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Adaptive Laboratory Evolution for Multistress Tolerance, including Fermentability at High Glucose Concentrations in Thermotolerant *Candida tropicalis*

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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.

Keywords: *Candida tropicalis*; fermenting yeast; adaptive laboratory evolution; fermentability at high glucose concentrations; multistress tolerance

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1. Introduction

Solutions for the problems of global warming and the unsustainability of future energy supplies are crucial for continued economic development [1,2]. Much interest has been shown in biomass as carbon sources for biofuel production due to the environmentally friendly low carbon emissions of biofuels [3–6]. Biomass conversion has been performed by fermentation or other biomass utilization technologies, particularly fast pyrolysis. For example, Hamouda et al. [7] converted sugarcane molasses by fermentation with

Candida tropicalis HSC-24 to ethanol. Li et al. [3,4] 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 [8–13]. Lignocellulose is composed of three major carbohydrate polymers: cellulose, hemicelluloses and lignin [1,8,9]. Hemicellulose hydrolysates contain xylose as an abundant sugar [14,15]. 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, the efficient co-fermentation of glucose and xylose is a major challenge in achieving the conversion of lignocellulosic biomass to biofuels [15]. 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 [1,9]. 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 [1,16,17].

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 [18–24]. 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 [25]. 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 [26,27], improving the durability of xylose-fermenting yeast strains to high concentrations of glucose is important for the 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 [26] or high temperatures [27], 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 to 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. Materials and Methods

2.1. Yeast Strains

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 [28]. *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.

2.2. Media and Growth Conditions

YPD medium (10 g·L^{-1} yeast extract, 20 g·L^{-1} peptone and 20 g·L^{-1} glucose) was used for pre-culture at 30 °C under a shaking condition at 160 rpm and YP medium (10 g·L^{-1} yeast extract, 20 g·L^{-1} peptone) containing 200 g·L^{-1} glucose was used for adaptation. YP agar plates supplemented with 20 g·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·L^{-1} glucose (YP16D), 20 g·L^{-1} glucose (YP2D), 20 g·L^{-1} xylose (YP2X), 50 g·L^{-1} xylose (YP5X), both 2 g·L^{-1} glucose and 20 g·L^{-1} xylose (YP0.2D2X), 20 g·L^{-1} glucose and 20 g·L^{-1} xylose (YP2D2X), 40 g·L^{-1} glucose and 20 g·L^{-1} xylose (YP4D2X), 20 g·L^{-1} glucose and 50 g·L^{-1} xylose (YP2D5X) xylose, and 50 g·L^{-1} glucose and 50 g·L^{-1} xylose (YP5D5X) as a carbon source.

2.3. 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·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.

2.4. 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.

2.5. 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.

2.6. 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 [29] 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 [30]. Different sequence sites in both strains were searched for using the Genome Analysis Toolkit (GATK) v3.6-0-g89b7209 [31]. Accession numbers of sequence data are DRR328071 for X-17 and DRR32807 for X-17.2b.

2.7. RNA-Seq Analysis

RNA for RNA-Seq analysis was prepared, as described previously [32]. 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 [33]. 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 [34]. 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 [35]. 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.

2.8. Hydropathy Analysis

Hydropathy analysis was performed by using the algorithm of Kyte and Doolittle [36] with the normalized consensus hydrophobicity value of Eisenberg [37] 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.

3. Results

3.1. 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_{600} 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.

3.2. 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 [18–21]. Effects of various stresses on growth of X-17.2b were therefore examined by spot tests (Figure 1).

