1 **Lactose fermentation by engineered *Saccharomyces cerevisiae* capable of fermenting**
2 **cellobiose**

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Abstract

Lactose is an inevitable byproduct of the dairy industry. In addition to cheese manufacturing, the growing Greek yogurt industry generates excess acid whey, which contains lactose. Therefore, rapid and efficient conversion of lactose to fuels and chemicals would be useful for recycling the otherwise harmful acid whey. *Saccharomyces cerevisiae*, a popular metabolic engineering host, cannot natively utilize lactose. However, we discovered that an engineered *S. cerevisiae* strain (EJ2) capable of fermenting cellobiose can also ferment lactose. This finding suggests that a cellobiose transporter (CDT-1) can transport lactose and a β -glucosidase (GH1-1) can hydrolyze lactose by acting as a β -galactosidase. While the lactose fermentation by the EJ2 strain was much slower than the cellobiose fermentation, a faster lactose-fermenting strain (EJ2e8) was obtained through serial subcultures on lactose. The EJ2e8 strain fermented lactose with a consumption rate of 2.16 g/L·h. The improved lactose fermentation by the EJ2e8 strain was due to the increased copy number of *cdt-1* and *ghl-1* genes. Looking ahead, the EJ2e8 strain could be exploited for the production of other non-ethanol fuels and chemicals from lactose through further metabolic engineering.

Keywords: *Saccharomyces cerevisiae*; Lactose fermentation; Adaptive evolution; *cdt-1*; *ghl-1*.

1. Introduction

Due to their superior capability to anaerobically ferment sugars into ethanol, *Saccharomyces cerevisiae* yeast strains have been widely used for decades for industrial ethanol production (Nielsen et al., 2013). First generation bioethanol is based on fermenting hexoses derived from sugarcane and corn starch into ethanol. However, increased production of first generation bioethanol has intensified the food-versus-fuel debate. To address this issue, *S. cerevisiae* has been engineered to ferment pentose sugars which are abundant in inedible lignocellulosic biomass (Bettiga et al., 2009; Kim et al., 2013; Wisselink et al., 2007; Zhou et al., 2012). Using engineered yeast, direct fermentation of the glucose-glucose dimer, cellobiose, instead of glucose, has been proposed in order to enhance the utilization of cellulosic sugars in plant cell wall hydrolysates (Galazka et al., 2010; Ha et al., 2011). Specifically, the introduction and expression of a cellodextrin transporter (CDT-1) and a β -glucosidase (GH1-1) from *Neurospora crassa* into *S. cerevisiae* enabled direct fermentation of cellodextrin by engineered yeast (Galazka et al., 2010; Ha et al., 2011). One advantageous trait of a cellodextrin-utilizing yeast is the alleviation of glucose repression when mixtures of cellobiose and other sugars, such as xylose or galactose are fermented (Ha et al., 2011; Ha et al., 2013; Wei et al., 2015).

Lactose, which has a similar structure to cellobiose, is a potential substrate for bioethanol production because cheese manufacturing processes can generate 9 kg of cheese whey as a waste product when producing 1 kg of cheese (Gonzalez-Siso et al., 2015). Whey contains substantial amounts of lactose so that direct whey disposal to environments can cause serious problems due to high biological oxygen demand (BOD)

and chemical oxygen demand (COD) (Guimaraes et al., 2010). As such, bioconversion of lactose to fuels and chemicals not only solves the pollution problem, but also offers economic production of value-added products.

Kluyveromyces marxianus (Christensen et al., 2011; Fonseca et al., 2008; Sansonetti et al., 2011; Sansonetti et al., 2013) and *Kluyveromyces lactis* (Gonzalez-Siso et al., 2015; Maullu et al., 1999; Ornelas et al., 2008) are naturally lactose-fermenting yeasts (Sheetz and Dickson, 1981; Sreekrishna and Dickson, 1985). However, there are some drawbacks for using *K. marxianus* and *K. lactis*. They cannot grow under strict anaerobic conditions, which prohibit their utilization in cost-effective industrial-scale applications (Bellaver et al., 2004; Lane and Morrissey, 2010; Zeeman et al., 2000). Moreover, *K. marxianus* and *K. lactis* have a low ethanol tolerance, making it difficult to employ these yeasts for fermenting concentrated cheese whey with high lactose concentrations (Silveira et al., 2005). As a result, the ethanol yields from lactose by *K. marxianus* and *K. lactis* are low because they cannot ferment high concentrations of lactose under anaerobic conditions. In contrast, *S. cerevisiae* has long been used for producing ethanol and it can bypass all aforementioned drawbacks of *K. marxianus* and *K. lactis*. Unfortunately, wild-type *S. cerevisiae* cannot assimilate lactose due to the lack of endogenous lactose assimilation pathways. Previous studies addressed the introduction of lactose-consuming pathways into *S. cerevisiae*. Specifically, the introduction of *LAC4* coding for β -galactosidase and *LAC12* coding for lactose transporter from *K. marxianus* and *K. lactis* into *S. cerevisiae* enabled direct fermentation of lactose by engineered *S. cerevisiae* (Domingues et al., 2001; Guimaraes et al., 2008a; Guimaraes et al., 2008b; Sreekrishna and Dickson, 1985; Zou et al., 2013).

In the present study, we demonstrate that the existing engineered cellobiose-consuming yeast strain EJ2 (expressing *cdt-1* and *ghl-1*) (Oh et al., 2016), is capable of naturally fermenting lactose. While lactose fermentation by the EJ2 strain was much slower than cellobiose fermentation, a faster lactose-fermenting strain (EJ2e8) was obtained through serial subcultures on lactose. Here, we discuss our methods, results, and future potential impact of the EJ2e8 engineered yeast strain which is capable of efficiently fermenting lactose into ethanol.

