation can be continued during distillation increasing ethanol yields. Although further experiments for its evaluation are required, the system including HTF is expected to be one of next-generation fermentation technologies.

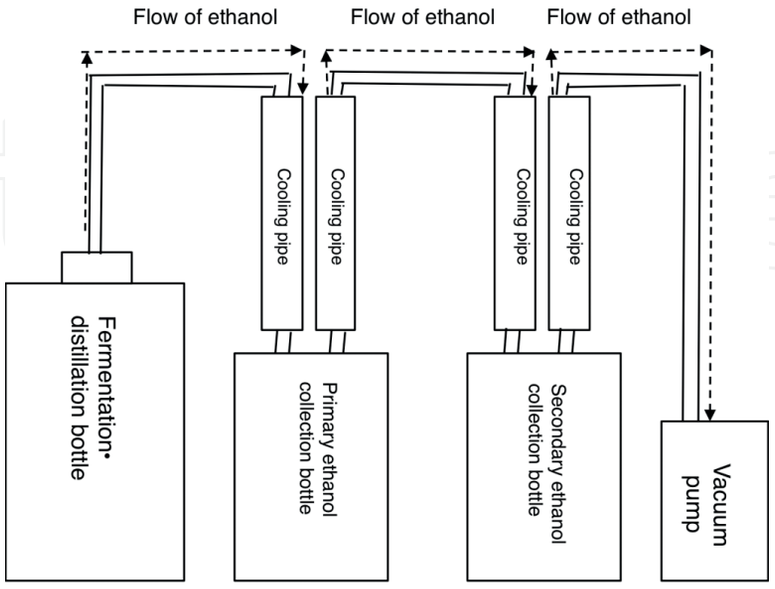

Figure 5. Apparatus for fermentation and distillation under a low pressure. This apparatus consists of a fermentation and distillation tank, primary and secondary recovery bottles, a drain unit, and a vacuum pump.

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References

[1] Wright MM, Brown RC. Comparative economics of biorefineries based on the biochemical and thermochemical platforms. Biofuels, Bioproducts and Biorefining. 2007;**1**:49-56. DOI: 10.1002/bbb.8

[2] Rees J. The Renewable Fuel Standard: Issues for 2014 and Beyond. USA: Report of Congressional Budget Office; 2014

[3] Rozakis S, Haque MI, Natsis A, Borzecka-Walker M, Mizak K. Cost-effectiveness of bioethanol policies to reduce carbon dioxide emissions in Greece. The International Journal of Life Cycle Assessment. 2013;**18**:306-318. DOI: 10.1007/s11367-012-0471-2

[4] Tesfaw A, Assefa F. Current trends in bioethanol production by *Saccharomyces cerevisiae*: Substrate, inhibitor reduction, growth variables, coculture, and immobilization. International Scholarly Research Notices. 2014;**2014**:532852. DOI: 10.1155/2014/532852

[5] Reis VR, Antonangelo ATBF, Bassi APG, Colombi D, Ceccato-Antonini SR. Bioethanol strains of *Saccharomyces cerevisiae* characterised by microsatellite and stress resistance. Brazilian Journal of Microbiology. 2017;**48**:268-274. DOI: 10.1016/j.bjm.2016.09.017

[6] Banat IM, Nigam P, Singh D, Marchant R, McHale AP. Ethanol production at elevated temperatures and alcohol concentrations: Part I–yeasts in general. World Journal of Microbiology and Biotechnology. 1998;**14**:809-821

[7] Naik SN, Goud VV, Rout PK, Dalai AK. Production of first and second generation biofuels: A comprehensive review. Renewable and Sustainable Energy Reviews. 2010;**14**: 578-597. DOI: 10.1016/j.rser.2009.10.003

[8] Lee J. Biological conversion of lignocellulosic biomass to ethanol. Journal of Biotechnology. 1997;**56**:1-24

[9] Young E, Lee SM, Alper H. Optimizing pentose utilization in yeast: The need for novel tools and approaches. Biotechnology for Biofuels. 2010;**3**:24. DOI: 10.1186/1754-6834-3-24

[10] Trumbly RJ. Glucose repression in the yeast *Saccharomyces cerevisiae*. Molecular Microbiology. 1992;**6**:15-21

[11] Nevoigt E. Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Reviews. 2008;**72**:379-412. DOI: 10.1128/mmbr.00025-07

[12] Liu Y, Zhang G, Sun H, Sun X, Jiang N, Rasool A, Lin Z, Li C. Enhanced pathway efficiency of *Saccharomyces cerevisiae* by introducing thermo-tolerant devices. Bioresource Technology. 2014;**170**:38-44. DOI: 10.1016/j.biortech.2014.07.063

[13] Sato TK, Tremaine M, Parreiras LS, Hebert AS, Myers KS, Higbee AJ, Sardi M, McIlwain SJ, Ong IM, Breuer RJ, Avanasi Narasimhan R, McGee MA, Dickinson Q, La Reau A, Xie D, Tian M, Reed JL, Zhang Y, Coon JJ, Hittinger CT, Gasch AP, Landick R. Directed evolution reveals unexpected epistatic interactions that alter metabolic regulation and enable anaerobic xylose use by *Saccharomyces cerevisiae*. PLoS Genetics. 2016;**12**:e1006372. DOI: 10.1371/journal.pgen.1006372

[14] Matsushita K, Azuma Y, Kosaka T, Yakushi T, Hoshida H, Akada R, Yamada M. Genomic analyses of thermotolerant microorganisms used for high-temperature fermentations. Bioscience, Biotechnology, and Biochemistry. 2016;**80**:655-668. DOI: 10.1080/09168451.2015.1104235

