Development of xylose-fermenting microbes for high-temperature fermentation of ethanol and 2,3-butanediol

(エタノール及び 2,3 ブタンジオール高温発酵のためのキシロース発酵微生物の開発)

PhD Thesis

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CHAPTER 1

General Introduction

In recent years, the need for renewable energy is rapidly increasing due to the increase in human population, the progress of industrialization, the continuous increase in energy demand and the decrease in fossil and traditional fuels. Human has used traditional energy sources such as coal, oil and natural gas to cause environmental pollution. As a result, many countries are facing environmental degradation due to the use of fossil fuels and have a keen interest in renewable energy (Saxena *et al*. 2009). When considering renewable energy sources that can replace fossils and traditional fuels, inexpensive and renewable alternative energy resources such as solar, wind, heat, hydroelectric and biomass that do not cause pollution are an important and utmost need.

Biomass is a renewable, sustainable and relatively environmentally friendly source of energy due to low carbon emission (Othmer 1980; White *et al*. 1981). Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin, and the former two compounds provide several sugars that can be converted into biofuels and chemical products via fermentation by microorganisms. (Moreno *et al.* 2019; Rodrussamee *et al*. 2018). Therefore, the microbial conversion of lignocellulosic biomass requires microbes with highly efficient utilization ability of both glucose and xylose.

Several microorganisms are able to produce ethanol or 2,3-butnaediol as the main products. However, most fermenting microbes are sensitive to high concentrations of substrate, high temperatures and glucose repression. In addition, lignocellulose-derived compounds such as aliphatic acids, furan derivatives and several phenolic compounds can be generated during its pretreatment, which have inhibitory effects on enzyme activities, cell viability and fermentation performance. Other relevant stress factors affecting fermentation are end-products such as ethanol and acetic acid. These inhibitors decrease in fermentable sugar yields and negatively impact on subsequent productions (Kim *et al*. 2018).

There were two main objectives of study. The first objective was adaptive laboratory evolution of thermotolerant and xylose-fermenting *Candida tropicalis* for improvement of tolerance to multistress including high glucose concentrations. The evolutionary adaptation was conducted by repetitive long-term cultivation with gradual increase of temperature (RLCGT) in the presence of a high concentration of glucose. Stress resistances and ethanol production of adapted strain were examined, and the mechanism of tolerance to multistress including high glucose concentrations was examined by transcriptome analysis or hydropathy analysis. As the second objective, screening of strains, which were isolated from Lao PDR, with highly efficient fermentation ability in xylose medium was performed, and one strain was found to produce 2, 3-butanediol (2,3-BD) from xylose or glucose. The strain was identified as *Cronobacter sakazakii*, and subjected to fermentation experiments to see the production ability of 2,3-BD at high temperatures in glucose and xylose media. Considering these results, these procedures may be useful for improving microbial tolerance to multistress or as methodology to find out microorganisms that have potentials for industrial application.

CHAPTER 2

Adaptive laboratory evolution for multistress tolerance, including fermentability at high glucose concentrations in thermotolerant *Candida tropicalis*

2.1 Abstract

Candida tropicalis, a xylose-fermenting yeast, has the potential for converting cellulosic biomass to ethanol. Thermotolerant *C. tropicalis* X-17, which was isolated in Laos, was subjected to repetitive long-term cultivation with a gradual increase in temperature (RLCGT) in the presence of a high concentration of glucose, which exposed cells to various stresses in addition to the high concentration of glucose and high temperatures. The resultant adapted strain demonstrated increased tolerance to ethanol, furfural and hydroxymethylfurfural at high temperatures and displayed improvement in fermentation ability at high glucose concentrations and xylose-fermenting ability. Transcriptome analysis revealed the up-regulation of a gene for a glucose transporter of the major facilitator superfamily and genes for stress response and cell wall proteins. Additionally, hydropathy analysis revealed that three genes for putative membrane proteins with multiple membrane-spanning segments were also up-regulated. From these findings, it can be inferred that the up-regulation of genes, including the gene for a glucose transporter, is responsible for the phenotype of the adaptive strain. This study revealed part of the mechanisms of fermentability at high glucose concentrations in *C. tropicalis* and the results of this study suggest that RLCGT is an effective procedure for improving multistress tolerance.

2.2 Introduction

Solutions for the problems of global warming and the unsustainability of future energy supplies are crucial for continued economic development (Moreno *et al*. 2019; Hoekman *et al*. 2009). Much interest has been shown in biomass as carbon sources for

biofuel production due to the environmentally friendly low carbon emissions of biofuels (Li *et al*. 2020; Li *et al*. 2020; Manyuchi *et al*. 2018; Patel *et al*. 2021). Biomass conversion has been performed by fermentation or other biomass utilization technology, particularly fast pyrolysis. For example, Hamouda *et al.* (Hamouda *et al*. 1015) converted sugarcane molasses by fermentation with *Candida tropicalis* HSC-24 to ethanol. Li et al. (Li *et al*. 2020; Li *et al*. 2020) cultivated *Desmodesmus sp*. in anaerobic digested wastewater and then converted it by fast pyrolysis to biofuel. In recent years, increasing interest has been shown in lignocellulose biomass as renewable and sustainable energy resources. Bioethanol, a biofuel from lignocellulosic biomass, is gaining increasing attention as an alternative fuel due to fluctuations in oil prices, reduced oil reserves, and important environmental issues associated with greenhouse gas emissions (Nweze *et al*. 2019; Rodrussamee *et al*. 2018; Panwar *et al*. 2011; Mano *et al*. 2018; Chen *et al.* 2016; Triwahyuni *et al*. 2015). Lignocellulose is composed of three major carbohydrate polymers: cellulose, hemicelluloses and lignin (Moreno *et al*. 2019; Nweze *et al*. 2019; Rodrussamee *et al*. 2018). Hemicellulose hydrolysates contain xylose as an abundant sugar (Nitiyon *et al*. 2016; Nguyen *et al*. 2020). Therefore, the utilization of lignocellulosic biomass as a substrate for ethanol production requires yeast strains that are capable of efficiently converting xylose to ethanol. Nevertheless, due to glucose repression, efficient co-fermentation of glucose and xylose is a major challenge in achieving the conversion of lignocellulosic biomass to biofuels (Nguyen *et al*. 2020). In addition, lignocellulose has a complex molecular structure in which chains of the three carbohydrate polymers are intertwined, requiring a pretreatment step to obtain sugars for fermentation. The steps for biochemical conversion of lignocellulose to useful products such as ethanol include (1) pretreatment to open, unique and cumbersome structures, (2) enzymatic hydrolysis for the saccharification of carbohydrate polymers and (3) the microbial fermentation of hydrolyzed sugars (Moreno *et al*. 2019; Rodrussamee *et al*. 2018). However, additional lignocellulose-derived compounds, such as aliphatic acids, furan derivatives and phenolic compounds, can be generated during pretreatment and have inhibitory effects on enzymatic performance. These inhibitors reduce the yield of fermentable sugars and adversely affect subsequent ethanol productions (Moreno *et al*. 2019; Kim *et al*. 2018; Sánchez *et al*. 2008).

Furthermore, during the ethanol fermentation process, yeast cells are simultaneously and continuously exposed to various stresses, such as high concentrations of sugar substrates, final products including ethanol and elevated temperature, in addition to the lignocellulose-derived compounds (Gibson *et al*. 2007; Zhao *et al*. 2009; Puligundla *et al*. 2011; Auesukaree 2017; Azhar *et al*. 2017; Kamoldeen *et al*. 2017; Greetham *et al*. 2016). Specifically, fermentation with high concentrations of sugar to obtain high yields of ethanol exacerbates the negative effects of stress on both the substrate and the product (Cheng *et al*. 2009). In general, compared to *Saccharomyces cerevisiae*, xylose-fermenting yeasts are sensitive to high concentrations of glucose. As with the development of various stress-tolerant strains (Alba *et al*. 2015; Wang *et al*. 2021), improving the durability of xylose-fermenting yeast strains to high concentrations of glucose is important for efficient conversion of lignocellulosic biomass.

Candida tropicalis X-17, which was isolated in Laos, is a thermotolerant xylose-fermenting yeast with similar growth rates in glucose and xylose media. However, similarly to most other xylose-fermenting yeasts, this strain is sensitive to high concentrations of glucose. In this study, we attempted to improve the sensitiveness of the strain to high concentrations of glucose by repetitive long-term cultivation with a gradual increase in temperature (RLCGT) (Pattanakittivorakul *et al*. submitted) in the presence of a high concentration of glucose. RLCGT is an efficient laboratory adaptation procedure for the development of adapted strains because it allows cells to be exposed to various stresses, such as stresses from metabolites including ethanol and organic acids, by-products formed by chemical reactions, nutrient starvation, high temperatures and oxidative stress. As adapted strains were successfully obtained by laboratory adaptation procedures in the presence of main stress factors of high concentrations of substrate (Alba *et al*. 2015) or high temperatures (Wang *et al*. 2021), RLCGT containing these factors may have advantages for acquiring adapted strains compared to the previous procedures. As expected, an adapted strain isolated by RLCGT in the presence of 200 $g \cdot L^{-1}$ glucose was found to be resistant not only to high concentrations of glucose but also other stresses. This was the first trial of RLCGT performed in the presence of high levels of

glucose, and its success may encourage us to further challenge cell capacity improvement for achieving a cost-effective method for lignocellulosic bioethanol production.

2.3 Materials and methods

2.3.1 Yeast strain

The yeast strains used in this study were thermotolerant, xylose-fermenting *C. tropicalis* X-17, its derivative strain and thermotolerant *Kluyveromyces marxianus* DMKU 3-1042 (Limtong *et al*. 2007). *C. tropicalis* X-17 was isolated from a fruit by the non-enrichment method in Laos and its isolation with other strains will be reported elsewhere.

2.3.2 Media and growth conditions

YPD medium (10 $g \cdot L^{-1}$ yeast extract, 20 $g \cdot L^{-1}$ peptone and 20 $g \cdot L^{-1}$ glucose) was used for pre-culture at 30 ˚C under a shaking condition at 160 rpm and YP medium (10 $g \cdot L^{-1}$ yeast extract, 20 $g \cdot L^{-1}$ peptone) containing 200 $g \cdot L^{-1}$ glucose was used for adaptation. YP agar plates supplemented with 20 $g \cdot L^{-1}$ of mannose, galactose, xylose or sucrose were named YPMan, YPGal, YPXyl and YPSuc, respectively. In some cases, 2-deoxyglucose (2-DOG) as a glucose analog was further supplemented. Fermentation was carried out in YP medium supplemented with 160 $g \cdot L^{-1}$ glucose (YP16D), 20 $g \cdot L^{-1}$ glucose (YP2D), 20 $g \cdot L^{-1}$ xylose (YP2X), 50 $g \cdot L^{-1}$ xylose (YP5X), both 2 $g \cdot L^{-1}$ glucose and 20 $g \cdot L^{-1}$ xylose (YP0.2D2X), 20 $g \cdot L^{-1}$ glucose and 20 $g \cdot L^{-1}$ xylose (YP2D2X), 40 $g \cdot L^{-1}$ glucose and 20 $g \cdot L^{-1}$ xylose (YP4D2X), 20 $g \cdot L^{-1}$ glucose and 50 $g \cdot L^{-1}$ xylose (YP2D5X) xylose, and 50 $g \cdot L^{-1}$ glucose and 50 $g \cdot L^{-1}$ xylose (YP5D5X) as a carbon source.

2.3.3 Evolutionary adaptation by RLCGT

Evolutionary adaptation was performed by RLCGT (Pattanakittivorakul *et al*., submitted) in the presence of a high concentration of glucose. *C. tropicalis* X-17 cells were pre-cultured in YPD medium at 30 ˚C for 16 h under a shaking condition at 160 rpm. The pre-culture was inoculated into 5 ml of YP liquid medium containing 200

$g \cdot L^{-1}$ glucose in five test tubes at an optical density (OD_{660}) of 0.1 and subjected to long-term cultivation at 40 ˚C under a shaking condition at 100 rpm for 7 days. After that, the cells were transferred to a fresh medium at OD_{660} of 0.1 and cultivation was repeated under the same conditions. After cultivation twice at 40 ˚C, at the second time the culture was transferred to a fresh medium and cultivated at 41 ˚C and 100 rpm for 7 days. Cultivation was repeated with a gradual increase in temperature from 40 ˚C to 44.5 ˚C (Figure S1). Cultivation was performed two or three times at each temperature. Finally, the RLCGT culture that survived at 44.5 ˚C was streaked on an YPD plate and a single colony was isolated as an adapted strain. The stability of the adaptive strain was tested by transferring it from low glucose concentration plates to high glucose concentration media several times, and a reproduced phenotype of growth was observed.

2.3.4 Analysis of stress resistance and effects of 2-DOG on utilization of various sugars

Cells grown in YPD medium at 30 ˚C under a shaking condition at 160 rpm for 16 h were washed and suspended in sterile distilled water with adjustment of OD_{660} to 1. The cell suspension was 10-fold serially diluted and spotted onto YP agar plates supplemented with 35% of glucose, 6% ethanol, 10 mM furfural or 15 mM HMF at a final concentration. These plates were then incubated at different temperatures for 48 h. The serially diluted cell suspensions were also spotted on YPD, YPMan, YPGal, YPXyl or YPSuc agar plates with or without 0.01%, 0.05% or 0.1% 2-DOG. These plates were then incubated at 30 ˚C for 48 h.

2.3.5 Analysis of ethanol fermentation

To examine abilities for ethanol production and glucose and xylose utilization, yeast strains were pre-cultured in YPD medium at 30 ˚C under a shaking condition at 160 rpm for 16 h. The pre-culture was inoculated into a 100-mL flask containing 30 mL of YP16D, YP5X or YP2D2X at OD_{660} of 0.1, followed by incubation at different temperatures. Cell density was determined by measurement on a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Fermentation parameters were analyzed on a high-performance liquid chromatography (HPLC) system (Hitachi, Tokyo, Japan)

consisting of a Hitachi Model D-2000 Elite HPLC system Manager, L-2130 column oven, L-2130 pump, L-2200 auto-sampler and L-2490 RI detector equipped with a GL-C610H-S gel pack column at 60 ˚C with 0.5 mL/min eluent of 0.1% phosphoric acid.