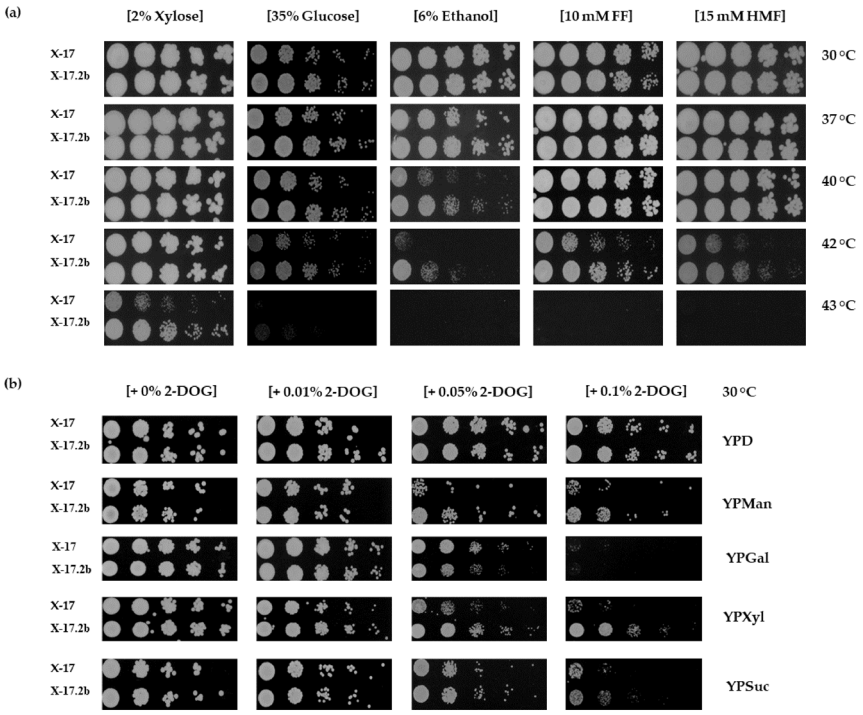

Figure 1. Stress resistance and effects of 2-DOG on sugar utilization of *C. tropiclis* X-17 and adapted strain *C. tropiclis* X-17.2b. Serially diluted cells were spotted onto (**a**) YP2X, YP35D and YP2X agar plates supplemented with 6% ethanol, 10 mM furfural or 15 mM HMF and (**b**) YPD, YPMan, YPGal, YPXyl and YPSuc agar plates supplemented with or without 0.01%, 0.05% or 0.1% 2-DOG. The plates were incubated (**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 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 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.

3.3. 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). 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 [38].

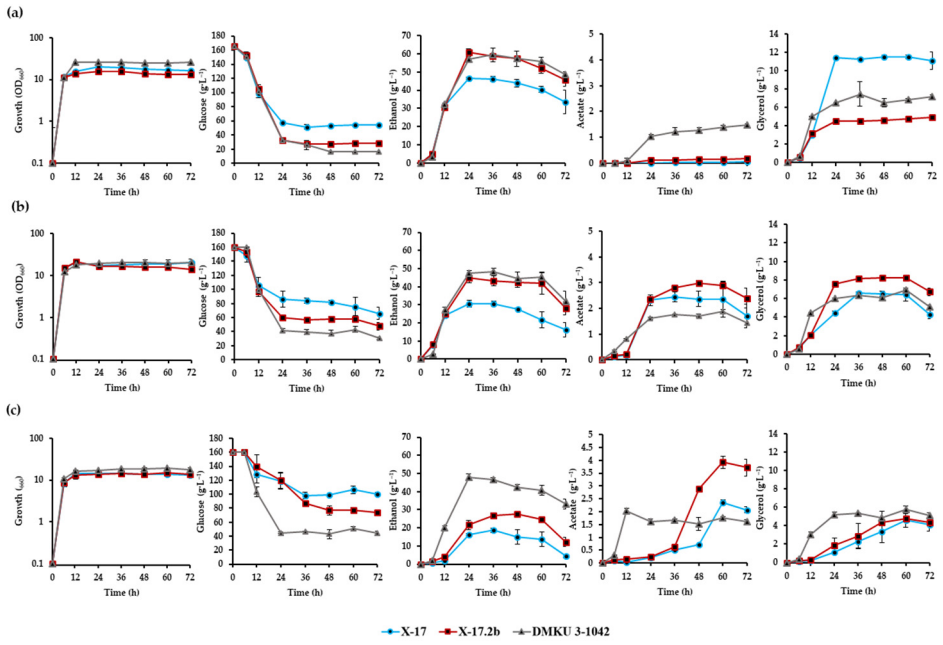

Figure 2. Growth and metabolite profiles of *C. tropiclis* X-17 (*filled circles*), adapted strain *C. tropicalis* X-17.2b (*filled squares*) and *K. marxianus* DMKU 3-1042 (*filled triangles*). Cells are grown in YP media containing 160 g·L^{-1} glucose at (**a**) 37 °C, (**b**) 40 °C and (**c**) 42 °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·L^{-1}, 46.5 g·L^{-1} and 56.9 g·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·L^{-1}, 30.5 g·L^{-1} and 47.6 g·L^{-1}, respectively, at 40 °C, and 27.8 g·L^{-1}, 14.9 g·L^{-1} and 42.3 g·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·L^{-1} xylose of X-17.2b was examined at 35 °C, 37 °C and 42 °C (Figure 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·L^{-1}, 4.40 g·L^{-1} and 3.0 g·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 3a,b). X-17.2b consumed xylose and produced a larger amount of ethanol than did X-17 even at 42 °C (Figure 3c). Therefore, it is likely that the adapted strain has enhanced xylose-fermentation ability.