[15] Adachi O, Moonmangmee D, Toyama H, Yamada M, Shinagawa E, Matsushita K. New developments in oxidative fermentation. Applied Microbiology and Biotechnology. 2003;**60**:643-653. DOI: 10.1007/s00253-002-1155-9

[16] Dantán-González E, Vite-Vallejo O, Martínez-Anaya C, Méndez-Sánchez M, González MC, Palomares LA, Folch-Mallol J. Production of two novel laccase isoforms by a thermotolerant strain of *Pycnoporus sanguineus* isolated from an oil-polluted tropical habitat. International Microbiology. 2008;**11**:163-169

[17] Arora R, Behera S, Sharma NK, Kumar S. Bioprospecting thermostable cellulosomes for efficient biofuel production from lignocellulosic biomass. Bioresources and Bioprocessing. 2015;**2**:38

[18] Yamamoto H, Shima T, Yamaguchi M, Mochizuki Y, Hoshida H, Kakuta S, Kondo-Kakuta C, Noda NN, Inagaki F, Itoh T, Akada R, Ohsumi Y. The Thermotolerant yeast *Kluyveromyces marxianus* is a useful organism for structural and biochemical studies of autophagy. The Journal of Biological Chemistry. 2015;**290**:29506-29518. DOI: 10.1074/jbc.M115.684233

[19] Saini JK, Agrawal R, Satlewal A, Saini R, Gupta R, Mathur A, Tuli D. Second generation bioethanol production at high gravity of pilot-scale pretreated wheat straw employing newly isolated thermotolerant yeast *Kluyveromyces marxianus* DBTIOC-35. RSC Advances. 2015;**5**:37485-37494

[20] Yuangsaard N, Yongmanitchai W, Yamada M, Limtong S. Selection and characterization of a newly isolated thermotolerant *Pichia kudriavzevii* strain for ethanol production at high temperature from cassava starch hydrolysate. Antonie Van Leeuwenhoek. 2013;**103**:577-588. DOI: 10.1007/s10482-012-9842-8

[21] Hu N, Yuan B, Sun J, Wang SA, Li FL. Thermotolerant *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* strains representing potentials for bioethanol production from Jerusalem artichoke by consolidated bioprocessing. Applied Microbiology and Biotechnology. 2012;**95**:1359-1368. DOI: 10.1007/s00253-012-4240-8

[22] Limtong S, Srisuk N, Yongmanitchai W, Yurimoto H, Nakase T, Kato N. *Pichia thermomethanolica* sp. nov., a novel thermotolerant, methylotrophic yeast isolated in Thailand. International Journal of Systematic and Evolutionary Microbiology. 2005;**55**:2225-2229. DOI: 10.1099/ijs.0.63712-0

[23] Limtong S, Srisuk N, Yongmanitchai W, Kawasaki H, Yurimoto H, Nakase T, Kato N. Three new thermotolerant methylotrophic yeasts, *Candida krabiensis* sp. nov., *Candida sithepensis* sp. nov., and *Pichia siamensis* sp. nov., isolated in Thailand. The Journal of General and Applied Microbiology. 2004;**50**:119-127

[24] Abdel-Fattah WR, Fadil M, Nigam P, Banat IM. Isolation of thermotolerant ethanologenic yeasts and use of selected strains in industrial scale fermentation in an Egyptian distillery. Biotechnology and Bioengineering. 2000;**68**:531-535

[25] Banat IM, Nigam P, Marchant R. Isolation of thermotolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C. World Journal of Microbiology and Biotechnology. 1992;**8**:259-263. DOI: 10.1007/BF01201874

[26] Dhaliwal SS, Oberoi HS, Sandhu SK, Nanda D, Kumar D, Uppal SK. Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*. Bioresource Technology. 2011;**102**:5968-5975. DOI: 10.1016/j.biortech.2011.02.015

[27] Arora R, Behera S, Sharma NK, Kumar S. A new search for thermotolerant yeasts, its characterization and optimization using response surface methodology for ethanol production. Frontiers in Microbiology. 2015;**6**:889. DOI: 10.3389/fmicb.2015.00889

[28] Keo-oudone C, Nitiyon S, Sotitham P, Tani A, Lertwattanasakul N, Yuangsaard N, Bounphanmy S, Limtong S, Yamada M. Isolation and characterization of thermotolerant ethanol-fermenting yeasts from Laos and application of whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis for their quick identification. African Journal of Biotechnology. 2016;**15**:153-164. DOI: 10.5897/AJB2015.14984

[29] Fonseca GG, Heinzle E, Wittmann C, Gombert AK. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Applied Microbiology and Biotechnology. 2008;**79**: 339-354. DOI: 10.1007/s00253-008-1458-6

[30] Lachance MA. *Kluyveromyces* van der Walt (1971). In: Kurtzman CP, Fell JW, Boekhout T, editors. The Yeasts. 5th ed. Amsterdam: Elsevier; 2010. p. 471-481. DOI: 10.1016/B978-0-444-52149-1.00035-5

[31] Limtong S, Sringiew C, Yongmanitchai W. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. Bioresource Technology. 2007;**98**:3367-3374. DOI: 10.1016/j.biortech.2006.10.044

[32] Rodrussamee N, Lertwattanasakul N, Hirata K, Suprayogi, Limtong S, Kosaka T, Yamada M. Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. Applied Microbiology and Biotechnology. 2011;**90**:1573-1586. DOI: 10.1007/s00253-011-3218-2

[33] Lertwattanasakul N, Rodrussamee N, Suprayogi, Limtong S, Thanonkeo P, Kosaka T, Yamada M. Utilization capability of sucrose, raffinose and inulin and its less-sensitiveness to glucose repression in thermotolerant yeast *Kluyveromyces marxianus* DMKU 3-1042. AMB Express. 2011;**1**:20. DOI: 10.1186/2191-0855-1-20

[34] Jeong H, Lee D-H, Kim SH, Kim H-J, Lee K, Song JY, Kim BK, Sung BH, Park JC, Sohn JH, Koo HM, Kim JF. Genome sequence of the thermotolerant yeast *Kluyveromyces marxianus* var. *marxianus* KCTC 17555. Eukaryotic Cell. 2012;**11**:1584-1585. DOI: 10.1128/ec.00260-12

[35] Lertwattanasakul N, Kosaka T, Hosoyama A, Suzuki Y, Rodrussamee N, Matsutani M, Murata M, Fujimoto N, Suprayogi, Tsuchikane K, Limtong S, Fujita N, Yamada M.