2.3.6 Preparation of genomic DNA, genomic sequencing, and determination of mutations

The genome DNAs of *C. tropicalis* X-17.2b and *C. tropicalis* X-17 as the parent strain were extracted as described previously (Sambrook *et al*. 2001) from cells grown in YPD medium for 18 h under a shaking condition at 30 ˚C and further purified using a Genomic-tip 20 kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In order to identify the mutation sites of X-17.2b, we performed genome sequencing of both X-17 and X-17.2b using the Illumina NextSeq 500 platform and mapped this against the complete genome sequence of *C. tropicalis* MYA-3404, which is available at DDBJ/EMBL/GenBank, accession number GCA_000006335.3. The quantity and purity of genomic DNA were assessed by using a Qubit 2.0 Fluorometer with a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a NanoDrop One spectrophotometer (Thermo Fisher Scientific). A genomic DNA library for Illumina sequencing was prepared using NEBNext Ultra II DNA Library Prep Kit for Illumina according to the manufacturer's instructions with 9 PCR cycles (New England BioLabs Inc., Ipswich, MA, USA). Genome sequencing was carried out with Illumina NextSeq 500 platform. A total of 27,724,872 and 28,307,864 sequence pairs of 76-bp paired-end nucleotide reads from X-17 and X-17.2b, respectively, were obtained, which yielded approximately 144-fold and 147-fold sequence coverage, respectively. The Illumina sequencing reads of both X-17 and X-17.2b were aligned with the MYA-3404 genome sequence using BWA (Li *et al*. 2009). Different sequence sites in both strains were searched for using the Genome Analysis Toolkit (GATK) v3.6-0-g89b7209 (McKenna *et al*. 2010). Accession numbers of sequence data are DRR328071 for X-17 and DRR32807 for X-17.2b.

2.3.7 RNA-Seq analysis

RNA for RNA-Seq analysis was prepared, as described previously (Nurcholis *et al*. 2019). The parental strain X-17 and adapted strain X-17.2b were cultivated in 30 mL of YP16D medium in a 100-mL Erlenmeyer flask on a rotary shaker at 100 rpm and 37 ˚C for 12 h. The cells were harvested by centrifugation at 5000 rpm for 5 min at 4 ˚C and subjected to an RNA preparation process. RNA was prepared by a modified procedure on the basis of the procedure reported previously (Lertwattanasakul *et al*. 2015). The RNA samples then were subjected to RNase-free DNase treatment. All RNA samples were purified by using an RNeasy plus mini kit (QIAGEN, Hilden, Germany) according to the protocol provided by the supplier.

The purified RNA samples were analyzed on an Illumina NextSeq at the Research Center of Yamaguchi University. The detailed procedure for RNA-Seq has been described previously (Kim *et al*. 2013). The sequencing results were analyzed using CLC Genomic Workbench version 10.1.1. All mapped reads at exons were counted, and the numbers were converted to unique exon reads. The unique exon reads (15,531,789 and 20,065,386) from two biological replicates of X-17.2b were compared to those (17,191,058 and 20,374,059) of the parental strain. Accession numbers of sequence data are DRR324097 and DRR324098 for X-17 and DRR324099 and DRR324100 for X-17.2b.

Gene expression profiles of X-17.2b and the parental strain were compared to find differentially expressed genes (DEGs) based on unique exon read values from CLC genomic workbench outputs using DESeq2 R package (Andars *et al.* 2010). The resulting *P*-values were adjusted using Benjamin-Hochberg's method for controlling the false discovery rate. Genes with adjusted *P*-values less than 0.01 ($P_{adj} < 0.01$) and $\log_2$ (fold change) values greater than 2 or lower than -2 were assigned as significant DEGs.

Functions of significantly up-regulated or down-regulated genes were searched by BLAST and Uni-Prot.

2.3.8 Hydropathy analysis

Hydropathy analysis was performed by using the algorithm of Kyte and Doolittle (Kyte *et al*. 1982) with the normalized consensus hydrophobicity value of Eisenberg (Eisenberg *et al*. 1984) and a window of 21 amino acids. The predicted amino acid sequences of putative transporters were plotted using the ExPASy-Expasy website (https://web.expasy.org/protscale/, 10 December 2021). Sequences with a hydrophobicity value of more than 0.42 were identified as membrane-spanning segments.

2.4 Results

2.4.1 Evolutionary adaptation of *C. tropicalis* X-17 by RLCGT

In order to improve the sensitivity of *C. tropicalis* X-17 to high glucose concentrations, we applied the RLCGT established previously to acquire multi-stress tolerance with the modification of addition of a high concentration of glucose; that is, the strain was subjected to RLCGT in the presence of 200 $g \cdot L^{-1}$ glucose under a shaking condition (Figure S1). The treatment was initiated at 40 ˚C after cells had been inoculated into five different test tubes, and cell survival was observed by monitoring the optical density at 660 nm (OD_{660}). Three of the five tubes showed no increase in OD_{660} at 43.5 ˚C and one tube showed no increase at 44.5 ˚C. Cells in the remaining tube after incubation at 44.5 ˚C three times were spread on an agar plate, and one larger colony named X-17.2b was selected. X-17.2b was then used as an adapted strain in the following experiments.

2.4.2 Effects of various stresses on growth of X-17.2b

During RLCGT, yeast cells were expected to be exposed to various stresses as in a general fermentation process (Gibson *et al*. 2007; Zhao *et al*. 2009; Puligundla *et al*. 2011; Auesukaree 2017). Effects of various stresses on growth of X-17.2b were therefore examined by spot tests (Figure 2.1).

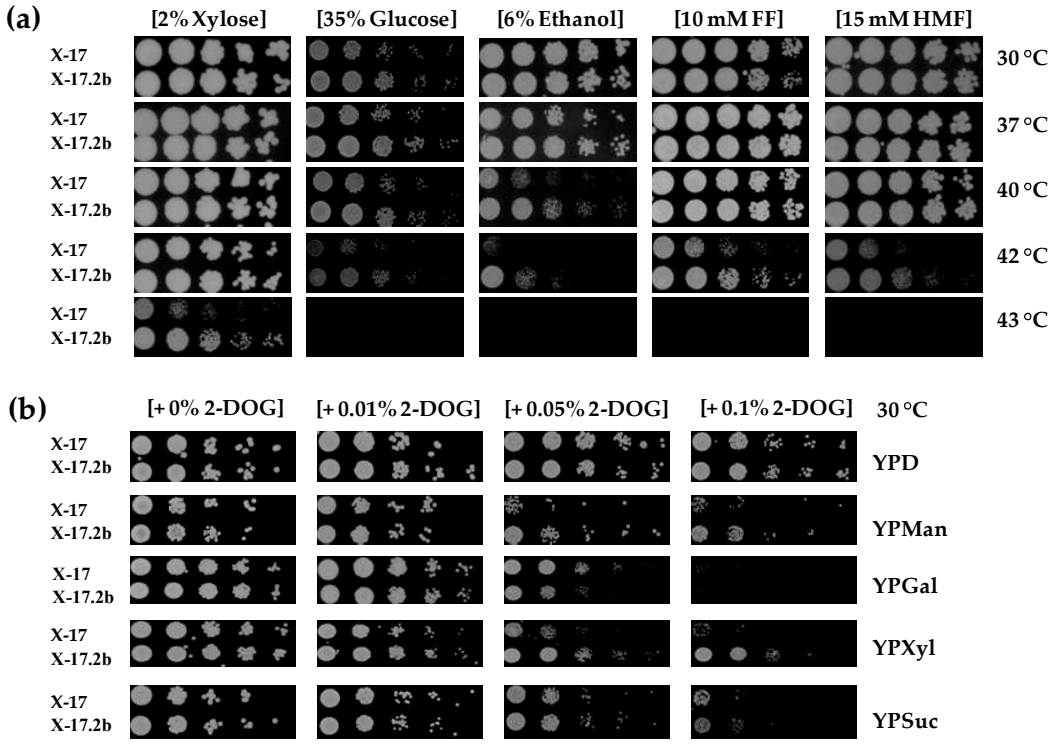

Figure 2.1 Stress resistance and effect of 2-DOG on sugar utilization of *C. tropiclis* X-17 and adapted strain *C. tropiclis* X-17.2b. Serially diluted cells were spotted onto **(a)** YP2X, YP35D and YP2X agar plates supplemented with 6% ethanol, 10 mM furfural or 15 mM HMF **(b)** YPD, YPMan, YPGal, YPXyl and YPSuc agar plates supplemented with or without 0.01%, 0.05% or 0.1% 2-DOG. The plates were incubated at **(a)** at 30 ˚C, 37 ˚C, 40 ˚C, 42 ˚C and 43 ˚C and **(b)** at 30 ˚C for 48 h.

Pre-cultures were serially diluted and spotted onto YP agar plates supplemented with 35% glucose, 2% xylose, 6% ethanol, 10 mM furfural or 10 mM hydroxymethylfurfural and incubated at five different temperatures for 48 h. When compared with X-17 as the parental strain, X-17.2b showed better growth after 48-h incubation at 40 ˚C, 42 ˚C or 43 ˚C (Figure 2.1a). Similarly, X-17.2b showed better growth than that of the parental strain on various sugar-containing plates supplemented with 0.05% or 0.1% 2-DOG except for galactose-containing plates (Figure 2.1b), indicating that X-17.2b may exhibit a relatively weak glucose repression in cases of mannose, xylose and sucrose. These findings suggest that the adapted strain had resistance to various stresses at high temperatures.

2.4.3 Ethanol fermentation ability of X-17.2b

Efficient ethanol fermentation with high concentrations of sugars at high temperatures is crucial for industrial applications. The ethanol fermentation ability of X-17.2b was therefore examined in the presence of high concentrations of glucose at high temperatures (Figure 2.2). Pre-culture was inoculated into YP medium containing 160 g·L^{-1} glucose and cultured at 37 ˚C, 40 ˚C and 42 ˚C for 72 h, and fermentation parameters were compared with those of the parental strain and *K. marxianus* DMKU 3-1042, which is an efficient fermenting yeast at high temperatures (Rodrussamee *et al*. 2011).

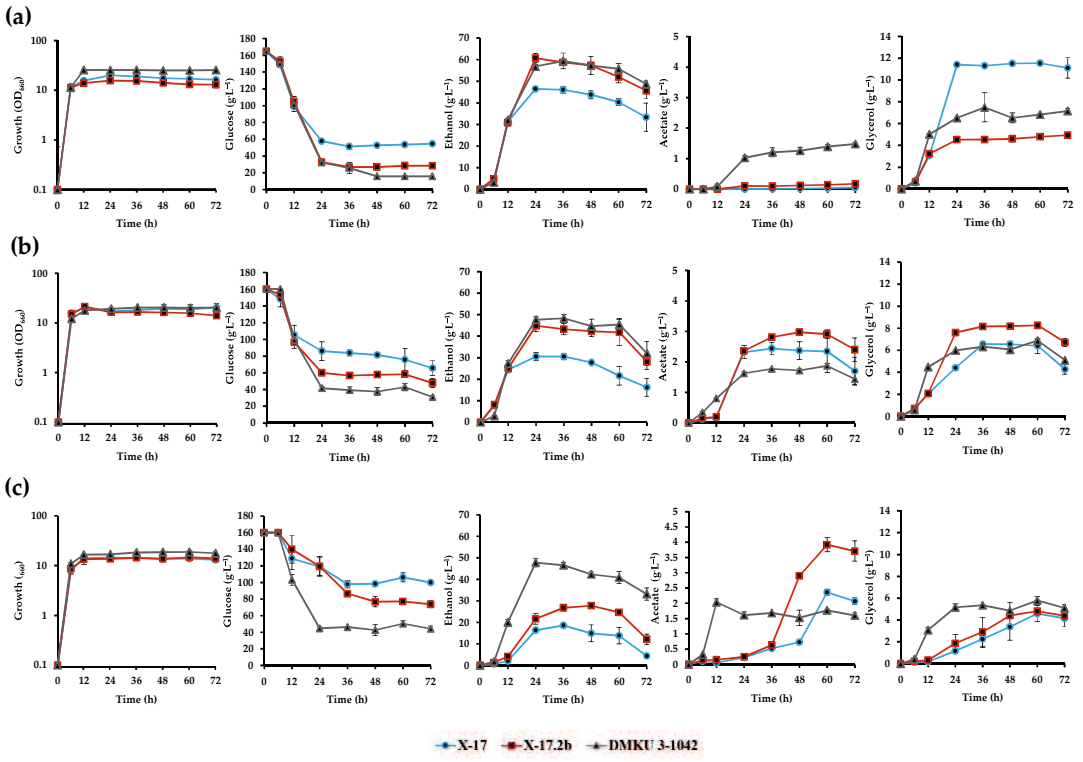

Figure 2.2 Growth and metabolite profiles of *C. tropiclis* X-17 (*filled circles*), adapted strain *C.tropicalis* X-17.2b (*filled squares*) and *K. marxianus* DMKU 3-1042 (*filled triangles*) in high concentration of glucose. Cells are grown in YP media containing 160 g·L^{-1} glucose at **(a)** 37 ˚C, **(b)** 40 ˚C and **(c)** 42 ˚C under a shaking condition at 100 rpm, and samples were taken at 6 h and every 12 h until 72 h of incubation. Bars represent the ±SD of values from experiments performed in triplicate.

At 37 ˚C, X-17.2b showed levels of glucose consumption and ethanol production similar to those of DMKU 3-1042 and much higher than those of the parental strain. Maximum ethanol concentrations of X-17.2b, X-17 and DMKU 3-1042 were 60.7 $g \cdot L^{-1}$, 46.5 $g \cdot L^{-1}$ and 56.9 $g \cdot L^{-1}$, respectively. Interestingly, X-17.2b and X-17 produced almost no acetate and X-17.2b showed much lower glycerol accumulation than that of the parental strain. With increases in temperature, 40 ˚C and 42 ˚C, the glucose consumption and ethanol production levels of X-17.2b were lower than those of DMKU 3-1042 but still higher than those of the parental strain. Maximum ethanol concentrations of X-17.2b, X-17 and DMKU 3-1042 were 44.9 $g \cdot L^{-1}$, 30.5 $g \cdot L^{-1}$ and 47.6 $g \cdot L^{-1}$, respectively, at 40 ˚C and 27.8 $g \cdot L^{-1}$, 14.9 $g \cdot L^{-1}$ and 42.3 $g \cdot L^{-1}$, respectively, at 42 ˚C. The acetate levels of X-17.2b were higher than those of the parental strain, and the acetate production of X-17.2b and X-17 was delayed compared to that of DMKU 3-1042. These results suggest that the adapted strain can achieve efficient fermentation with high concentrations of glucose at high temperatures.

Next, fermentation ability with 50 $g \cdot L^{-1}$ xylose of X-17.2b was examined at 35 ˚C, 37 ˚C and 42 ˚C (Figure 2.3). The adapted strain showed higher ethanol concentrations at 36 h to 60 h than those of the parental strain, and both strains accumulated large amounts of xylitol. Maximum ethanol concentrations of X-17.2b at 35 ˚C, 37 ˚C and 42 ˚C were 5.34 $g \cdot L^{-1}$, 4.40 $g \cdot L^{-1}$ and 3.0 $g \cdot L^{-1}$, respectively. Notably, *C. tropicalis* X-17 and its adapted strain consumed xylose and produced ethanol faster and in larger amounts than did *K. marxianus* DMKU 3-1042 and accumulated much lower concentrations of acetate at 35 ˚C and 37 ˚C (Figure 2.3a, b). X-17.2b consumed xylose and produced a larger amount of ethanol than did X-17 even at 42 ˚C (Figure 2.3c). Therefore, it is likely that the adapted strain has enhanced xylose-fermentation ability.