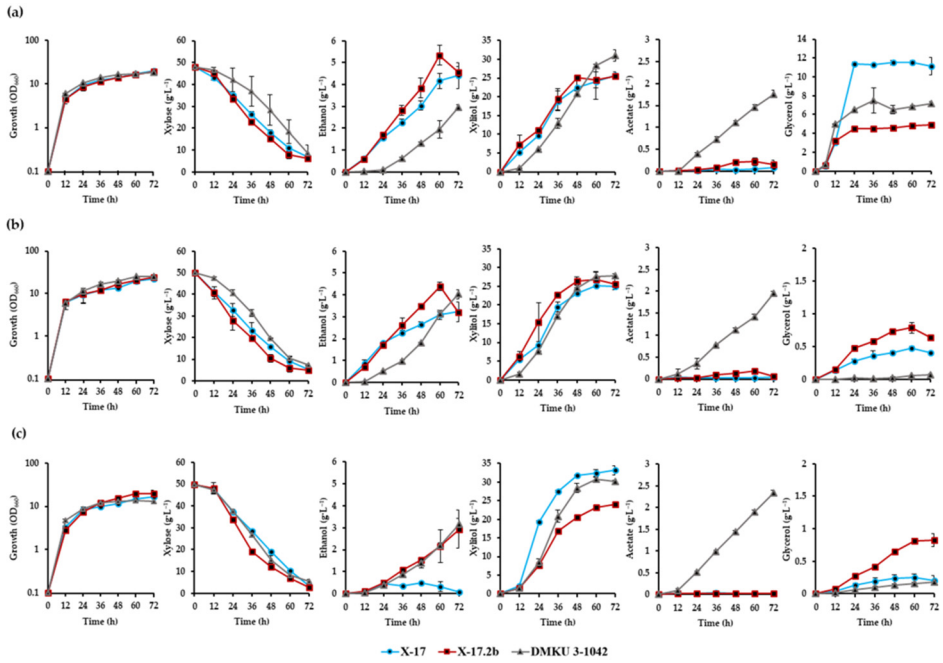

Figure 3. Growth and metabolite profiles of *C. tropiclis* X-17 (*filled circles*), adapted strain *C. tropicalis* X-17.2b (*filled squares*) and *K. marxianus* DMKU 3-1042 (*filled triangles*). Cells were grown in YP media containing 50 g·L^{-1} xylose at (**a**) 35 °C, (**b**) 37 °C and (**c**) 42 °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 4), considering the optimum temperatures for ethanol production in the case of glucose and xylose (Figures 2 and 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 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·L^{-1} xylose (Figure 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·L^{-1},

10 g·L^{-1} and 9.48 g·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.