Genetic basis of the highly efficient yeast *Kluyveromyces marxianus*: complete genome sequence and transcriptome analyses. Biotechnol Biofuels. 2015;**8**:47. DOI: 10.1186/s13068-015-0227-x

[36] Nonklang S, Abdel-Banat BM, Cha-aim K, Moonjai N, Hoshida H, Limtong S, Yamada M, Akada R. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. Applied and Environmental Microbiology. 2008;**74**:7514-7521. DOI: 10.1128/AEM.01854-08

[37] Nitiyon S, Keo-Oudone C, Murata M, Lertwattanasakul N, Limtong S, Kosaka T, Yamada M. Efficient conversion of xylose to ethanol by stress-tolerant *Kluyveromyces marxianus* BUNL-21. Springerplus. 2016;**5**:185. DOI: 10.1186/s40064-016-1881-6

[38] Abdel-Banat BM, Hoshida H, Ano A, Nonklang S, Akada R. High-temperature fermentation: How can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast. Applied Microbiology and Biotechnology. 2010;**85**:861-867. DOI: 10.1007/s00253-009-2248-5

[39] Murata M, Nitiyon S, Lertwattanasakul N, Sootsuwan K, Kosaka T, Thanonkeo P, Limtong S, Yamada M. High-temperature fermentation technology for low-cost bioethanol. Journal of the Japan Institute of Energy. 2015;**94**:1154-1162

[40] Anderson PJ, McNeil K, Watson K. High-efficiency carbohydrate fermentation to ethanol at temperatures above 40°C by *Kluyveromyces marxianus* var. *marxianus* isolated from sugar mills. Applied and Environmental Microbiology. 1986;**51**:1314-1320

[41] Xu K, Lv B, Huo YX, Li C. Toward the lowest energy consumption and emission in biofuel production: Combination of ideal reactors and robust hosts. Current Opinion in Biotechnology. 2018;**50**:19-24. DOI: 10.1016/j.copbio.2017.08.011

[42] Mohd Azhar SH, Abdulla R, Jambo SA, Marbawi H, Gansau JA, Mohd Faik AA, Rodrigues KF. Yeasts in sustainable bioethanol production: A review. Biochemistry and Biophysics Reports. 2017;**10**:52-61. DOI: 10.1016/j.bbrep.2017.03.003

[43] Figueroa-Torres GM, Pittman JK, Theodoropoulos C. Kinetic modelling of starch and lipid formation during mixotrophic, nutrient-limited microalgal growth. Bioresource Technology. 2017;**241**:868-878

[44] Bekirogullari M, Fragkopoulos IS, Pittman JK, Theodoropoulos C. Production of lipid-based fuels and chemicals from microalgae: An integrated experimental and model-based optimization study. Algal Research. 2017;**23**:78-87

[45] Kumar S, Singh SP, Mishra IM, Adhikari DK. Ethanol and xylitol production from glucose and xylose at high temperature by *Kluyveromyces* sp. IIPE453. Journal of Industrial Microbiology & Biotechnology. 2009;**36**:1483-1489. DOI: 10.1007/s10295-009-0636-6

[46] Yanase S, Hasunuma T, Yamada R, Tanaka T, Ogino C, Fukuda H, Kondo A. Direct ethanol production from cellulosic materials at high temperature using the thermotolerant yeast *Kluyveromyces marxianus* displaying cellulolytic enzymes. Applied Microbiology and Biotechnology. 2010;**88**:381-388. DOI: 10.1007/s00253-010-2784-z

[47] Pilap W, Thanonkeo S, Klanrit P, Thanonkeo P. The potential of the newly isolated thermotolerant *Kluyveromyces marxianus* for high-temperature ethanol production using sweet sorghum juice. 3 Biotech. 2018;**8**:126. DOI: 10.1007/s13205-018-1161-y

[48] Koutinas M, Patsalou M, Stavrinou S, Vyrides I. High temperature alcoholic fermentation of orange peel by the newly isolated thermotolerant *Pichia kudriavzevii* KVMP10. Letters in Applied Microbiology. 2016;**62**:75-83. DOI: 10.1111/lam.12514

[49] Chamnipa N, Thanonkeo S, Klanrit P, Thanonkeo P. The potential of the newly isolated thermotolerant yeast *Pichia kudriavzevii* RZ8-1 for high-temperature ethanol production. Brazilian Journal of Microbiology. 2018;**49**:378-391. DOI: 10.1016/j.bjm.2017.09.002

[50] Sree NK, Sridhar M, Suresh K, Banat IM, Rao LV. Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. Bioresource Technology. 2000;**72**:43-46. DOI: 10.1016/S0960-8524(99)90097-4

[51] Auesukaree C, Koedrith P, Saenpayavai P, Asvarak T, Benjaphokee S, Sugiyama M, Kaneko Y, Harashima S, Boonchird C. Characterization and gene expression profiles of thermotolerant *Saccharomyces cerevisiae* isolates from Thai fruits. Journal of Bioscience and Bioengineering. 2012;**114**:144-149. DOI: 10.1016/j.jbiosc.2012.03.012