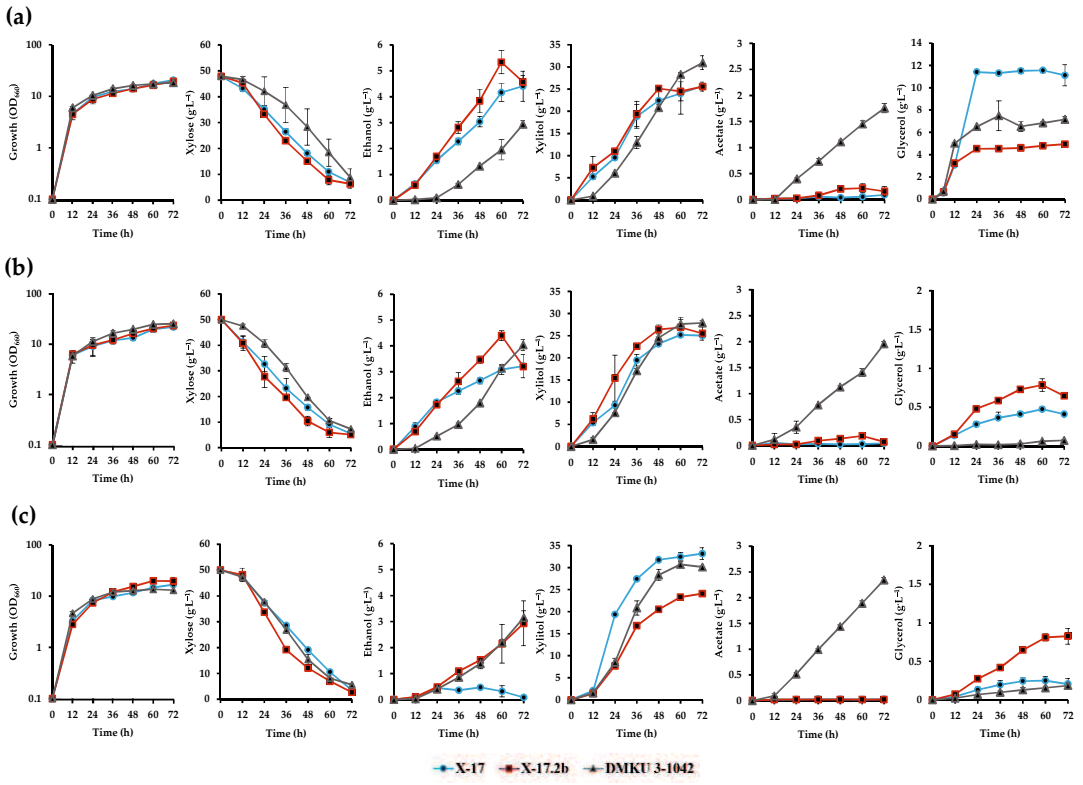

Figure 2.3 Growth and metabolite profiles of *C. tropiclis* X-17 (*filled circles*), adapted strain *C. tropiclis* X-17.2b (*filled squares*) and *K. marxianus* DMKU 3-1042 (*filled triangles*) in high concentration of xylose. Cells were grown in YP media containing 50 g·L^{-1} xylose at **(a)** 35 ˚C, **(b)** 37 ˚C and **(c)** 42 ˚C under a shaking condition at 100 rpm, and samples were taken every 12 h until 72 h of incubation. Bars represent the ±SD of values from experiments performed in triplicate.

Fermentation with mixed sugars of xylose and glucose was also carried out at 37 ˚C (Figure 2.4), considering the optimum temperatures for ethanol production in the case of glucose and xylose (Figures 2.2 and 2.3). In the case of 20 g·L^{-1} xylose and 2 g·L^{-1} glucose, X-17.2b and X-17 demonstrated patterns of xylose utilization that were similar to and ethanol production levels that were higher than those in the case of only 20 g·L^{-1} xylose, and they demonstrated maximum ethanol concentrations of 3.77 g·L^{-1} and 2.82 g·L^{-1}, respectively (Figure 2.4a, b). On the other hand, DMKU 3-1042 produced a lower concentration of ethanol and a higher concentration of xylitol than those in the case of only 20 g·L^{-1} xylose. In the case of 20 g·L^{-1} xylose and 20 g·L^{-1} glucose, X-17.2b and X-17 demonstrated slightly delayed xylose utilization and

DMKU 3-1042 demonstrated greatly reduced xylose utilization compared to those in the case of only 20 $g \cdot L^{-1}$ xylose (Figure 2.4a, c). Both strains demonstrated faster glucose consumption and higher level of ethanol production than those of DMKU 3-1042. DMKU 3-1042 appeared to quickly convert ethanol to acetate after 12 h. Maximum ethanol concentrations of X-17.2b, X-17 and DMKU 3-1042 were 10.72 $g \cdot L^{-1}$, 10 $g \cdot L^{-1}$ and 9.48 $g \cdot L^{-1}$, respectively. These results suggest that both of the *C. tropicalis* strains have a resistant xylose metabolism to glucose repression compared to that of *K. marxianus* DMKU 3-1042 and are suitable for ethanol fermentation with lignocellulosic biomass at high temperatures.

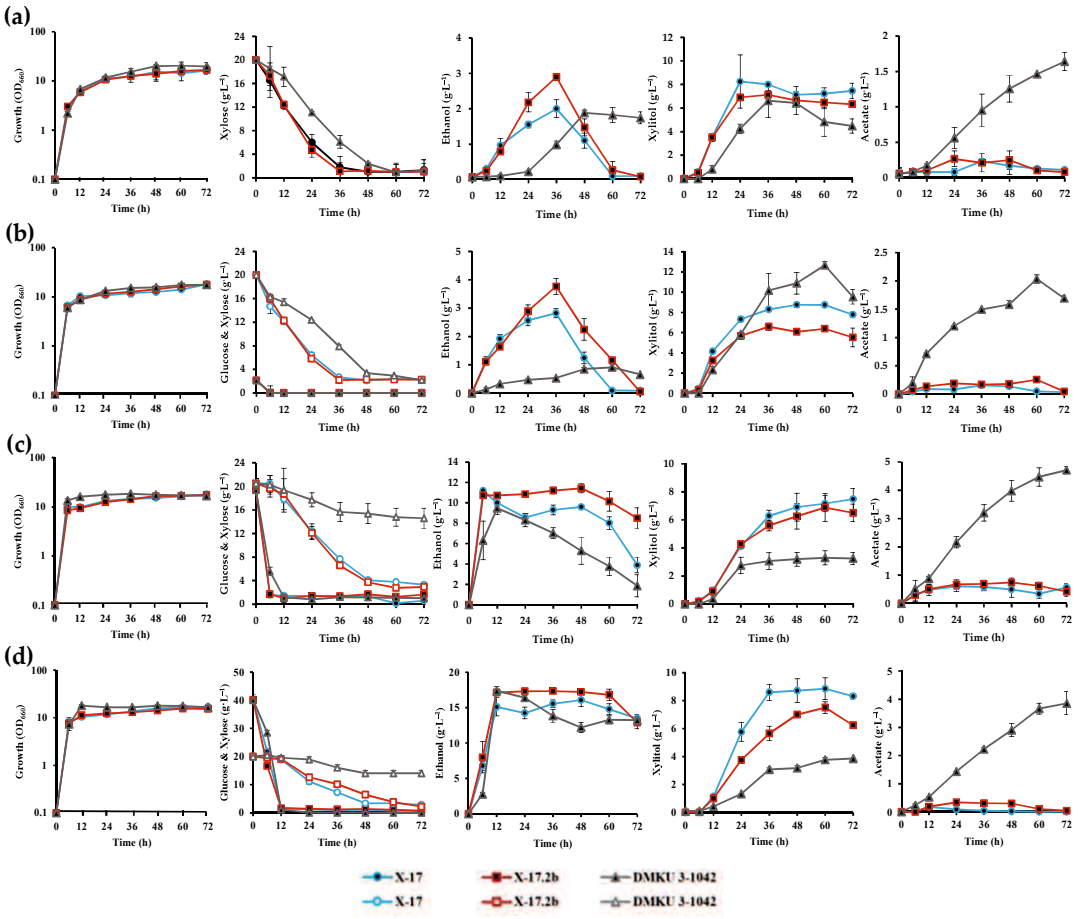

Figure 2.4 Growth and metabolite profiles of *C. tropiclis* X-17 (*filled circles* or *open circles*), adapted strain *C. tropiclis* X-17.2b (*filled squares* or *open squares*) and *K. marxianus* DMKU 3-1042 (*filled triangles* or *open triangles*) in mixed sugars. Cells were grown in YP media containing **(a)** 20 $g \cdot L^{-1}$ xylose and a mixed sugar medium containing **(b)** 2 $g \cdot L^{-1}$ glucose + 20 $g \cdot L^{-1}$ xylose or **(c)** 20 $g \cdot L^{-1}$ glucose

+ 20 g·L^{-1} xylose or **(d)** 40 g·L^{-1} glucose + 20 g·L^{-1} xylose at 37 ˚C under a shaking condition at 100 rpm, and samples were taken at 6 h and every 12 h until 72 h of incubation. In the Glucose & Xylose panels, filled symbols represent glucose consumption and opened symbols represent xylose consumption. Bars represent the ±SD of values from experiments performed in triplicate.

Moreover, the 2:1 ratio of glucose and xylose was examined, on the basis of their existence in lignocellulosic biomass after complete hydrolysis (Guragain *et al*. 2017; Ji *et al*. 2009; Yan *et al*. 2009; Stephanopoulos 2007). When cells were grown in a medium containing mixed sugars of 40 g·L^{-1} glucose and 20 g·L^{-1} xylose (Figure 2.4d), the consumption of glucose and xylose by X-17.2b was almost the same as that by X-17, and X-17.2b and DMKU 3-1042 produced a larger amount of ethanol than that produced by X-17 at 12 h and 24 h, while the xylitol accumulation in X-17.2b was lower than that in X-17. However, both *C. tropicalis* strains showed weak glucose repression on xylose utilization compared to DMKU 3-1042. Therefore, these results suggest that adapted X-17.2b is superior to the parent strain in ethanol production with both a single sugar and mixed sugars and that X-17.2b may have a great potential for lignocellulosic biomass fermentation.

2.4.4 Transcriptome analysis

Genome analysis using the Illumina NextSeq 500 platform was performed, but mutation points in X-17.2b were unable to be identified because more than 98,000 putative mutation points were found probably due to the lack of the complete genome sequence of the parent, although the genome data of X-17.2b and the parent were individually compared to those of *C. tropicalis* MYA-3404 in the NCBI database. We thus carried out transcriptome analysis of X-17.2b and the parental strain as a control by RNA-Seq using total RNAs prepared from cells grown in YP16D medium at 37 ˚C for 12 h, which was the glucose-consuming phase before a slow-down of glucose consumption (see Figure 2.2a). Reads per kilobase of exon per million (RPKM) of each gene were estimated as a transcript abundance. The difference of each gene in X-17.2b from that in the parent was reflected as the ratio of the RPKM value in X-17.2b to that in the parent. To further explore the transcriptional changes, analysis of

differentially expressed genes (DEGs) based on the RNA-Seq data was conducted. DEGs showed significant changes at the transcription level with $\log_2$ (fold change) > 2 and $\log_2$ (fold change) < -2. Sixty six genes were significantly up-regulated and 18 genes were significantly down-regulated in X-17.2b (Table 2.1, S1 and Figure S3). KEGG enrichment analysis and GO function enrichment analysis were performed, but no notable results were obtained.

Table 2.1 Significantly up-regulated genes ($\log_2$ > 2) in the adapted strain

Name	$\log_2$ Fold Change	Product
CTRG_06057	4.95	Hypothetical protein
CTRG_06055	4.07	Hypothetical protein
CTRG_06056	3.84	Hypothetical protein
CTRG_06100	3.63	Maltose permease
CTRG_06311	3.31	tRNA
CTRG_00691	3.31	Hypothetical protein
CTRG_04447	3.23	Hypothetical protein
CTRG_02794	3.12	Hypothetical protein
CTRG_03584	3.08	Opaque-phase-specific protein OP4 precursor
CTRG_06401	3.06	tRNA
CTRG_02358	3.05	Resistance to glucose repression protein 1
CTRG_00749	3.02	Hypothetical protein
CTRG_03295	2.86	Hypothetical protein
CTRG_06346	2.82	tRNA
CTRG_05401	2.77	Ornitine carbamoyltransferase
CTRG_05272	2.73	Aga1p
CTRG_00349	2.71	Cell wall protein RHD3
CTRG_05402	2.61	Methylglyoxal reductase (NADPH-dependent)
CTRG_00711	2.60	White-opaque regulator 3
CTRG_01483	2.57	Hypothetical protein
CTRG_05080	2.56	Hypothetical protein
CTRG_04732	2.55	Histone H3
CTRG_01407	2.55	Hypothetical protein
CTRG_00808	2.54	Hypothetical protein
CTRG_06299	2.54	tRNA
CTRG_05490	2.52	Hypothetical protein

CTRG_06416	2.51	tRNA
CTRG_03294	2.51	Hypothetical protein
CTRG_04755	2.51	Hypothetical protein
CTRG_06250	2.50	Glucose transporter of major facilitator superfamily
CTRG_02210	2.42	Acetylornithine aminotransferase, mitochondrial precursor
CTRG_03885	2.29	Lipase 8
CTRG_00519	2.28	Hypothetical protein
CTRG_00291	2.28	Hypothetical protein
CTRG_03791	2.27	Hypothetical protein
CTRG_00298	2.27	Hypothetical protein
CTRG_05031	2.25	Hypothetical protein
CTRG_00623	2.24	Hypothetical protein
CTRG_02946	2.24	Peroxiredoxin HYR1
CTRG_06103	2.23	Hypothetical protein
CTRG_00350	2.22	Cell wall protein PGA31
CTRG_06301	2.20	tRNA
CTRG_05078	2.17	Hypothetical protein
CTRG_03785	2.16	Cell wall protein PGA31
CTRG_00604	2.15	Hypothetical protein
CTRG_01139	2.14	Hypothetical protein
CTRG_02833	2.12	Vacuolar basic amino acid transporter 5
CTRG_00842	2.11	Peroxisomal membrane protein LPX1
CTRG_00266	2.10	Hypothetical protein
CTRG_00233	2.10	Hypothetical protein
CTRG_02278	2.10	Thiol-specific monooxygenase
CTRG_01965	2.10	Hypothetical protein
CTRG_01779	2.09	4-hydroxyphenylpyruvate dioxygenase
CTRG_04145	2.07	Hypothetical protein
CTRG_00102	2.07	Hypothetical protein
CTRG_03730	2.07	NAG4
CTRG_06102	2.07	Hypothetical protein
CTRG_02773	2.06	Hypothetical protein
CTRG_03597	2.05	Hypothetical protein
CTRG_06383	2.05	tRNA
CTRG_05709	2.04	Carboxylic acid transporter
CTRG_00500	2.03	Hypothetical protein
CTRG_06026	2.02	Hypothetical protein
CTRG_00590	2.02	Stress response regulator protein 1

CTRG_04524	2.01	Hypothetical protein
CTRG_06404	2.01	tRNA

In order to understand the function of significantly up-regulated gene products, BLAST searching was performed, and the top ranked proteins in *C. tropicalis*, *Candida albicans*, *Candida dubiniensis* and *Spathaora passalidarum* were listed in Table 2.1. These proteins were found to be classified as follows: (1) glucose uptake and regulation, including a glucose transporter and negative regulator of glucose repression, (2) stress response, including stress response regulator protein 1, peroxiredoxin HYR1 and NADPH-dependent methylglyoxal reductase, (3) urea cycle and its association, including ornitine carbamolyltransferase and acetylornithine aminotransferease, (4) cell wall proteins, including RHD3, PGA31 encoded by *CTRG_00350*, PGA31 encoded by *CTRG_03785* and AGA1, (5) amino acid degradation, including vacuolar basic amino acid transporter 5 and 4-hydroxyphenylpyruvate dioxygenase, (6) other transporters, including multidrug transporter (NAG4) and carboxylic acid transporter, (7) lipase, including lipase 8 and peroxisomal membrane protein LPX1 and (8) transcriptional regulator, including white-opaque regulator 3. The up-regulation of a gene for the glucose transporter of the major facilitator superfamily (MFS) may be related to the phenotype of improved utilization capability of high concentrations of glucose (Figure 2.1). On the other hand, there were 36 genes for hypothetical proteins. To examine whether there were additional MFS members, hydropathy analysis was performed (Figure S2). The analysis revealed that three proteins in those hypothetical proteins might be integral membrane proteins with several possible membrane-spanning segments. The up-regulation of genes for stress response or cell wall proteins may contribute to the multistress tolerance of the adapted strain.