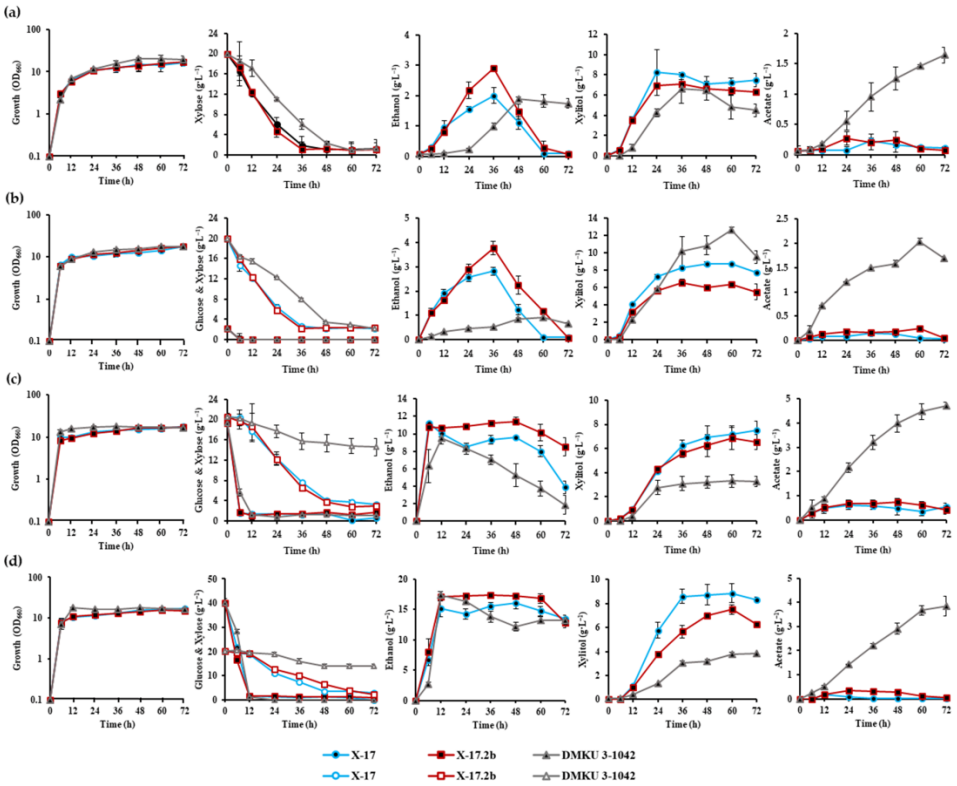

Figure 4. Growth and metabolite profiles of *C. tropiclis* X-17 (*filled circles* or *open circles*), adapted strain *C. tropicalis* X-17.2b (*filled squares* or *open squares*) and *K. marxianus* DMKU 3-1042 (*filled triangles* or *open triangles*). Cells were grown in YP media containing (**a**) 20 g·L^{-1} xylose and a mixed sugar medium containing (**b**) 2 g·L^{-1} glucose + 20 g·L^{-1} xylose or (**c**) 20 g·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 [39–42]. When cells were grown in a medium containing mixed sugars of 40 g·L^{-1} glucose and 20 g·L^{-1} xylose (Figure 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.

3.4. 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 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 (Tables 1 and S1 and Figure S3). KEGG enrichment analysis and GO function enrichment analysis were performed, but no notable results were obtained.

Table 1. Significantly up-regulated genes ($\log_2$ > 2) in the adapted strain.

Name	$\mathbf{Log_2}$ 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

Table 1. *Cont.*

Name	Log_2 Fold Change	Product
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 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 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.

4. Discussion

Generally, *C. tropicalis* is superior to *K. marxianus* in the ability to convert xylose to ethanol but inferior in 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 the 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 1–3). 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).

Table 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 Xyl 16.0 ± 0.1	3.8 ± 0.3	6.6 ± 0.4	-	0.2 ± 0.0	0.2 ± 0.0	0.10 ± 0.0	This study
	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	[14]
	30	Glc 20: Xyl 20	60	Glc~0.0 Xyl~7	~8.0	~2.00	NR	NR	~0.20	~0.13	[14]
C. tropicalis W103	35	Glc 50 + Xyl 50	75	Glc~50 Xyl~25	~20	NR	NR	NR	~0.2	~0.26	[43]
C. tropicalis MTCC 25057	32	Glc 100	48	Glc~100	36	NR	NR	NR	~0.36	~0.75	[44]
	32	Glc 50 + Xyl 50	24	Glc~50 Xyl~00	18.8 ± 0.8	NR	NR	NR	~0.2	~0.78	[44]
C. tropicalis UFMGBX12-a	30	Glc 18 + Xyl 2	30	Glc~00 Xyl~0.7	1.5	12	NR	NR	0.1	0.05	[45]
C. tropicalis CICC1779	34	Glc 30 + Xyl 30	72	Glc 27.5 Xyl 16.7	17.6	NR	NR	NR	~0.3	~0.24	[46]
C. tropicalis M9	42	Xyl 50	168	NR	~12	NR	NR	NR	~0.2	~0.07	[8]

NR not reported. ± 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 [44]. 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 [8]. 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 [43,44]. 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). *C. tropicalis* UFMGBX12 consumed 35% of xylose in a medium containing 2 $g \cdot L^{-1}$ xylose and 18 $g \cdot L^{-1}$ glucose [45]. *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 [46] 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 [14]. Therefore, it is likely that *C. tropicalis* X-17 and X-17.2b are less sensitive in xylose utilization to glucose repression.

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.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en15020561/s1, 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 200 $g \cdot L^{-1}$ 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 [36]. 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. Table S1: Significantly down-regulated genes ($\log_2 < -2$) in the adapted strain.

Author Contributions: K.P. performed phenotypic analysis, analyzed data, and wrote the original manuscript. K.P. and M.N. performed adaptation experiments. C.K.-o., N.V., M.C., V.S. and S.B. screened and characterized the parental strain. M.M. performed genome analysis. T.K. gave advice for genome and transcriptome analyses. S.L. gave advice for enrichment culture. M.Y. organized the study and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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