[52] Nuanpeng S, Thanonkeo S, Yamada M, Thanonkeo P. Ethanol production from sweet sorghum juice at high temperatures using a newly isolated thermotolerant yeast *Saccharomyces cerevisiae* DBKKU Y-53. Energies. 2016;**9**:253. DOI: 10.3390/en9040253

[53] Techaparin A, Thanonkeo P, Klanrit P. High-temperature ethanol production using thermotolerant yeast newly isolated from Greater Mekong Subregion. Brazilian Journal of Microbiology. 2017;**48**:461-475. DOI: 10.1016/j.bjm.2017.01.006

[54] Nigam PS, Singh A. Production of liquid biofuels from renewable resources. Progress in Energy and Combustion Science. 2011;**37**:52-68

[55] Lin Y, Tanaka S. Ethanol fermentation from biomass resources: Current state and prospects. Applied Microbiology and Biotechnology. 2006;**69**:627-642. DOI: 10.1007/s00253-005-0229-x

[56] de Farias Silva CE, Bertucco A. Bioethanol from microalgae and cyanobacteria: A review and technological outlook. Process Biochemistry. 2016;**51**:1833-1842

[57] Chen C-Y, Zhao X-Q, Yen H-W, Ho S-H, Cheng C-L, Lee D-J, Bai F-W, Chang J-S. Microalgae-based carbohydrates for biofuel production. Biochemical Engineering Journal. 2013;**78**:1-10

[58] Brethauer S, Studer MH. Biochemical conversion processes of lignocellulosic biomass to fuels and chemicals – A review. Chimia (Aarau). 2015;**69**:572-581. DOI: 10.2533/chimia.2015.572

[59] van Maris AJ, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MA, Wisselink HW, Scheffers WA, van Dijken JP, Pronk JT. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: Current status. Antonie Van Leeuwenhoek. 2006;**90**:391-418. DOI: 10.1007/s10482-006-9085-7

[60] Zabed H, Sahu JN, Boyce AN, Faruq G. Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches. Renewable and Sustainable Energy Reviews. 2016;**66**:751-774

[61] Kim SR, Ha SJ, Wei N, Oh EJ, Jin YS. Simultaneous co-fermentation of mixed sugars: A promising strategy for producing cellulosic ethanol. Trends in Biotechnology. 2012; **30**:274-282. DOI: 10.1016/j.tibtech.2012.01.005

[62] Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology. 2005;**96**:673-686. DOI: 10.1016/j.biortech.2004.06.025

[63] Kumar S, Gupta R, Kumar G, Sahoo D, Kuhad RC. Bioethanol production from *Gracilaria verrucosa*, a red alga, in a biorefinery approach. Bioresource Technology. 2013;**135**:150-156. DOI: 10.1016/j.biortech.2012.10.120

[64] Harun R, Danquah MK, Forde GM. Microalgal biomass as a fermentation feedstock for bioethanol production. Journal of Chemical Technology and Biotechnology. 2010; **85**:199-203

[65] Isono N, Hayakawa H, Usami A, Mishima T, Hisamatsu M. A comparative study of ethanol production by *Issatchenkia orientalis* strains under stress conditions. Journal of Bioscience and Bioengineering. 2012;**113**:76-78. DOI: 10.1016/j.jbiosc.2011.09.004

[66] Kwon YJ, Ma AZ, Li Q, Wang F, Zhuang GQ, Liu CZ. Effect of lignocellulosic inhibitory compounds on growth and ethanol fermentation of newly-isolated thermotolerant *Issatchenkia orientalis*. Bioresource Technology. 2011;**102**:8099-8104. DOI: 10.1016/j.biortech.2011.06.035

[67] Ishchuk OP, Voronovsky AY, Abbas CA, Sibirny AA. Construction of *Hansenula polymorpha* strains with improved thermotolerance. Biotechnology and Bioengineering. 2009;**104**:911-919. DOI: 10.1002/bit.22457

[68] Huang Y, Qin X, Luo X-M, Nong Q, Yang Q, Zhang Z, Gao Y, Lv F, Chen Y, Yu Z. Efficient enzymatic hydrolysis and simultaneous saccharification and fermentation of sugarcane bagasse pulp for ethanol production by cellulase from *Penicillium oxalicum* EU2106 and thermotolerant *Saccharomyces cerevisiae* ZM1-5. Biomass and Bioenergy. 2015;**77**:53-63

[69] Prasetyo J, Naruse K, Kato T, Boonchird C, Harashima S, Park EY. Bioconversion of paper sludge to biofuel by simultaneous saccharification and fermentation using a cellulase of paper sludge origin and thermotolerant *Saccharomyces cerevisiae* TJ14. Biotechnol Biofuels. 2011;**4**:35. DOI: 10.1186/1754-6834-4-35

[70] Faga BA, Wilkins MR, Banat IM. Ethanol production through simultaneous saccharification and fermentation of switchgrass using *Saccharomyces cerevisiae* D(5)A and thermotolerant *Kluyveromyces marxianus* IMB strains. Bioresource Technology. 2010;**101**:2273-2279. DOI: 10.1016/j.biortech.2009.11.001

[71] Harun R, Jason WSY, Cherrington T, Danquah MK. Exploring alkaline pre-treatment of microalgal biomass for bioethanol production. Applied Energy. 2011;**88**:3464-3467

[72] Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R. Hemicelluloses for fuel ethanol: A review. Bioresource Technology. 2010;**101**:4775-4800. DOI: 10.1016/j.biortech.2010.01.088

[73] Kasavi C, Finore I, Lama L, Nicolaus B, Oliver SG, Oner ET, Kirdar B. Evaluation of industrial *Saccharomyces cerevisiae* strains for ethanol production from biomass. Biomass and Bioenergy. 2012;**45**:230-238