Significantly down-regulated genes included genes for oxidoreductase, fructose-bisphosphate aldolase, RNA polymerase II transcription factor B subunit 5, transporter protein SMF1/ESP1, meiotic sister chromatid recombination protein 1, putative diacetyl reductase 2, NAD-dependent alcohol dehydrogenase, glutathione S-transferase 1, respiratory supercomplex factor 2 and sodium transport ATPase 2 (Table

S1). The down-regulation of genes for RNA polymerase II transcription factor B subunit 5 or respiratory supercomplex factor 2 might reduce transcription and respiratory activity, saving cellular energy for use in stress tolerance.

2.5 Discussion

Generally, *C. tropicalis* is superior to *K. marxianus* in the ability to convert xylose to ethanol but inferior in the fermentation ability at high glucose concentrations. We thus attempted to improve the inferior property of *C. tropicalis*. Thermotolerant *C. tropicalis* X-17, which was isolated via non-enrichment culture at 37 ˚C, was subjected to RLCGT for adaptive evolution under a high glucose concentration condition. RLCGT exposes cells to various stresses such as stresses from metabolites including ethanol or organic acids, by-products formed by chemical reactions, nutrient starvation, high temperatures and oxidative stress in addition to large changes in substrate sugar concentration, and it has been shown to be a simple and efficient procedure for the development of robust strains (Pattanakittivorakul *et al*. submitted). In this study, we first performed RLCGT in the presence of a high glucose concentration and successfully obtained a strain with improved fermentation ability at high glucose concentrations from cultivation of five different test tubes. Therefore, it is likely that RLCGT is an effective evolutionary adaptation procedure and can be modified with additional factors, such as high sugar concentrations.

X-17.2b, obtained as an adapted strain, was found to have several beneficial properties, including improved tolerance to ethanol, furfural and hydroxymethylfurfural at high temperatures, and enhanced ability for fermentation of high concentrations of glucose and ability for fermentation of xylose (Figures 2.1 and 2.3). The improvement in fermentation capacity was very remarkable. In comparison with the data for the parent, glucose consumption was increased by 15.6% and ethanol production increased by 30.7% when cultured in YP16D at 37 ˚C and ethanol production was increased by 44.9% when cultured in YP2X at 37 ˚C. Transcriptome analysis provided clues for understanding the mechanisms of these properties. Up-regulation of a gene for a glucose transporter may be responsible for increasing glucose consumption ability of the adapted strain. It is possible that some of the putative

membrane proteins derived from up-regulated genes also act as glucose transporters or support glucose uptake. Several up-regulated genes for stress response and cell wall proteins may be related to enhanced multistress tolerance of the adapted strain. In addition, it is assumed that up-regulation of genes for amino acid degradation and urea cycle and its association may provide energy and that down-regulation of RNA polymerase II transcription factor B subunit 5 or respiratory supercomplex factor 2 may save energy. On the other hand, most of the up-regulated gene products, except for unknown proteins, can be divided to eight groups (transporter, stress response, cell wall protein, transcriptional regulator, urea cycle, lipase, amino acid metabolism and others) and the finding that each group consisted of two or three members suggests that specific transcriptional factors regulate the expression of these genes.

The fermentation capacity of the adapted strain appears to be superior to those of other *C. tropicalis* strains, including its parental strain. In a medium containing 160 $g \cdot L^{-1}$ glucose, *C. tropicalis* X-17, X-17.2b and DMKU 3-1042 produced ethanol at 1.94 $g \cdot L^{-1} \cdot h^{-1}$, 2.53 $g \cdot L^{-1} \cdot h^{-1}$ and 2.37 $g \cdot L^{-1} \cdot h^{-1}$, respectively (Table 2.2).

Table 2.2 Summary of fermentation abilities of *C. tropicalis* X-17, X-17.2b and *K. marxianus* DMKU 3-1042 and comparison with those of other *C. tropicalis* strains.

Strains	Temp. (°C)	Sugars Conc. ($g \cdot L^{-1}$)	Time (h)	Sugars Consumption ($g \cdot L^{-1}$)	Ethanol Production ($g \cdot L^{-1}$)	Xylitol Production ($g \cdot L^{-1}$)	Glycerol Production ($g \cdot L^{-1}$)	Acetic Acid Production ($g \cdot L^{-1}$)	Ethanol Yield ($g \cdot g^{-1}$)	Ethanol Productivity ($g \cdot L^{-1} \cdot h^{-1}$)	Reference
C. tropicalis X-17	37	Glc 20	6	Glc 15.6 ± 2.9	8.2 ± 0.6	–	–	0.0 ± 0.0	0.4 ± 0.0	1.37 ± 0.1	This study
	37	Glc 160	24	Glc 107.2 ± 1.3	46.5 ± 0.7	–	11.4 ± 0.2	0.0 ± 0.0	0.3 ± 0.0	1.94 ± 0.0	This study
	37	Xyl 20	36	Xyl 17.1 ± 3.1	2.0 ± 0.3	8.0 ± 0.2	–	0.2 ± 0.0	0.1 ± 0.0	0.06 ± 0.0	This study
	37	Xyl 50	60	Xyl 37.9 ± 1.6	3.1 ± 0.1	25.2 ± 0.1	0.5 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.05 ± 0.0	This study
	37	Glc 2 + Xyl 20	36	Glc 2.1 ± 0.0 Xly 15.6 ± 0.1	2.8 ± 0.2	8.3 ± 0.2	–	0.2 ± 0.0	0.1 ± 0.0	0.08 ± 0.0	This study
	37	Glc 20 + Xyl 20	60	Glc 19.2 ± 2.0 Xyl 16.7 ± 1.1	8.0 ± 0.6	7.2 ± 0.6	–	0.3 ± 0.2	0.2 ± 0.0	0.13 ± 0.0	This study
	37	Glc 20 + Xyl 50	72	Glc 21.6 ± 1.7 Xyl 45.1 ± 1.0	10.6 ± 0.7	30.3 ± 5.9	0.4 ± 0.2	0.1 ± 0.1	0.1 ± 0.0	0.15 ± 0.0	This study
	35	Glc 50 + Xyl 50	72	Glc 50.0 ± 0.0 Xyl 39.1 ± 0.0	25.2 ± 0.0	20.0 ± 0.0	–	0.1 ± 0.0	0.2 ± 0.0	0.42 ± 0.0	This study
C. tropicalis X-17.2b	37	Glc 20	6	Glc 16.2 ± 4.2	8.1 ± 0.9	–	–	0.0 ± 0.0	0.4 ± 0.0	1.35 ± 0.1	This study
	37	Glc 160	24	Glc 132.2 ± 1.4	60.7 ± 2.1	–	4.5 ± 0.1	0.1 ± 0.0	0.4 ± 0.0	2.53 ± 0.1	This study
	37	Xyl 20	36	Xyl 17.8 ± 2.7	2.9 ± 0.1	7.1 ± 0.4	–	0.2 ± 0.1	0.1 ± 0.0	0.08 ± 0.0	This study
	37	Xyl 50	60	Xyl 41.1 ± 1.4	4.4 ± 0.1	26.9 ± 1.5	0.8 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.07 ± 0.0	This study
	37	Glc 2 + Xyl 20	36	Glc 2.1 ± 0.0	3.8 ± 0.3	6.6 ± 0.4	–	0.2 ± 0.0	0.2 ± 0.0	0.10 ± 0.0	This study

				Xyl 16.0 ± 0.1							
	37	Glc 20 + Xyl 20	60	Glc 18.1 ± 1.9 Xyl 17.8 ± 1.2	10.1 ± 0.9	6.9 ± 1.0	–	0.6 ± 0.1	0.3 ± 0.0	0.17 ± 0.0	This study
	37	Glc 20 + Xyl 50	72	Glc 20.8 ± 0.7 Xyl 45.7 ± 1.0	13.6 ± 0.5	25.4 ± 2.3	0.4 ± 0.3	0.7 ± 0.7	0.2 ± 0.0	0.19 ± 0.0	This study
	35	Glc 50 + Xyl 50	72	Glc 50.00 ± 0.0 Xyl 40.6 ± 0.0	24.6 ± 0.9	22.7 ± 0.0	–	0.2 ± 0.0	0.2 ± 0.0	0.40 ± 0.0	This study
K. marxianus DMKU 3-1042	37	Glc 20	6	Glc 6.6 ± 3.2	3.9 ± 0.7	–	–	0.4 ± 0.0	0.2 ± 0.0	0.65 ± 0.1	This study
	37	Glc 160	24	Glc 132.4 ± 1.2	56.8 ± 0.8	–	6.5 ± 0.0	1.0 ± 0.1	0.3 ± 0.0	2.37 ± 0.0	This study
	37	Xyl 20	36	Xyl 12.8 ± 2.3	1.0 ± 0.1	6.6 ± 1.4	–	0.9 ± 0.2	0.1 ± 0.0	0.03 ± 0.0	This study
	37	Xyl 50	60	Xly 36.3 ± 0.4	3.1 ± 0.1	27.6 ± 0.7	0.1 ± 0.0	1.4 ± 0.0	0.1 ± 0.0	0.05 ± 0.0	This study
	37	Glc 2 + Xyl 20	36	Glc 2.1 ± 0.0 Xyl 10.3 ± 0.2	0.5 ± 0.0	10.2 ± 1.7	–	1.5 ± 0.0	0.0 ± 0.00	0.01 ± 0.0	This study
	37	Glc 20 + Xyl 20	60	Glc 18.3 ± 1.9 Xyl 5.7 ± 2.3	3.8 ± 0.9	3.3 ± 0.5	–	4.5 ± 0.3	0.1 ± 0.0	0.06 ± 0.0	This study
	37	Glc 20 + Xyl 50	72	Glc 22.0 ± 1.7 Xyl 15.5 ± 5.5	3.3 ± 1.5	3.5 ± 1.1	0.9 ± 0.1	4.6 ± 0.7	0.1 ± 0.02	0.05 ± 0.0	This study
	30	Xyl 20	48	Xyl 19.2 ± 1.09	1.7 ± 0.4	2.2 ± 0.5	NR	7.2 ± 0.4	0.1 ± 0.0	~0.03	Nitiyon *et al.* 2016
	30	Glc 20 : Xyl 20	60	Glc~0.0 Xyl~7	~8.0	~2.00	NR	NR	~0.20	~0.13	Nitiyon *et al.* 2016
C. tropicalis W103	35	Glc 50 + Xyl 50	75	Glc~50 Xyl~25	~20	NR	NR	NR	~0.2	~0.26	Cheng *et al*. 2014
C. tropicalis MTCC 25057	32	Glc 100	48	Glc~100	36	NR	NR	NR	~0.36	~0.75	Mattam *et al*. 2016

	32	Glc 50 + Xyl 50	24	Glc~50 Xyl~00	18.8 ± 0.8	NR	NR	NR	~0.2	~0.78	Mattam *et al*. 2016
C. tropicalis UFMGBX12-a	30	Glc 18 + Xyl 2	30	Glc~00 Xyl~0.7	1.5	12	NR	NR	0.1	0.05	Antunes *et al*. 2021
C. tropicalis CICC1779	34	Glc 30 + Xyl 30	72	Glc 27.5 Xyl 16.7	17.6	NR	NR	NR	~ 0.3	~ 0.24	Li *et al*. 2017
C. tropicalis M9	42	Xyl 50	168	NR	~ 12	NR	NR	NR	~ 0.2	~ 0.07	Nweze *et al*. 2019

NR not report. ± Standard deviation of values from experiments in triplicate.

The ethanol productivity of *C. tropicalis* X-17 and X-17.2b was much higher than that of *C. tropicalis* MTCC 25057, producing ethanol at 0.75 $g \cdot L^{-1} \cdot h^{-1}$ in a medium containing 100 $g \cdot L^{-1}$ glucose (Mattam *et al*. 2016). At 50 $g \cdot L^{-1}$ of xylose, ethanol productivity of *C. tropicalis* X-17.2b (0.07 $g \cdot L^{-1} \cdot h^{-1}$) was nearly the same as that of *C. tropicalis* M9 (Nweze *et al.* 2019). When glucose and xylose co-existed at the same concentration, *C. tropicalis* X-17 and X-17.2b consumed about 90% of xylose within 36-48 h and converted it to ethanol at 25.2 $g \cdot L^{-1}$ and 25.6 $g \cdot L^{-1}$, respectively, while *C. tropicalis* W103 and MTCC 25057 consumed about 50% (within 75 h) and 0% of xylose, respectively, and converted it to ethanol at 20 $g \cdot L^{-1}$ and 18.8 $g \cdot L^{-1}$, respectively (Cheng *et al*. 2014; Mattam *et al*. 2016). In connection with glucose repression on xylose utilization, *C. tropicalis* X-17 and X-17.2b consumed 77.8%-90.2% and 80.2%-91.4% of xylose, respectively, in media containing 20 $g \cdot L^{-1}$ or 50 $g \cdot L^{-1}$ xylose and 2 $g \cdot L^{-1}$ or 20 $g \cdot L^{-1}$ glucose (Table 2.2). *C. tropicalis* UFMGBX12 consumed 35% of xylose in a medium containing 2 $g \cdot L^{-1}$ xylose and 18 $g \cdot L^{-1}$ glucose (Antunes *et al*. 2021). *C. tropicalis* CICC1779 utilized 55.8% of xylose in a medium containing 30 $g \cdot L^{-1}$ xylose and 30 $g \cdot L^{-1}$ glucose (Li *et al*. 2017) and *K. marxianus* DMKU 3-1042 utilized 35% of xylose in a medium containing 20 $g \cdot L^{-1}$ xylose and 20 $g \cdot L^{-1}$ glucose (Nitiyon *et al*. 2016). Therefore, it is likely that *C. tropicalis* X-17 and X-17.2b are less sensitive in xylose utilization to glucose repression.