[74] Toivola A, Yarrow D, van den Bosch E, van Dijken JP, Scheffers WA. Alcoholic fermentation of d-xylose by yeasts. Applied and Environmental Microbiology. 1984;**47**:1221-1223

[75] Chandel AK, Kapoor RK, Singh A, Kuhad RC. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresource Technology. 2007;**98**:1947-1950. DOI: 10.1016/j.biortech.2006.07.047

[76] Jeffries TW, Fady JH, Lightfoot EN. Effect of glucose supplements on the fermentation of xylose by Pachysolen tannophilus. Biotechnology and Bioengineering. 1985;**27**:171-176. DOI: 10.1002/bit.260270211

[77] Charoensopharat K, Thanonkeo P, Thanonkeo S, Yamada M. Ethanol production from Jerusalem artichoke tubers at high temperature by newly isolated thermotolerant inulin-utilizing yeast *Kluyveromyces marxianus* using consolidated bioprocessing. Antonie Van Leeuwenhoek. 2015;**108**:173-190. DOI: 10.1007/s10482-015-0476-5

[78] Wu WH, Hung WC, Lo KY, Chen YH, Wan HP, Cheng KC. Bioethanol production from taro waste using thermo-tolerant yeast *Kluyveromyces marxianus* K21. Bioresource Technology. 2016;**201**:27-32. DOI: 10.1016/j.biortech.2015.11.015

[79] Pessani NK, Atiyeh HK, Wilkins MR, Bellmer DD, Banat IM. Simultaneous saccharification and fermentation of Kanlow switchgrass by thermotolerant *Kluyveromyces marxianus* IMB3: The effect of enzyme loading, temperature and higher solid loadings. Bioresource Technology. 2011;**102**:10618-10624. DOI: 10.1016/j.biortech.2011.09.011

[80] Suryawati L, Wilkins MR, Bellmer DD, Huhnke RL, Maness NO, Banat IM. Simultaneous saccharification and fermentation of Kanlow switchgrass pretreated by hydrothermolysis using *Kluyveromyces marxianus* IMB4. Biotechnology and Bioengineering. 2008; **101**:894-902. DOI: 10.1002/bit.21965

[81] Ballesteros I, Ballesteros M, Cabañas A, Carrasco J, Martín C, Negro MJ, Saez F, Saez R. Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. Applied Biochemistry and Biotechnology. 1991;**28-29**:307-315

[82] Castro RC, Roberto IC. Selection of a thermotolerant *Kluyveromyces marxianus* strain with potential application for cellulosic ethanol production by simultaneous saccharification and fermentation. Applied Biochemistry and Biotechnology. 2014;**172**:1553-1564. DOI: 10.1007/s12010-013-0612-5

[83] Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Girones R, Koutsoumanis K, Herman L, Lindqvist R, Nørrung B. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 5: Suitability of taxonomic units notified to EFSA until September 2016. EFSA Journal. 2017;**15**(3):4663. DOI: 10.2903/j.efsa.2017.4663

[84] Lane MM, Morrissey JP. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. Fungal Biology Reviews. 2010;**24**:17-26. DOI: 10.1016/j.fbr.2010.01.001

[85] Kobayashi Y, Sahara T, Suzuki T, Kamachi S, Matsushika A, Hoshino T, Ohgiya S, Kamagata Y, Fujimori KE. Genetic improvement of xylose metabolism by enhancing the expression of pentose phosphate pathway genes in *Saccharomyces cerevisiae* IR-2 for high-temperature ethanol production. Journal of Industrial Microbiology & Biotechnology. 2017;**44**:879-891. DOI: 10.1007/s10295-017-1912-5

[86] Guimarães PMR, Teixeira JA, Domingues L. Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnology Advances. 2010;**28**:375-384. DOI: 10.1016/j.biotechadv.2010.02.002

[87] Lane MM, Burke N, Karreman R, Wolfe KH, O'Byrne CP, Morrissey JP. Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. Antonie Van Leeuwenhoek. 2011;**100**:507-519. DOI: 10.1007/s10482-011-9606-x

[88] Groeneveld P, Stouthamer AH, Westerhoff HV. Super life – How and why 'cell selection' leads to the fastest-growing eukaryote. FEBS Journal. 2009;**276**:254-270. DOI: 10.1111/j.1742-4658.2008.06778.x

[89] Hughes SR, Qureshi N, López-Núñez JC, Jones MA, Jarodsky JM, Galindo-Leva LÁ, Lindquist MR. Utilization of inulin-containing waste in industrial fermentations to produce biofuels and bio-based chemicals. World Journal of Microbiology and Biotechnology. 2017;**33**. DOI: 10.1007/s11274-017-2241-6

[90] Lin Y-J, Chang J-J, Lin H-Y, Thia C, Kao Y-Y, Huang C-C, Li W-H. Metabolic engineering a yeast to produce astaxanthin. Bioresource Technology. 2017;**245**:899-905. DOI: 10.1016/j.biortech.2017.07.116

[91] Wang Y-J, Ying B-B, Shen W, Zheng R-C, Zheng Y-G. Rational design of *Kluyveromyces marxianus* ZJB14056 aldo-keto reductase KmAKR to enhance diastereoselectivity and activity. Enzyme and Microbial Technology. 2017;**107**:32-40

[92] Simoness O, Murilol B, Carlosr R, Paulalde A, Mariackv R, Franciscorde A-N, Soreleb F, Luizars D. Asymmetric bioreduction of β-ketoesters derivatives by *Kluyveromyces marxianus*: Influence of molecular structure on the conversion and enantiomeric excess. Anais da Academia Brasileira de Ciências. 2017;**89**:1403-1415. DOI: 10.1590/0001-3765201720170118

[93] Lee JW, In JH, Park J-B, Shin J, Park JH, Sung BH, Sohn J-H, Seo J-H, Park J-B, Kim SR, Kweon D-H. Co-expression of two heterologous lactate dehydrogenases genes in *Kluyveromyces marxianus* for L-lactic acid production. Journal of Biotechnology. 2017;**241**:81-86. DOI: 10.1016/j.jbiotec.2016.11.015

[94] Gombert AK, Madeira JV, Cerdán ME, González-Siso MI. *Kluyveromyces marxianus* as a host for heterologous protein synthesis. Applied Microbiology and Biotechnology. 2016;**100**:6193-6208. DOI: 10.1007/s00253-016-7645-y

[95] Suzuki T, Hoshino T, Matsushika A. Draft genome sequence of *Kluyveromyces marxianus* strain DMB1, isolated from sugarcane bagasse hydrolysate. Genome Announcements. 2014;**2**. DOI: 10.1128/genomeA.00733-14

[96] Silveira WB, Diniz RH, Cerdán ME, González-Siso MI, Souza RA, Vidigal PM, Brustolini OJ, de Almeida Prata ER, Medeiros AC, Paiva LC, Nascimento M, Ferreira EG, Dos Santos VC, Bragança CR, Fernandes TA, Colombo LT, Passos FM. Genomic sequence of the yeast *Kluyveromyces marxianus* CCT 7735 (UFV-3), a highly lactose-fermenting yeast isolated from the Brazilian dairy industry. Genome Announcements. 2014;**2**. DOI: 10.1128/genomeA.01136-14

[97] Inokuma K, Ishii J, Hara KY, Mochizuki M, Hasunuma T, Kondo A. Complete genome sequence of *Kluyveromyces marxianus* NBRC1777, a nonconventional thermotolerant yeast. Genome Announcements. 2015;**3**:e00389-15

[98] Quarella S, Lovrovich P, Scalabrin S, Campedelli I, Backovic A, Gatto V, Cattonaro F, Turello A, Torriani S, Felis GE. Draft genome sequence of the probiotic yeast *Kluyveromyces marxianus fragilis* B0399. Genome Announcements. 2016;**4**. DOI: 10.1128/genomeA.00923-16

[99] Schabort DTW, Letebele PK, Steyn L, Kilian SG, du Preez JC. Differential RNA-seq, multi-network analysis and metabolic regulation analysis of *Kluyveromyces marxianus* reveals a compartmentalised response to xylose. PLoS One. 2016;**11**:e0156242. DOI: 10.1371/journal.pone.0156242

[100] Ortiz-Merino RA, Varela JA, Coughlan AY, Hoshida H, da Silveira WB, Wilde C, Kuijpers NGA, Geertman J-M, Wolfe KH, Morrissey JP. Ploidy variation in *Kluyveromyces marxianus* separates dairy and non-dairy isolates. Frontiers in Genetics. 2018;**9**. DOI: 10.3389/fgene.2018.00094

[101] Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, Stewart A, Sgouros J, Peat N, Hayles J, Baker S, Basham D, Bowman S, Brooks K, Brown D, Brown S, Chillingworth T, Churcher C, Collins M, Connor R, Cronin A, Davis P, Feltwell T, Fraser A, Gentles S, Goble A, Hamlin N, Harris D, Hidalgo J, Hodgson G, Holroyd S, Hornsby T, Howarth S, Huckle EJ, Hunt S, Jagels K, James K, Jones L, Jones M, Leather S, McDonald S, McLean J, Mooney P, Moule S, Mungall K, Murphy L, Niblett D, Odell C, Oliver K, O'Neil S, Pearson D, Quail MA, Rabbinowitsch E, Rutherford K, Rutter S, Saunders D, Seeger K, Sharp S, Skelton J, Simmonds M, Squares R, Squares S, Stevens K, Taylor K, Taylor RG, Tivey A, Walsh S, Warren T, Whitehead S, Woodward J, Volckaert G, Aert R, Robben J, Grymonprez B, Weltjens I, Vanstreels E, Rieger M, Schäfer M, Müller-Auer S, Gabel C, Fuchs M, Düsterhöft A, Fritzc C, Holzer E, Moestl D, Hilbert H, Borzym K, Langer I, Beck A, Lehrach H, Reinhardt R, Pohl TM, Eger P, Zimmermann W, Wedler H, Wambutt R, Purnelle B, Goffeau A, Cadieu E, Dréano S, Gloux S, Lelaure V, Mottier S, Galibert F, Aves SJ, Xiang Z, Hunt C, Moore K, Hurst SM, Lucas M, Rochet M, Gaillardin C, Tallada VA, Garzon A, Thode G, Daga RR, Cruzado L, Jimenez J, Sánchez M, del Rey F, Benito J, Domínguez A, Revuelta JL, Moreno S, Armstrong J, Forsburg SL, Cerutti L, Lowe T, McCombie WR, Paulsen I, Potashkin J, Shpakovski GV, Ussery D, Barrell BG, Nurse P, Cerrutti L. The genome sequence of *Schizosaccharomyces pombe*. Nature. 2002;**415**:871-880. DOI: 10.1038/nature724

[102] Butler G, Rasmussen MD, Lin MF, Santos MAS, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, Agrafioti I, Arnaud MB, Bates S, Brown AJP, Brunke S, Costanzo MC, Fitzpatrick DA, de Groot PWJ, Harris D, Hoyer LL, Hube B, Klis FM, Kodira C, Lennard N, Logue ME, Martin R, Neiman AM, Nikolaou E, Quail MA,

Quinn J, Santos MC, Schmitzberger FF, Sherlock G, Shah P, Silverstein KAT, Skrzypek MS, Soll D, Staggs R, Stansfield I, Stumpf MPH, Sudbery PE, Srikantha T, Zeng Q, Berman J, Berriman M, Heitman J, Gow NAR, Lorenz MC, Birren BW, Kellis M, Cuomo CA. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature. 2009;**459**:657-662. DOI: 10.1038/nature08064