2.6 Conclusion

This study provided one adapted strain of *C. tropicalis* via RLCGT as an effective evolutionary adaptation procedure. The strain can ferment a high concentration of glucose more efficiently than can the parental strain or other *C. tropicalis* strains reported. The adapted strain gained multistress tolerance including efficient xylose utilization under glucose repression. These beneficial properties may be useful for industrial ethanol production using lignocellulosic biomass as a substrate.

CHAPTER 3

Highly efficient production of 2,3-butanediol from xylose and glucose by newly isolated thermotolerant *Cronobacter sakazakii*

3.1 Abstract

2,3-Butanediol (2,3-BD), a valuable compound used for chemicals, cosmetics, pesticides and pharmaceuticals, has been produced by various microbes. However, no high-temperature fermentation of the compound at high productivity has been reported. Thermotolerant xylose-utilizing microbes were isolated from 6 different districts in Laos and screened for a low accumulation of xylitol in a xylose medium at 37 ˚C. One isolate was found to produce 2,3-BD and identified by 16S rDNA sequencing. The 2,3-BD fermentation capacity was investigated at different temperatures using xylose and glucose as carbon sources, and the fermentation parameters were determined by a high-performance liquid chromatography system. By screening for a low accumulation of xylitol in a xylose medium, one isolate that accumulated almost no xylitol was obtained. Further analyses revealed that the isolate is *Cronobacter sakazakii* and that it has the ability to produce 2,3-BD at high temperatures. When xylose and glucose were used, this strain, named *C. sakazakii* OX-25, accumulated 2,3-BD in a short period before the complete consumption of these sugars and then appeared to convert 2,3-BD to acetoin. The optimum temperature of the 2,3-BD fermentation was 42 ˚C to 45 ˚C, and the maximum yields of 2,3-BD were 0.3 g/g at 12 h in 20 g/l xylose medium and 0.4 g/g at 6 h in 20 g/l glucose medium at 42 ˚C. The 2,3-BD productivity of the strain was higher than the 2,3-BD productivities of other non-genetically engineered microorganisms reported previously, and the highest productivity was 0.6 g/l·h and 1.2 g/l·h for xylose and glucose, respectively. Among thermotolerant microbes isolated in Laos, we discovered a strain, *C. sakazakii* OX-25, that can convert xylose and glucose to 2,3-BD with high efficiency and high productivity at high temperatures, suggesting that *C. sakazakii* OX-25 has the potential for industrial application to produce 2,3-BD as an important platform chemical.

3.2 Introduction

In recent years, attention has been given to the use of lignocellulosic biomass as a renewable source for biotechnology because of reduced petroleum resources, greenhouse gas emission risks and fluctuations in crude oil market prices (Phommachan *et al*. 2022; Nweze *et al*. 2019). Most petrochemically synthesized chemicals can be produced using microbial biocatalysts (Hazeena *et al*. 2020). Of the various microbial fermentation products, 2,3-butanediol (2,3-BD) is a valuable bulk chemical that is used in various application such as production of chemicals, cosmetics, pesticides, foods and pharmaceuticals (Hazeena *et al*. 2020; Qi *et al*. 2014; Xie *et al*. 2022; Yang *et al*. 2022; Amraoui *et al*. 2022; Mailaram *et al*. 2022). 2,3-BD is also a widely used antifreeze agent and a valuable fuel additive with a heating value of 27.2 kJ/g, comparable to those of methanol (22.1 kJ/g), n-butanol (33.1 kJ/g) and ethanol (29.1 kJ/g). Due to its high octane number, BD now functions as a gasoline octane booster (Amraoui *et al*. 2022; Mailaram *et al*. 2022; Białkowska *et al*. 2015; Celi´nska *et al*. 2009). Furthermore, 2,3-BD can be converted by dehydration to methyl-ethyl-ketone (MEK), a potent fuel additive with higher combustion values and industrial solvents, or to 1,3-butadiene, an important monomer in synthetic rubber production. On the other hand, acetoin, the precursor of or product from 2,3-BD, can be oxidized to diacetyl, which is used as a high-value flavoring of food that gives food a buttery taste and as a bacteriostatic additive for food processing (Hazeena *et al*. 2020; Białkowska *et al*. 2015). In the global market, 2,3 BD is expected to grow at a compound annual growth rate of 3% from 2019 to 2027, reaching US $ 220 million by 2027, and the current market price of 2,3-BD is higher than that of 1,4-butanediol (Yang *et al*. 2022; Maina *et al*. 2022; Tinco *et al* 2021). The downstream products of BD have the potential of a global market of around 32 million tons per annually, worth US $ 43 billion (Amraoui *et al*. 2022; Mailaram *et al*. 2022). Therefore, the production of bio-based organic chemicals such as BD represents the potential for renewable, sustainable energy for the planet.

In most studies on microbial production of 2,3-BD, food-based materials, including glucose and sucrose, have been used as feedstocks (Ji *et al*. 2011; Sheehan *et al*. 1999), which affect food security. Therefore, 2,3-BD production using lignocellulosic biomass is promising because of its abundant availability, no direct competition with the

food supply, and environmental benefits (Prado *et al*. 2016; Guragain *et al.* 2017). Since industrial production and use of 2,3-BD are limited by the high cost of its petro-based production, many 2,3-BD-producing microorganisms including bacteria and yeast have been isolated and metabolically engineered to improve 2,3-BD productivity (Xie *et al*. 2022; Song *et al*. 2019; Qin *et al*. 2014). Most microorganisms prefer glucose as a carbon source when a mixture of different sugars such as sucrose, glucose and fructose is applied (Yang *et al*. 2022; Jung *et al*. 2015). However, there have been a few studies on 2,3-BD production from xylose and responsible microorganisms. Moreover, although high-temperature fermentation is known to have several advantages for reduction of running cost in fermentation (Murata *et al*. 2015; Kosaka *et al*. 2018), most 2,3-BD production has been performed at 30 ˚C and 37 ˚C with xylose and glucose as carbon sources, respectively. In addition to negative effects of high temperature, inhibition of production generally occurs at high substrate concentrations (Song *et al*. 2019).

In this study, we found a strain that quickly utilized xylose but produced almost no xylitol in a screening process for efficient ethanol-producing microorganisms in a xylose medium. 16S rRNA sequencing revealed that the strain was *Cronobacter sakazakii*. The genus *Cronobacter* is generally positive for acetoin production (Voges-Proskauer test) and negative for the methyl red test indicating 2,3-BD rather than mixed acid fermentation (Iversen *et al*. 2007), but no further study on 2,3-BD production in *C. sakazakii* has been reported. The strain found in this study showed highly efficient production of 2,3-BD using xylose as well as glucose as a carbon source at high temperatures, indicating potential industrial applications.

3.3 Materials and methods

3.3.1 Strain, media and growth conditions

C. sakazakii OX-25 was isolated by enrichment culture at 37 ˚C from rotten grass in Laos and its isolation with other strains will be reported elsewhere. Cells were grown in YP medium (10 g/l yeast extract (Difco) and 20 g/l peptone (Difco) containing 20 g/l glucose (YP2D) or 20 g/l xylose (YP2X) at 37 ˚C, 40 ˚C or 42 ˚C under a shaking condition at 160 rpm. The thermotolerance was examined by checking the growth at 37

˚C, 40 ˚C, 42 ˚C and 45 ˚C on YPD or YPX plates as described previously (Keo-oudone *et al*. 2016).

3.3.2 Isolation of xylose-utilizing microorganisms

Xylose-utilizing microorganisms were isolated from various samples in six provinces in Laos (latitude and longitude is 18° 00'N and 105° 00' E): LuangPhrabang (lat 16° 27' 21" N; lng 108° 38'10" E), Oudomxay (lat 20° 30' 15" N; lng 101° 50' 22" E) and Xiengkhuang (lat 19° 37' 0" N; lng 103° 33' 0" E) that are located at the northern part of Laos; Vientiane (lat 18° 04' 05" N; lng 102° 40' 43" E) and Bolikhamxay (lat 18° 24' 2" N; lng 104° 14' 2" E) that are located at the central part; Champasak (lat 15° 3' 32" N; lng 106° 39' 11" E) that is located at the southern part of the country. The samples included vegetables, dried banana and papaya leaves, grass, rice straw, sawdust, coconut and other fruit shells, and rice starch waste of noodles. Isolation was carried out at 37 ˚C by enrichment cultures for all samples except for ripened fruits, which were subjected to non-enrichment cultures as described previously (Limtong *et al*. 2007; Keo-oudone *et al*. 2016). In the enrichment cultures, samples (5 to 10 g) were pressed into small pieces and transferred into 100-ml Erlenmeyer flasks containing 25 ml of YPD medium and then incubated at 37 ˚C for 24-48 h under a shaking condition at 160 rpm. In the non-enrichment cultures, samples (5 to 10 g) of ripened fruits were pressed into small pieces and transferred into small glass bottles. Distilled water was then added, and the bottles were covered with aluminum foil and incubated at 37 ˚C for 24-48 h. Both of the cultures were then streaked on YPX agar plates and incubated at 37 ˚C for 24 to 48 h, and single colonies were obtained for further experiments.

3.3.3 Analysis of 2,3-BD fermentation

To examine growth and fermentation parameters, YP medium supplemented with glucose at 20 g/l (YP2D), 40 g/l (YP4D), 60 g/l (YP6D) or 80 g/l (YP8D), YP medium supplemented with xylose at 20 g/l (YP2X), 40 g/l (YP4X), 60 g/l (YP6X) or 80 g/l (YP8X) and YP medium supplemented with 20 g/l glucose + 20 g/l xylose (YP2D2X), 40 g/l glucose + 20 g/l xylose (YP4D2X) or 60 g/l glucose + 20 g/l xylose (YP6D2X) were used. Strains were pre-cultured in YPD medium at 30 ˚C under a shaking condition at 160 rpm for 18 h. The pre-culture was inoculated into a 100-ml flask containing 30 ml

of liquid medium at an optical density (OD_{600}) of 0.1, followed by incubation at temperatures of 37 ˚C, 40 ˚C, 42 ˚C and 45 ˚C. Cell density was determined by measurement on a UV-VIS spectrophotometer (Shimadzu, Japan). Fermentation parameters were analyzed by a high-performance liquid chromatography (HPLC) system (Hitachi, Japan), as described previously (Rodrussamee *et al*. 2011; Nurcholis *et al*. 2019), consisting of a Hitachi Model D-2000 Elite HPLC system Manager, L-2130 column oven, L-2130 pump, L-2200 auto-sampler and L-2490 RI detector equipped with a GL-C610H-S gel pack column at 60 ˚C with 0.5 mL/min eluent of 0.1% phosphoric acid. Authentic 2,3-BD (Wako, Japan) and acetoin (TCI, Japan) were used as controls.

3.3.4 Identification of selected strains

Isolation of genomic DNA and amplification and determination of 16S rDNA were performed according to the methods of Green and Sambrook (Green M *et al*. 2012). The region of 16S rDNA was amplified by PCR using Ex Taq polymerase (Takara, Japan) and primers that are generally utilized: rDNA forward, 5'-AGAGTTTGATCCTG GCTCAG-3' and reverse, 5'-GGTTACCTTGTTACGACTT-3', generating a 0.8-kb band in 1.6% agarose gel (Hiraishi 1992). The band was extracted by using a QIAquick gel extraction kit and subjected to DNA sequencing by the Sanger method (Sanger *et al*. 1977).

3.4 Results

3.4.1 Isolation of xylose-utilizing microbes

About 300 samples that were collected in six provinces of Lao PDR were subjected to enrichment culture at 37 ˚C in a YP2X medium, and the growth of isolated colonies was examined at 37 ˚C, 40 ˚C, 42 ˚C and 45 ˚C on YP2X or YP2D plates. Thirty isolates that were able to grow on YP2X plates at temperatures higher than 40 ˚C were further examined for xylitol accumulation in a test tube containing 2 mL of YP2X liquid medium at 37 ˚C under shaking condition at 100 rpm (Figure S4). Among almost no xylitol-accumulating strains, one strain was found to exhibit a unique profile of HPLC, and further analysis using authentic compounds as controls revealed that the large peaks at retention times of 22.94 min and 22.23 min of the sample collected at 48 h were 2,3-

BD and acetoin, respectively (Figure S5). 16S rDNA analysis revealed that the sequence of the strain is more than 99% identical to that of *Cronobacter sakazakii* in the GenBank database. Therefore, the strain, named *C. sakazakii* OX-25, was further characterized as follows.

3.4.2 2,3-BD and acetoin production in glucose and xylose medium at high temperatures

2,3-BD and acetoin production abilities in YP medium supplemented with 20 g/l glucose and 20 g/l xylose were examined at different temperatures under a shaking condition (Figures 3.1 and 3.2). In the case of 20 g/l glucose at temperatures of 37 ˚C, 40 ˚C and 42 ˚C (Figure 3.1), 2,3-BD increased along with a decrease of glucose, and maximum concentrations of 2,3-BD at the three temperatures were found at 6 h, when glucose was almost completely consumed. The concentrations of 2,3-BD were 7.9 g/l, 7.3 g/l, 7.1 g/l and the productivities were 1.3 g/l·h, 1.2 g/l·h, 1.2 g/l·h at temperatures of 37 ˚C, 40 ˚C and 42 ˚C, respectively. Maximum concentrations of acetoin at the three temperatures were found at 72 h. The increase in acetoin was biphasic, 3 h-24 h and 36 h-72 h at 37 ˚C and 40 ˚C and 3 h-24 h and 48 h-72 h at 42 ˚C. Interestingly, the decrease in 2,3-BD was biphasic, apparently complementary to the biphasic increase in acetoin. It is speculated that 2,3-BD produced may be a mixture of two forms among (2R, 3R), (2S, 3S) and (2R, 3S) configurations, and two different enzymes that oxidize the individual form are expressed at different conditions during cultivation. At 45 ˚C, the turbidity was reduced after 24 h, indicating cells lysis probably due to the negative effect of the high temperature on cell, while the concentration of 2,3-BD remained high compared to the concentrations at other temperatures tested.