[103] Jeffries TW, Grigoriev IV, Grimwood J, Laplaza JM, Aerts A, Salamov A, Schmutz J, Lindquist E, Dehal P, Shapiro H, Jin Y-S, Passoth V, Richardson PM. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. Nature Biotechnology. 2007;**25**:319-326. DOI: 10.1038/nbt1290

[104] Dietrich FS. The *Ashbya gossypii* genome as a tool for mapping the ancient *Saccharomyces cerevisiae* genome. Science. 2004;**304**:304-307. DOI: 10.1126/science.1095781

[105] Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, de Montigny J, Marck C, Neuvéglise C, Talla E, Goffard N, Frangeul L, Aigle M, Anthouard V, Babour A, Barbe V, Barnay S, Blanchin S, Beckerich J-M, Beyne E, Bleykasten C, Boisramé A, Boyer J, Cattolico L, Confanioleri F, de Daruvar A, Despons L, Fabre E, Fairhead C, Ferry-Dumazet H, Groppi A, Hantraye F, Hennequin C, Jauniaux N, Joyet P, Kachouri R, Kerrest A, Koszul R, Lemaire M, Lesur I, Ma L, Muller H, Nicaud J-M, Nikolski M, Oztas S, Ozier-Kalogeropoulos O, Pellenz S, Potier S, Richard G-F, Straub M-L, Suleau A, Swennen D, Tekaia F, Wésolowski-Louvel M, Westhof E, Wirth B, Zeniou-Meyer M, Zivanovic I, Bolotin-Fukuhara M, Thierry A, Bouchier C, Caudron B, Scarpelli C, Gaillardin C, Weissenbach J, Wincker P, Souciet J-L. Genome evolution in yeasts. Nature. 2004;**430**:35-44. DOI: 10.1038/nature02579

[106] Rubio-Texeira M. Endless versatility in the biotechnological applications of *Kluyveromyces* LAC genes. Biotechnology Advances. 2006;**24**:212-225

[107] Van Ooyen AJJ, Dekker P, Huang M, Olsthoorn MMA, Jacobs DI, Colussi PA, Taron CH. Heterologous protein production in the yeast *Kluyveromyces lactis*. FEMS Yeast Research. 2006;**6**:381-392

[108] Rocha SN, Abrahão-Neto J, Cerdán ME, Gombert AK, González-Siso MI. Heterologous expression of a thermophilic esterase in *Kluyveromyces* yeasts. Applied Microbiology and Biotechnology. 2011;**89**:375-385. DOI: 10.1007/s00253-010-2869-8

[109] Rocha SN, Abrahao-Neto J, Cerdan ME, Gonzalez-Siso MI, Gombert AK. Heterologous expression of glucose oxidase in the yeast *Kluyveromyces marxianus*. Microbial Cell Factories. 2010;**9**:4. DOI: 10.1186/1475-2859-9-4

[110] Jablonowski D, Schaffrath R. Zymocin, a composite chitinase and tRNase killer toxin from yeast. Biochemical Society Transactions. 2007;**35**:1533-1537. DOI: 10.1042/bst0351533

[111] Abranches J, Mendonça-Hagler LC, Hagler AN, Morais PB, Rosa CA. The incidence of killer activity and extracellular proteases in tropical yeast communities. Canadian Journal of Microbiology. 1997;**43**:328-336. DOI: 10.1139/m97-046

[112] Lertwattanasakul N, Shigemoto E, Rodrussamee N, Limtong S, Thanonkeo P, Yamada M. The crucial role of alcohol dehydrogenase Adh3 in *Kluyveromyces marxianus* mitochondrial metabolism. Bioscience, Biotechnology, and Biochemistry. 2009;**73**:2720-2726. DOI: 10.1271/bbb.90609

[113] Lertwattanasakul N, Sootsuwan K, Limtong S, Thanonkeo P, Yamada M. Comparison of the gene expression patterns of alcohol dehydrogenase isozymes in the thermotolerant yeast *Kluyveromyces marxianus*and their physiological functions. Bioscience, Biotechnology, and Biochemistry. 2007;**71**:1170-1182. DOI: 10.1271/bbb.60622

[114] Ishtar Snoek IS, Yde Steensma H. Why does *Kluyveromyces lactis* not grow under anaerobic conditions? Comparison of essential anaerobic genes of Saccharomyces cerevisiae with the *Kluyveromyces lactis* genome. FEMS Yeast Research. 2006;**6**:393-403

[115] Lulu L, Ling Z, Dongmei W, Xiaolian G, Hisanori T, Hidehiko K, Jiong H. Identification of a xylitol dehydrogenase gene from *Kluyveromyces marxianus* NBRC1777. Molecular Biotechnology. 2013;**53**:159-169. DOI: 10.1007/s12033-012-9508-9

[116] Zhang B, Zhang L, Wang D, Gao X, Hong J. Identification of a xylose reductase gene in the xylose metabolic pathway of *Kluyveromyces marxianus* NBRC1777. Journal of Industrial Microbiology & Biotechnology. 2011;**38**:2001-2010. DOI: 10.1007/s10295-011-0990-z

[117] van Dijken JP, Scheffers WA. Redox balances in the metabolism of sugars by yeasts. FEMS Microbiology Reviews. 1986;**1**:199-224

[118] Bakker BM, Overkamp KM, van Maris AJA, Kötter P, Luttik MAH, van Dijken JP, Pronk JT. Stoichiometry and compartmentation of NADH metabolism in*Saccharomyces cerevisiae*. FEMS Microbiology Reviews. 2001;**25**:15-37. DOI: 10.1111/j.1574-6976.2001.tb00570.x

[119] Cao J, Barbosa JM, Singh NK, Locy RD. GABA shunt mediates thermotolerance in*Saccharomyces cerevisiae*by reducing reactive oxygen production. Yeast. 2013;**30**:129-144. DOI: 10.1002/yea.2948

[120] Foukis A, Stergiou P-Y, Theodorou LG, Papagianni M, Papamichael EM. Purification, kinetic characterization and properties of a novel thermo-tolerant extracellular protease from *Kluyveromyces marxianus* IFO 0288 with potential biotechnological interest. Bioresource Technology. 2012;**123**:214-220. DOI: 10.1016/j.biortech.2012.06.090

[121] Raimondi S, Uccelletti D, Amaretti A, Leonardi A, Palleschi C, Rossi M. Secretion of *Kluyveromyces lactis* Cu/Zn SOD: Strategies for enhanced production. Applied Microbiology and Biotechnology. 2010;**86**:871-878. DOI: 10.1007/s00253-009-2353-5

[122] Carlson M. Glucose repression in yeast. Current Opinion in Microbiology. 1999;**2**: 202-207. DOI: 10.1016/S1369-5274(99)80035-6

[123] Gancedo JM. Yeast carbon catabolite repression. Microbiology and Molecular Biology Reviews. 1998;**62**:334-361

[124] Gethins L, Guneser O, Demirkol A, Rea MC, Stanton C, Ross RP, Yuceer Y, Morrissey JP. Influence of carbon and nitrogen source on production of volatile fragrance and flavour metabolites by the yeast *Kluyveromyces marxianus*. Yeast. 2015;**32**:67-76. DOI: 10.1002/yea.3047

[125] Lertwattanasakul N, Suprayogi MM, Rodrussamee N, Limtong S, Kosaka T, Yamada M. Essentiality of respiratory activity for pentose utilization in thermotolerant yeast *Kluyveromyces marxianus* DMKU 3-1042. Antonie Van Leeuwenhoek. 2013;**103**:933-945. DOI: 10.1007/s10482-012-9874-0

[126] Ahuatzi D, Herrero P, de la Cera T, Moreno F. The glucose-regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1-dependent. The Journal of Biological Chemistry. 2004;**279**:14440-14446. DOI: 10.1074/jbc.M313431200

[127] Nehlin JO, Ronne H. Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. The EMBO Journal. 1990;**9**:2891-2898

[128] Bergdahl B, Sandström AG, Borgström C, Boonyawan T, van Niel EW, Gorwa-Grauslund MF. Engineering yeast hexokinase 2 for improved tolerance toward xylose-induced inactivation. PLoS One. 2013;**8**:e75055. DOI: 10.1371/journal.pone.0075055

[129] Peláez R, Herrero P, Moreno F. Functional domains of yeast hexokinase 2. The Biochemical Journal. 2010;**432**:181-190. DOI: 10.1042/BJ20100663

[130] Ahuatzi D, Riera A, Peláez R, Herrero P, Moreno F. Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. The Journal of Biological Chemistry. 2007;**282**:4485-4493. DOI: 10.1074/jbc.M606854200

[131] Suprayogi, Murata M, Lertawattanasakul N, Kosaka T, Rodrussamee N, Yamada M. Characteristics of *kan*MX4-inserted mutants that exhibit 2-deoxyglucose resistance in thermotolerance yeast *Kluyveromyces marxianus*. The Open Biotechnology Journal. 2016;**10**:208-222. DOI: 10.2174/1874070701610010020 8S

[132] Suprayogi, Nguyen MT, Lertwattanasakul N, Rodrussamee N, Limtong S, Kosaka T, Yamada M. A *Kluyveromyces marxianus* 2-deoxyglucose-resistant mutant with enhanced activity of xylose utilization. International Microbiology. 2015;**18**:235-244. DOI: 10.2436/20.1501.01.255

[133] Zhou H-X, Xu J-L, Chi Z, Liu G-L, Chi Z-M. β-Galactosidase over-production by a mig1 mutant of *Kluyveromyces marxianus* KM for efficient hydrolysis of lactose. Biochemical Engineering Journal. 2013;**76**:17-24

[134] Zhou H-X, Xin F-H, Chi Z, Liu G-L, Chi Z-M. Inulinase production by the yeast *Kluyveromyces marxianus* with the disrupted MIG1 gene and the over-expressed inulinase gene. Process Biochemistry. 2014;**49**:1867-1874

[135] Nurcholis M, Nitiyon S, Suprayogi, Rodrussamee N, Lertwattanasakul N, Limtong S, Kosaka T, Yamada M. Functional Analysis in Thermotolerant Yeast *Kluyveromyces marxianus* of Mig1 and Rag5, which Are Ortholog of Mig1 and Hxk2, Respectively, Related to Glucose Repression in *Saccharomyces cerevisiae* (Unpublished)

[136] Wright T, Rahmanulloh A. Indonesia Biofuels Annual Report 2017. USDA Foreign Agricultural Service. 2017. https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual_Jakarta_Indonesia_6-20-2017.pdf

[137] Murdiyatmo U. Indonesian Bioethanol Industry: Current Condition and Opportunity for Development. Indonesian Ethanol Association (ASENDO); 2014. http://www.globalbioenergy.org/fileadmin/user_upload/gbep/docs/2015_events/3rd_Bioenergy_Week_25-29_May_Indonesia/28_5_12_MURDIYATMO.pdf

[138] Directorate General of New Energy, Renewable and Energy Conservation. Ministry of Energy and Mineral Resources of Republic Indonesia. 2007. https://www.esdm.go.id/

[139] Murdiyatmo U. Obstacles and challenges of bioethanol industry development in Indonesia. In: Surfactant and Bioenergy Research Center (SBRC) LPPM IPB. 2006. ISBN 978-979-1312-08-0

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