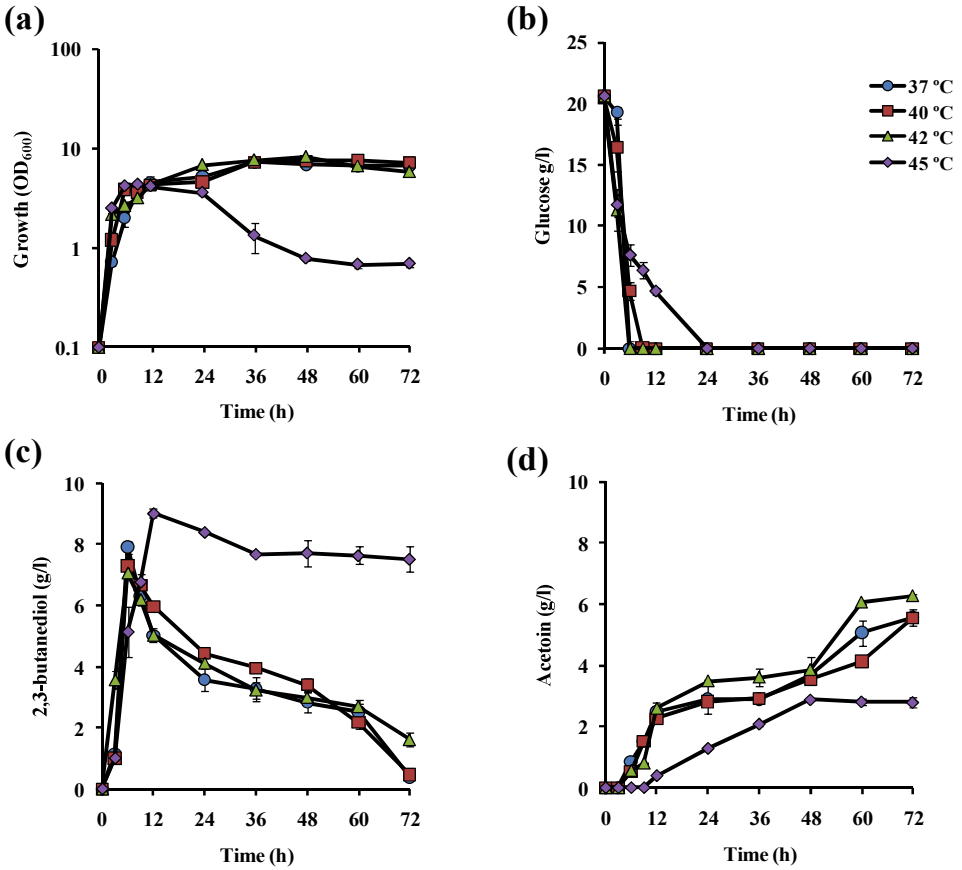

Figure 3.1 2,3-BD production by *C. sakazakii* OX-25 at different temperatures in a glucose medium. Cells were cultivated in YP medium supplemented with 20 g/l glucose at 37 ˚C (*filled circles*), 40 ˚C (*filled squares*), 42 ˚C (*filled triangles*) and 45 ˚C (*filled diamonds*) under a shaking condition at 100 rpm. **a** Turbidity (OD_{600}) and the concentrations of **b** glucose, **c** 2,3-BD and **d** acetoin in the culture medium were determined as described in Materials and methods. Error bars indicate standard deviation of three independent experiments.

In the case of 20 g/l xylose (Figure 3.2), the consumption of xylose was slow compared to that of glucose and the maximum concentrations of 2,3-BD at 37 ˚C, 40 ˚C and 42 ˚C were found at 12 h, when xylose was almost completely consumed. The concentrations of 2,3-BD were 6.1 g/l, 6.7 g/l and 6.6 g/l and the productivities were 0.5 g/l·h, 0.6 g/l·h and 0.6 g/l·h, at 37 ˚C, 40 ˚C and 42 ˚C, respectively. Maximum concentrations of acetoin at the three temperatures were found at 72 h. Decrease in 2,3-BD and increase in acetoin seemed to be biphasic as in the case of glucose as a carbon source. Reduction in turbidity after 24 h was observed as in the case of glucose. The peak

of the concentration of 2,3-BD at 45 ˚C was delayed compared to that at other temperatures tested and the accumulation of acetoin was monophasic with a peak at 60 h.

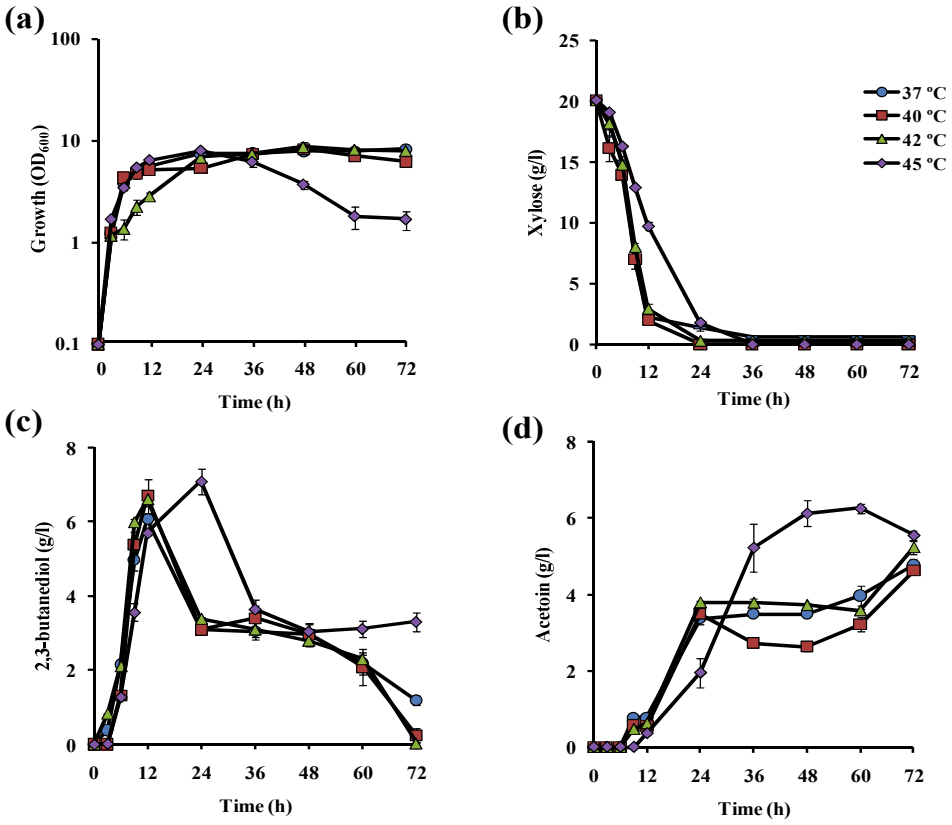

Figure 3.2 2,3-BD production by *C. sakazakii* OX-25 at different temperatures in a xylose medium. Cells were cultivated in YP medium supplemented with 20 g/l xylose at 37 ˚C (*filled circles*), 40 ˚C (*filled squares*), 42 ˚C (*filled triangles*) and 45˚C (*filled diamonds*) under a shaking condition at 100 rpm. **a** Turbidity (OD_{600}) and the concentration of **b** glucose, **c** 2,3-BD and **d** acetoin in the culture medium were determined as described in Materials and methods. Error bars indicate standard deviation of three independent experiments.

3.4.3 2, 3-BD and acetoin production with high concentrations of glucose or xylose

To further understand the production capacity of 2,3-BD and acetoin, higher concentrations of glucose or xylose were examined at 42 ˚C because the productivity of 2,3-BD was nearly the same as the productivities at 37 ˚C and 40 ˚ (Figure 3.1 and 3.2). When 40 g/l, 60 g/l and 80 g/l glucose were applied, 16 g/l and 19 g/l of 2,3-BD at 9 h and 22 g/l of 2,3-BD at 12 h were produced, respectively, and 9 g/l, 3 g/l and 2 g/l of

acetoin were produced at 48 h, respectively (Figure 3.3). However, 20 g/l and 36 g/l glucose remained in the medium under the condition with 60 g/l and 80 g/l glucose. The largest amounts of 2,3-BD were detected at 9 h with 80% and 63% of the theoretical yield and at 12 h with 56% of the theoretical yield under the conditions with 40 g/l, 60 g/l and 80 g/l glucose, respectively. The sum of 2,3-BD and acetoin at 12 h under the 80 g/L glucose condition was 59% of the theoretical yield, which was much lower than 88% and 66% at 9 h under the 40 g/l and 60 g/l glucose conditions, and the conversion of 2,3-BD to acetoin seemed to be stopped after 24 h, suggesting that the production of 2,3-BD and the conversion of 2,3-BD to acetoin were prevented. These findings suggest that about 22 g/l of 2,3-BD is the upper limit in the case of glucose as a carbon source. Notably, in contrast to the experiments with 20 g/l glucose, both the decrease in 2,3-BD and increase in acetoin were monophasic, and the 2,3-BD decrease was not significant in experiments with 60 g/l and 80 g/l glucose. It is presumed that the conversion of 2,3-BD to acetoin was prevented when glucose remained in the medium.

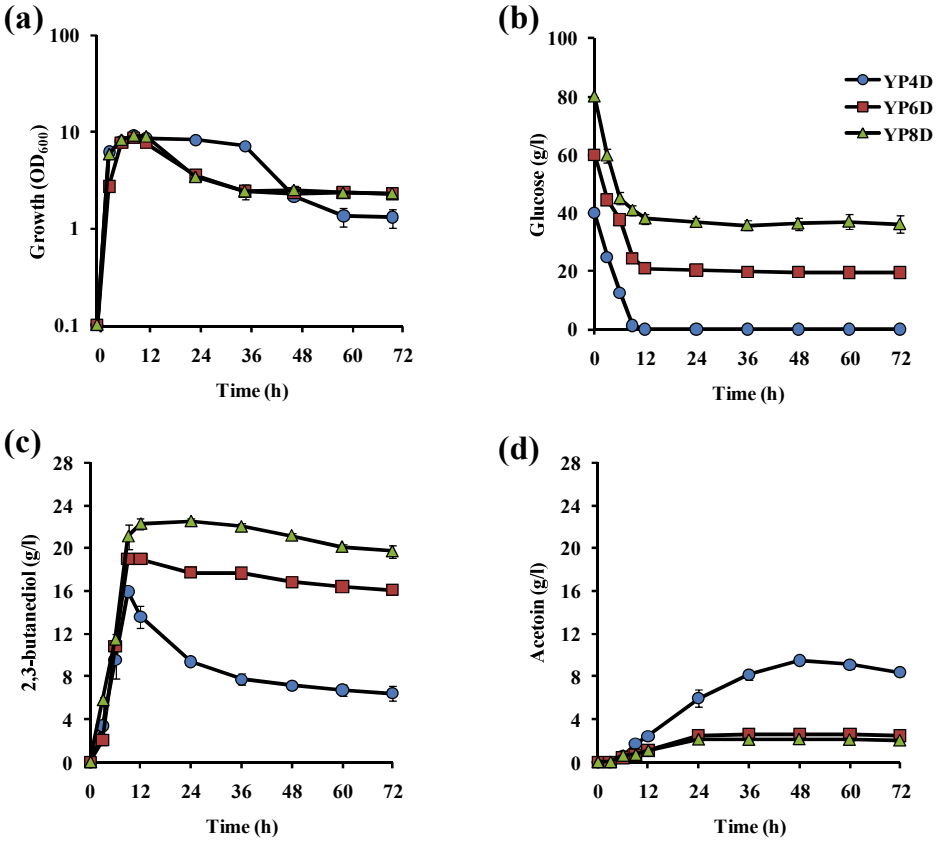

Figure 3.3 Effects of initial glucose concentrations on 2,3-BD production. Cells were cultivated in YP medium supplemented with 40 g/l (*filled circles*), 60 g/l (*filled squares*)

or 80 g/l (*filled triangles*) glucose at 42˚C under a shaking condition at 100 rpm. **a** Turbidity (OD_{600}) and the concentrations of **b** glucose, **c** 2,3-BD and **d** acetoin in the culture medium were determined as described in Materials and methods. Error bars indicate standard deviation of three independent experiments.

When 40 g/l, 60 g/l and 80 g/l xylose were applied, 13 g/l of 2,3-BD at 12 h, 22 g/l at 24 h and 29 g/l at 36 h were produced, respectively, and 8 g/l of acetoin at 48 h, 4 g/l at 48 h and 5 g/l at 60 h were produced, respectively (Figure 3.4). However, 4 g/l xylose and 7 g/l xylose remained in the medium at 48 h under the condition with 60 g/l and 80 g/l xylose, respectively. The largest amounts of 2,3-BD were found at 12 h with 53% of the theoretical yield, at 24 h with 60% of the theoretical yield and at 36 h with 59% of the theoretical yield under the conditions with 40 g/l, 60 g/l and 80 g/l xylose, respectively. The sums of 2,3-BD and acetoin under the 60 g/l and 80 g/l xylose conditions were 65% (24 h) and 65% (36 h) of the theoretical yield, respectively, which were much higher than 58% at 12 h under the 40 g/l xylose condition, but the conversion of 2,3-BD to acetoin at high xylose concentrations was greatly reduced. These findings suggest that this strain is able to produce about 29 g/l of 2,3-BD in the case of xylose as a carbon source. Therefore, it is assumed that the production of 2,3-BD with xylose is greater than that with glucose, although the production speed of the former is slower than that of the latter.

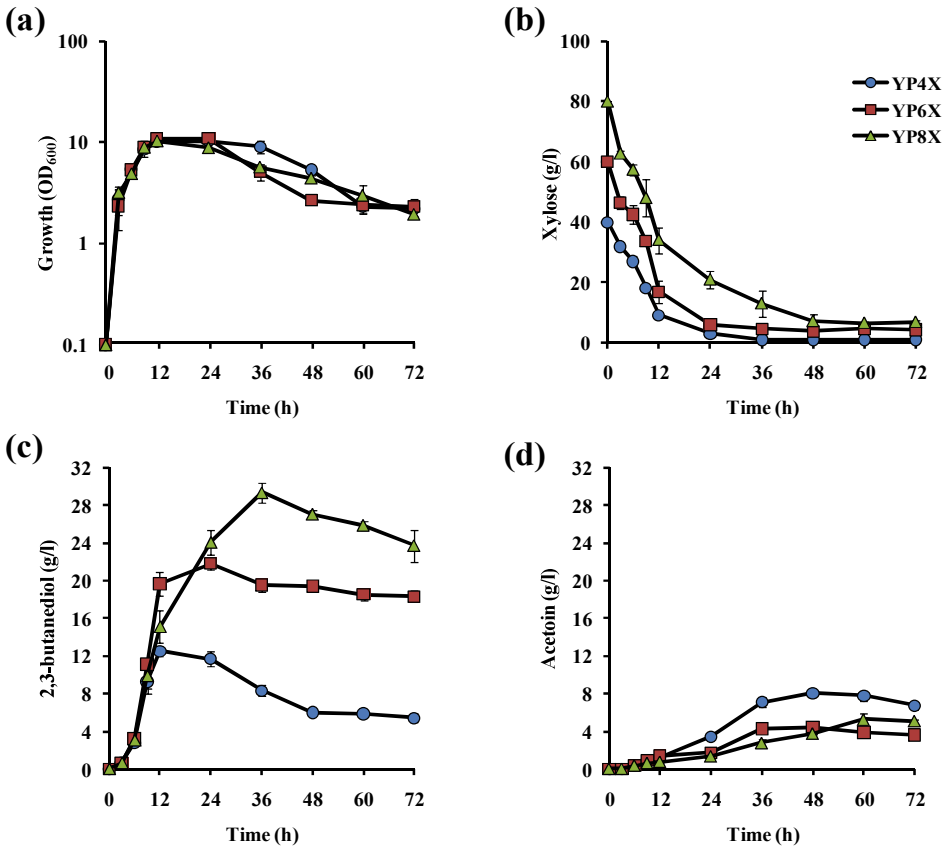

Figure 3.4 Effects of initial xylose concentrations on 2,3-BD production. Cells were cultivated in YP medium supplemented with 40 g/l (*filled circles*), 60 g/l (*filled squares*) or 80 g/l (*filled triangles*) xylose at 42 ˚C under a shaking condition at 100 rpm. **a** Turbidity (OD_{600}) and the concentrations of **b** glucose, **c** 2,3-BD and **d** acetoin in the culture medium were determined as described in Materials and methods. Error bars indicate standard deviation of three independent experiments.

3.4.4 2, 3-BD and acetoin production with mixed sugars of glucose and xylose

Considering cellulosic biomass, the production of 2,3-BD and acetoin from mixed sugars of glucose and xylose was examined using three different combinations of both sugars (Figure S6). Surprisingly, xylose was hardly utilized even after glucose had been completely consumed. When 20 g/l, 40 g/l and 60 g/l glucose in addition to 20 g/l xylose were applied, 8 g/l of 2,3-BD at 6 h, 17 g/l at 12 h and 22 g/l at 12 h were produced, respectively, and 4 g/l of acetoin at 24 h, 3 g/l at 24 h and 2 g/l at 24 h were produced, respectively. One of the reasons for xylose utilization inability in the presence of glucose may be glucose repression. Considering the findings that the strain was found to be potent in using xylose than glucose in fermentation using individual sugars, it is speculated that

enzymes related to xylose uptake and catabolism are negatively regulated in the presence of glucose, resulting in prevention of xylose utilization. Further research is required for improvement of the inability.

3.5 Discussion

During the process to isolate efficiently ethanol-fermenting thermotolerant microbes on xylose, we discovered a strain, *C. sakazakii* OX-25, that produced mainly 2,3-BD at the early growth phase. *C. sakazakii* OX-25 was then found to have several superior characteristics to other 2,3-BD-fermenting microbes. Compared to previously reported data for other wild-type microbes (Table 3.1), *C. sakazakii* OX-25 can utilize both glucose and xylose and showed the highest productivity when glucose or xylose was used as a carbon source. *Klebsiella pneumoniae* PM2 and *Paenibacillus polymyza* DSM 365 can also utilize both sugars, but their productivities were much lower than those of *C. sakazakii* OX-25 (Rehman *et al*. 2021; Okonkwo *et al*. 2021). *C. sakazakii* OX-25 was capable of efficiently fermenting 2,3-BD. Its production levels of 2,3-BD from glucose or xylose were higher than or equivalent to those of other microbes except for *P. polymyza* DSM 365. Moreover, this strain was able to produce the compound from both sugars at high temperatures. Fermentation experiments with other microbes were carried out at temperatures of 30 ˚C to 37 ˚C, except for *Bacillus licheniformis* YNP5-TSU (He *et al.* 2021). Recently, the production of 2,3-BD by *P. polymyxa* DSM 742 has been reported, and the titer of 2,3-BD was 10.57 g/l (0.26 g/g) at 30 ˚C for 40 h in a medium containing 30 g/l glucose (Ljubas *et al.* 2022). Furthermore, the metabolically engineered *E. ludwigii* has been shown to achieve 7.1 g/l (0.35 g/g) of 2,3-BD at 30 ˚C for 20 h in a medium containing 20 g/l of glucose (Amraoui *et al*. 2022). Meanwhile, *C. sakazakii* OX-25 achieved 7.1 g/l (0.4 g/g) at 42 ˚C for 6 h in a medium containing 20 g/l glucose. These results indicate that newly isolated *C. sakazakii* OX-25 has a high potential for 2,3-BD production.

Fermentation profiles revealed that *C. sakazakii* OX-25 accumulated 2,3-BD at the early growth phase and accumulated acetoin at a relatively late growth phase (Figures 3.1 and 3.2). Since both of the compounds are located next to each other in the metabolic pathway, 2,3-BD may be oxidized to acetoin. The conversion of 2,3-BD to acetoin was

initiated when glucose or xylose as the carbon source had been almost completely consumed, suggesting that accumulated 2,3-BD was taken up into cells. A part of the resultant acetoin may be further oxidized and the majority of acetoin was exported outside the cells. Notably, during cultivation, there were two-time reductions of 2,3-BD (for example, at 6 h-24 h and 36 h-72 h in the 20 g/l glucose medium at 37 ˚C) and at 12 h-24 h and 48 h-72 h in the 20 g/l xylose medium at 37 ˚C and two-time increases of acetoin (for example, at 3-24 h and 36-72 h in the case of the 20 g/l glucose medium at 37 ˚C, and at 6-24 h and 48-72 h in the 20 g/l xylose medium at 37 ˚C) (Figures 3.1 and 3.2). It is thus thought that accumulated 2,3-BD may be a mixture of at least two isomers as in most other microbes (Bialkowska *et al*. 2015; Jurchescu *et al*. 2013; Yang *et al*. 2011) and that one of them is first oxidized to acetoin and the other is oxidized later.

Table 3.1. Comparison of the amount of 2,3-BD produced by *C. sakazakii* OX-25 and the amounts produced by various microbes.

Strain	Conc. (g/l)	Temp. (C°)	Time (h)	2,3-BD (g/l)	2,3-BOD (g/g)	2,3-BOD (g/l·h)	Reference
Cronobacter sakazakii OX-25	Xyl: 20	42	12	6.6	0.3	0.6	This study
	Xyl: 40	42	12	12.5	0.3	1	This study
	Xly: 60	42	24	21.8	0.4	0.9	This study
	Xyl: 80	42	36	29.4	0.4	0.8	This study
Enterobacter ludwigii	Xyl: 20	30	17	7.6	0.4	0.4	Amraoui *et al*. 2021
	Xyl: 40	30	40	~12	0.3	0.3	Amraoui *et al*. 2021
	Xly: 60	30	40	~ 10	0.2	0.3	Amraoui *et al*. 2021
	Xyl: 80	30	40	~ 8	0.1	0.2	Amraoui *et al*. 2021
Klebsiella pneumoniae PM2	Xyl: 20	30	36	6	0.3	0.2	Rehman *et al*. 2021
	Xyl: 40	30	36	13	0.3	0.4	Rehman *et al*. 2021
	Xyl: 60	30	36	20	0.3	0.6	Rehman *et al*. 2021
	Xyl: 80	30	36	25	0.3	0.7	Rehman *et al*. 2021
Paenibacillus polymyxa DSM 365	Xyl: 55.9	35	70	29.4	0.3	0.4	Okonkwo *et al*. 2021
Cronobacter sakazakii OX-25	Glu: 20	42	6	7.1	0.4	1.2	This study
	Glu: 40	42	9	15.9	0.4	1.8	This study
	Glu: 60	42	9	19	0.3	2.1	This study
	Glu: 80	42	9	21.1	0.3	2.3	This study
Klebsiella oxytoca M1	Glu: 42.3	30	48	10.4	0.2	0.2	Cho *et al*. 2015
Bacillus licheniformis DSM 8785	Glu: 20	30	15	1.6	0.3	0.4	Jurchescu *et al*. 2013
	Glu: 40	30	20	2.0	0.4	0.7	Jurchescu *et al*. 2013
	Glu: 60	30	25	1.2	0.4	0.9	Jurchescu *et al*. 2013

	Glu: 80	30	31.5	1.5	0.4	1.0	Jurchescu *et al*. 2013
Klebsiella pneumoniae PM2	Glu: 20	30	24	8.0	~ 0.4	~ 0.3	Rehman *et al*. 2021
	Glu: 40	30	24	18.0	0.5	0.8	Rehman *et al*. 2021
	Glu: 60	30	36	21.8	0.4	0.6	Rehman *et al*. 2021
	Glu: 80	30	36	26.8	0.3	0.7	Rehman *et al*. 2021
Paenibacillus polymyxa DSM 365	Glu: 38.5	35	60	32.3	0.3	0.5	Okonkwo *et al*. 2021
Acillus amyloliquefaciens	Glu: 40	37	20	4.5	0.1	0.2	Okonkwo *et al*. 2021
	Glu: 60	37	26	11.5	0.2	0.4	Okonkwo *et al*. 2021
	Glu: 80	37	36	17.7	0.2	0.5	Okonkwo *et al*. 2021
Enterobacter cloacae SDM	Glu: 40	37	10	~18	0.5	1.8	Meng *et al*. 2021
Bacillus licheniformis YNP5-TSU	Glu: 48.3	50	18	20.5	0.4	0.29	He *et al*. 2021

When glucose was used as a carbon source, 2,3-BD production by the strain reached 80% of the theoretical yield at 9 h in the condition with 40 g/l glucose, but the amounts of 2,3-BD produced were reduced to 63% and 56% of the theoretical yield at 9 h and 12 h in the conditions with 60 g/l and 80 g/l glucose, respectively (Figure 3.3). On the other hand, in the case of xylose as a carbon source, the 2,3-BD production by the strain reached 59% of the theoretical yield at 36 h in the condition with 80 g/l xylose, but the amount of 2,3-BD produced was reduced to 53% of the theoretical yield at 12 h in the condition with 40 g/l xylose (Figure 3.4). Notably, cell turbidity declined after 12 h and 24 h in the glucose medium and xylose medium, respectively, at 45 ˚C (Figures 3.1 and 3.2) and after 12 h or 36 h in the media containing high sugar concentrations even at 42 ˚C (Figures 3.3 and 3.4), suggesting a negative impact of high temperatures or substrate inhibition. The latter is consistent with previous reports (Song *et al*. 2019).

Compared to the previously reported 2,3-BD fermentation by other microbes, *C. sakazakii* OX-25 was found to have several useful features including high productivity and good performance at high temperatures. Additionally, it showed almost the same yield as that of other efficient 2,3-BD-producing microbes. These features indicate that this strain is suitable and promising for the production of 2,3-BD. Considering the further conversion of 2,3-BD to acetoin, which may be due to consumption of sugars such as glucose or xylose in the medium, the production level of 2,3-BD could be maintained by keeping minimum amounts of sugars. *Klebsiella*, *Enterobacter*, and *Serratia* genera (Song *et al*. 2019) also produce about the same amount of 2,3-BD as that produced by *C. sakazakii* OX-25. However, their pathogenicity (genera of risk group 2) has been pointed out as an obstacle for industrial use. In this regard, *C. sakazakii* is less pathogenic and is classified as risk group 1 according to the guidelines of the US National Institutes of Health. Furthermore, *C. sakazakii* OX-25 was able to efficiently produce 2,3-BD at high temperatures, as opposed to most 2,3-BD fermentations with other microbes at 30 ˚C. Compared to low-temperature fermentation around 30 ˚C, high-temperature fermentation around 40 ˚C has several advantages such as reduction of operating costs for cooling systems, minimization of

the risk of contamination, and efficient achievement of simultaneous saccharification and fermentation (Limtong *et al*. 2007; Anderson *et al*. 1986; Rodrussamee *et al*. 2011). Taken together, the characteristics of *C. sakazakii* OX-25 enable energy-saving and cost-effective production of 2,3-BD. In particular, its high productivity and high temperature fermentation with the strain may be new important factors in 2,3-BD production in terms of process economics.

3.6 Conclusion

In the production of 2,3-BD, microbes that can utilize cellulosic biomass and ferment quickly and efficiently are extremely important for industrial applications. In this study, we succeeded in isolating a xylose-fermenting microbe, *C. sakazakii* OX-25, which has such potential. The microbe was able to utilize xylose and glucose as a carbon source and produced 2,3-BD at relatively high speed at high temperatures. When 20 g/l and 40 g/l glucose were applied, 7.1 g/l (0.4 g/g or 1.2 g/l·h) of 2,3-BD at 42 ˚C for 6 h and 15.9 g/l (0.4 g/g or 1.8 g/l·h) of 2,3-BD at 42 ˚C for 9 h, respectively, were achieved. In the cases of 20 g/l and 40 g/l xylose, 6.6 g/l (0.3 g/g or 0.6 g/l·h) of 2,3-BD at 42 ˚C for 12 h and 12.5 g/l (0.3 g/g or 1 g/l·h) of 2,3-BD at 42 ˚C for 12 h, respectively, were achieved. The theoretical yields reached 71%, 80%, 55% and 53% under the conditions with 20 g/l glucose, 40 g/l glucose, 20 g/l xylose and 40 g/l xylose, respectively. These results suggest that *C. sakazakii* OX-25 shows the highest productivity of previously reported non-genetically engineered microorganisms, suggesting that the strain has the potential for 2,3-BD production in industrial applications.

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LIST OF PUBLICATION

1. **Adaptive laboratory evolution for multistress tolerance, including fermentability at high glucose concentrations in thermotolerant *Candida tropicalis***

   Koudkeo Phommachan, Chansom Keo-oudone, Mochamad Nurcholis, Nookhao Vongvilaisak, Mingkhuan Chanhming, Vanhnavong Savanhnaly, Somchanh Bounphanmy, Minenosuke Matsutani, Tomoyuki Kosaka, Savitree Limtong and Mamoru Yamada

   Frontier in Energies, 2022, 15:561

2. **Highly efficient production of 2,3-butanediol from xylose and glucose by newly isolated thermotolerant *Cronobacter sakazakii***

   Chansom Keo-oudone, Koudkeo Phommachan, Orathai Suliya, Mochamad Nurcholis, Somchanh Bounphanmy, Tomoyuki Kosaka and Mamoru Yamada

   Frontier in BMC Microbiology, 2022, 22:164

APPENDIX

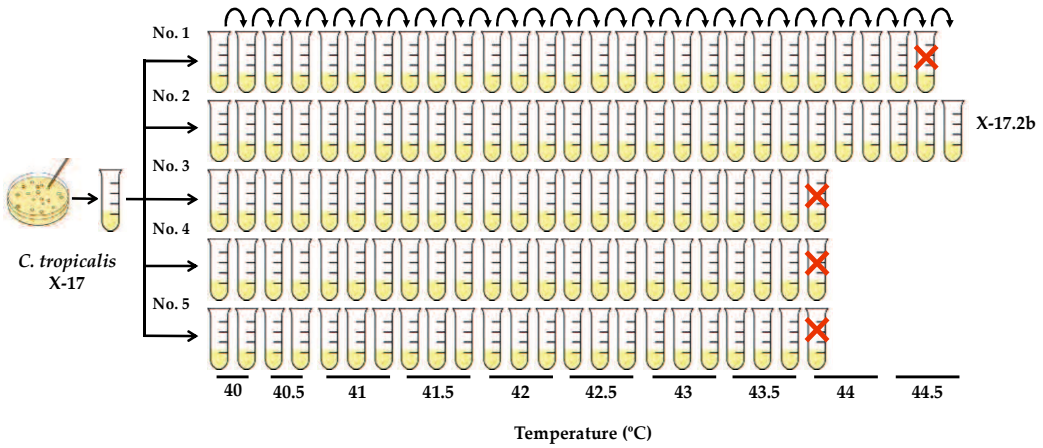

Figure S1 Schematic diagram of RLCGT for *C. tropicalis* X-17. *C. tropicalis* X-17 was cultured in five tubes in parallel in a 5-ml YP liquid medium containing 200g·L^{-1} of glucose under a shaking condition at 100 rpm for 7 days and the cultivation was repeated with gradually increasing temperature from 40 ˚C to 44.5 ˚C. At each step, cells were transferred to a fresh medium at the initial OD_{660} value of 0.1.

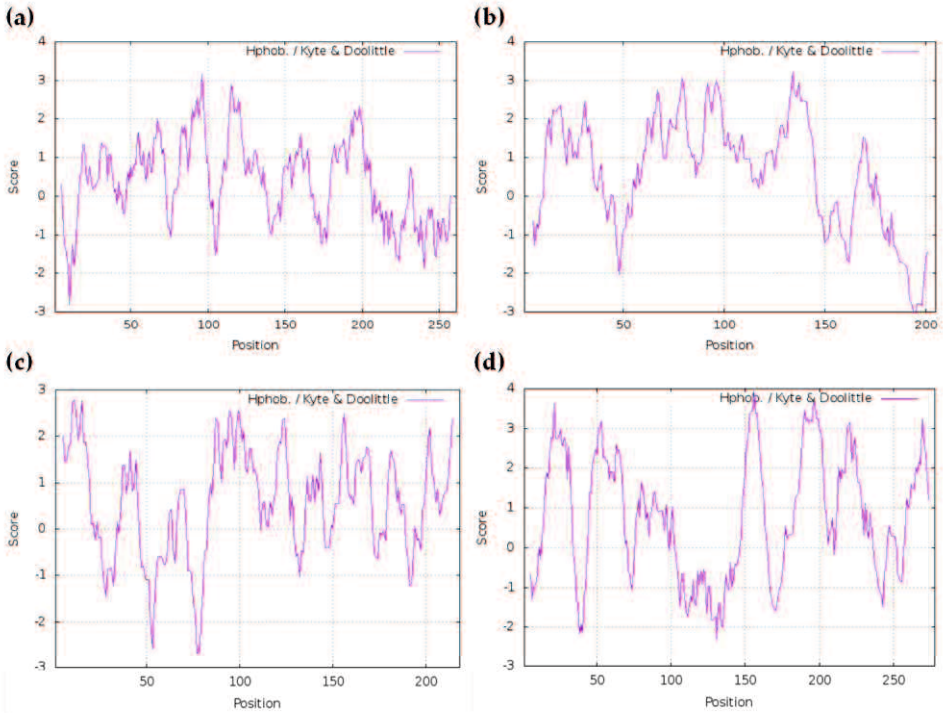

Figure S2 Hydropathy analysis of four hypothetical proteins of which genes were up-regulated in X-17.2b. Hydropathy profiles of a glucose transporter encoded by

CTRG_06250 **(a)**, a hypothetical protein encoded by *CTRG_06056* **(b)**, a hypothetical protein encoded by *CTRG_00691* **(c)** and a hypothetical protein encoded by *CTRG_01139* **(d)** are shown. Hydropathy analysis was performed by the method of Kyte and Doolittle (Kyte *et al*. 1928).

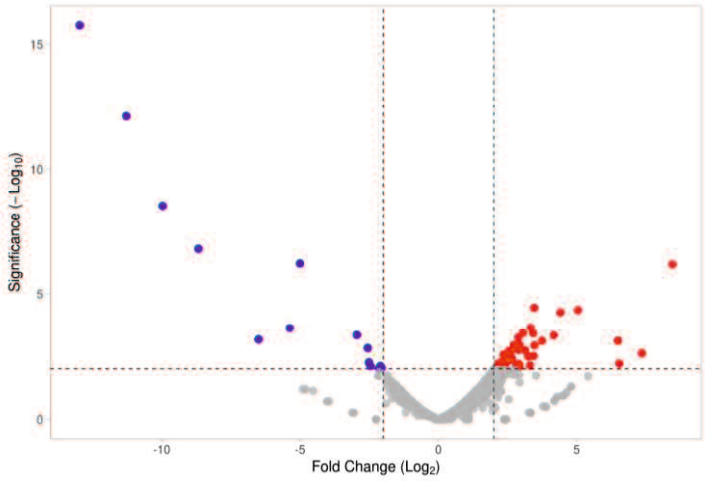

Figure S3 Volcano plots of differentially expressed genes (DEGs) of adapted strain X-17.2b versus the parental strain X-17 at 37 ˚C for 12 h. Genes with adjusted *P*-values less than 0.01 and $\log_2$ (fold change) values greater than 2 and less than -2 were assigned as genes with differential expression. Red symbols, significantly up-regulated genes; blue symbols, significantly down-regulated genes.

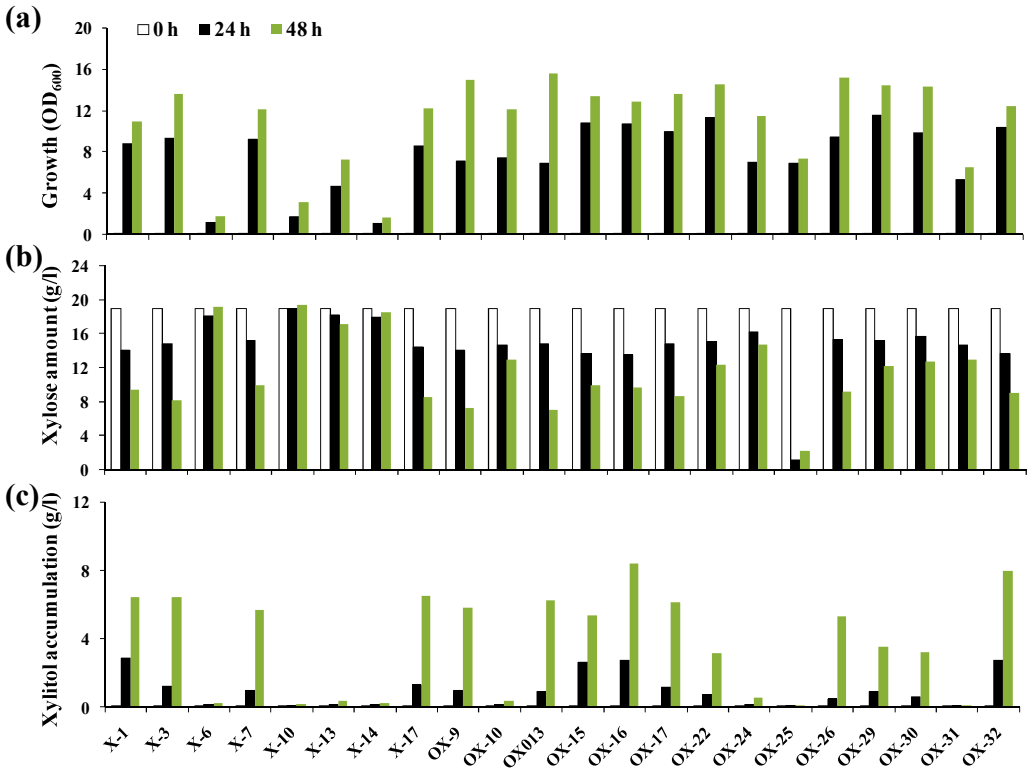

Figure S4 Screening strains with abilities for high xylose consumption and low xylitol accumulation. The screening was performed by cultivation in test tubes that each contained 3 mL of YP medium supplemented with 20 g/l xylose at 37 ˚C under a shaking condition at 100 rpm. **a** Turbidity (OD_{600}) and the concentrations of **b** xylose and **c** xylitol in the culture medium were determined as described in Materials and methods.

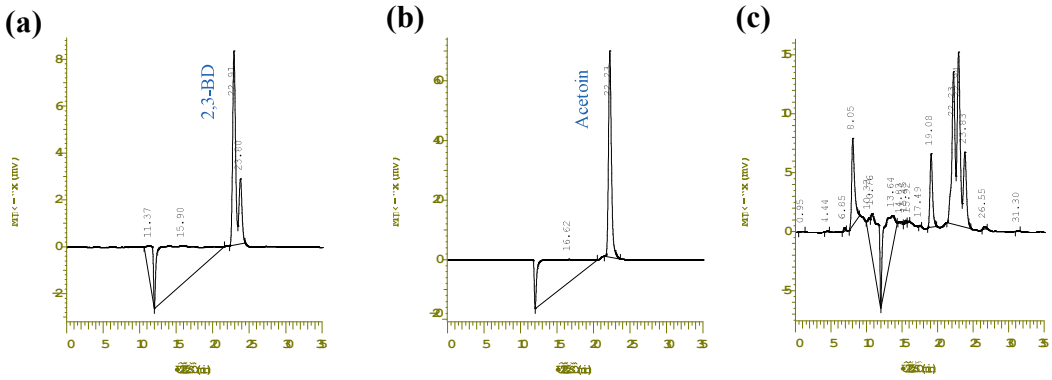

Figure S5 HPLC profiles showing the peaks of authentic 2,3-BD and acetoin and compounds in one sample. a HPLC profile showing a peak of authentic 2,3-BD, **b**

HPLC profile showing a peak of authentic acetoin and **c** HPLC profile of one sample taken at 48 h showing the peaks of 2,3-BD and acetoin at retention times of 22.94 min and 22.23 min, respectively.

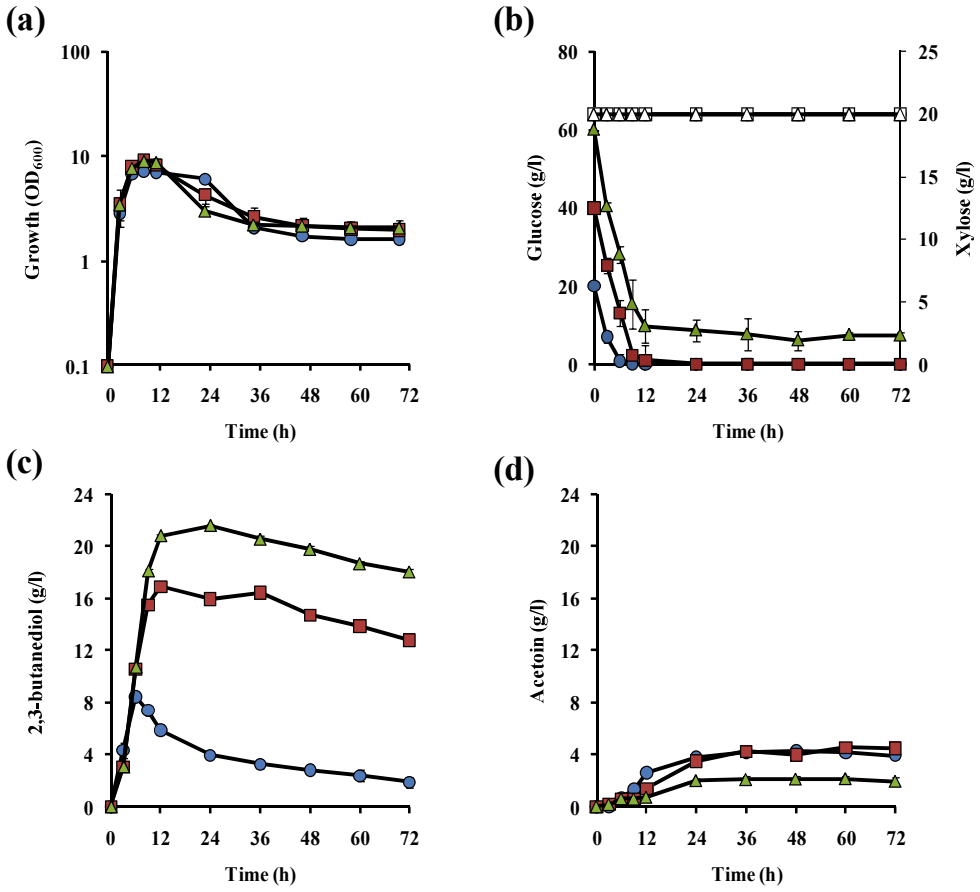

Figure S6 2,3-BD production by *C. sakazakii* OX-25 on mixed sugars at 42˚C. Cells were cultivated at 42˚C in YP medium supplemented with glucose and xylose at ratios of 1:1, 2:1 or 3:1 under a shaking condition at 100 rpm. The concentration of xylose was fixed at 20 g/l and the concentrations of glucose were 20 g/l (*filled circles* or *opened circles*), 40 g/l (*filled squares* or *opened squares*) and 60 g/l (*filled triangles* or *opened triangles*). **a** Turbidity (OD_{600}) and the concentrations of **b** glucose and xylose, **c** 2,3-BD and **d** acetoin in the culture medium were determined as described in Materials and methods. Closed symbols represent glucose consumption and opened symbols represent xylose consumption. Error bars indicate standard deviation of three independent experiments.

Table S1 Significantly down-regulated genes (log_2 < -2) in the adapted strain.

Name	Log_2 Fold Change	Product
CTRG_04849	-2.1	Oxidoreductase
CTRG_00109	-2.1	Hypothetical protein
CTRG_00211	-2.1	Fructose-bisphosphate aldolase
CTRG_05493	-2.2	RNA polymerase II transcription factor B subunit 5
CTRG_01742	-2.2	Transporter protein SMF1/ESP1
CTRG_05850	-2.2	Hypothetical protein
CTRG_05940	-2.3	Meiotic sister chromatid recombination protein 1
CTRG_04226	-2.3	Hypothetical protein
CTRG_00580	-2.3	Putative diacetyl reductase [(R)-acetoin forming] 2
CTRG_00882	-2.5	NAD-dependent alcohol dehydrogenase
CTRG_00284	-2.5	Glutathione S-transferase 1
CTRG_05790	-2.5	Respiratory supercomplex factor 2, mitochondrial
CTRG_01828	-2.6	Hypothetical protein
CTRG_01856	-2.7	Hypothetical protein
CTRG_03259	-2.8	Hypothetical protein
CTRG_03258	-3.3	Hypothetical protein
CTRG_04826	-5.2	Hypothetical protein
CTRG_04987	-5.8	Sodium transport ATPase 2