2. Materials and Methods

2.1 Strains, media and growth conditions

The yeast EJ2 strain was obtained from serial subcultures of EJ1 strain (D452-2 *leu2::LEU2 pRS405-ghl-1 ura3::URA3 pRS406-cdt-1*) on cellobiose (Oh et al., 2016). Thus, the EJ2 strain contains massively amplified copies of *cdt-1* and *ghl-1* in the genome, resulting in enhanced cellobiose fermentation. In this study, the yeast strains were pre-cultured at 30 °C in YP medium (10 g/L of yeast extract and 20 g/L of peptone) with 20 g/L of glucose or cellobiose. The yeast cells were precultured for 24-36 hours in YP medium containing 20 g/L cellobiose for the cellobiose or lactose fermentation. A 20 mL volume of YP medium containing cellobiose or lactose was used for oxygen-limited and anaerobic fermentations at 30 °C in 125 mL Erlenmeyer flasks at an initial OD₆₀₀ (optical density at 600 nm) equal to 1.0. For whey fermentation, a 25 g of whey from bovine milk (Sigma, St. Louis, MO) was dissolved in 100 mL hot water, resulting in medium containing 150 g/L of lactose. Yeast extract (10 g/L) and peptone (20 g/L) were

then added into the whey medium as necessary nutrients. Whey fermentations were carried out at 30 °C in 125 mL Erlenmeyer flasks at an initial OD₆₀₀ equal to 20. A control flask containing the same whey medium, but free of yeast cells, was used and sampled to monitor ethanol production or lactose consumption to verify whether contamination occurred (Ozmihci and Kargi, 2007). Due to the presence of insoluble proteins in whey powder, it is not possible to easily assess OD. All samples were centrifuged for 10 min to remove cell debris, and the supernatant was diluted 10-50 times for metabolite analysis using high-performance liquid chromatography (HPLC, 1200 series, Agilent Technologies, Santa Clara, CA).

2.2 Analysis of extracellular metabolites

The cell growth was monitored by OD₆₀₀ using a UV-visible spectrophotometer (Biomate 5, Thermo, NY). The concentrations of metabolites such as cellobiose, lactose, glycerol, acetate, and ethanol were determined by HPLC equipped with a refractive index detector using a Rezex ROA-Organic Acid H⁺ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 0.005 N of H₂SO₄ at a flow rate of 0.6 mL/min at 50 °C.

2.3 Enzyme activity assay

Yeast cells during lactose fermentation at the 12-hour time point were harvested by centrifugation at 12,000 rpm for 2 min at 4 °C. The cell pellets were washed and suspended in Y-PER solution (Pierce, Rockford, IL), used to lyse the cells, and then incubated at room temperature for 20 min. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was used for the enzyme activity assay. The

β -glucosidase activity was measured according to the previous method (Oh et al., 2016; Wei et al., 2015). The β -galactosidase activity was measured following the protocol from Ribeiro (Ribeiro et al., 2007) with slight changes. In the present study, *ortho*-Nitrophenyl- β -galactoside (ONPG) was used as a substrate. 20 μ L ONPG (4 mg/mL) was mixed with 80 μ L yeast samples of appropriate dilutions. The reaction was conducted at 30 °C and the OD₄₀₅ was read by a microplate reader (BioTek, Winooski, VT). One unit of enzyme activity is defined as the amount of enzyme that catalyzes one μ mol of substrate per min at 30 °C. The protein concentration of the yeast cell extract was determined by the BCA method (Pierce, Rockford, IL) following the manufacturer's instructions.

2.4 *ghl-1* and *cdt-1* copy numbers determination by quantitative PCR

Quantitative PCR was conducted to measure the copy numbers of *cdt-1* and *ghl-1*. Genomic DNA of the EJ2 and EJ2e8 strains was extracted by the YeaStar Genomic DNA Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. The concentration of genomic DNA was quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). Quantitative PCR was performed using SYBR Green I Master (Roche Applied Science, Indianapolis, IN) on a Lightcycler 480 instrument (Roche) following the manufacturer's instructions. The primers CDT1 qPCR-F / qPCR-F and GH1-1 qPCR-F / qPCR-F listed in Table 1 were used for the determination of *cdt-1* and *ghl-1* copy numbers, respectively. The genomic copy numbers of *cdt-1* and *ghl-1* were quantified by standard curves generated by the purified *cdt-1* (*Bam*HI-*Eco*RI) and *ghl-1* (*Spe*I-*Pst*I) fragments (0.01, 0.1, 1, and 10 pg/ μ l) from the plasmids pRS426-*cdt-1* and

pRS425-gh1-1, respectively. The genomic copy numbers of *cdt-1* and *gh1-1* were calculated as described by Kim et al. (2013). Briefly, the copy numbers (x) of the target genes in the genomic DNA were estimated by the following equation: $x=bc/ad$. a is the size of the target gene fragment (1.74 kb for *cdt-1* and 1.43 kb for *gh1-1*), b is the size of the whole genome of *S. cerevisiae* (12000 kb), c is the concentration of the target gene calculated by quantitative PCR (ng/ μ l), and d is the concentration of the genomic DNA samples (ng/ μ l).

3. Results and Discussion

3.1 The cellobiose-assimilating strain EJ2 can ferment lactose

Previously, we have constructed the EJ2 strain which is capable of efficiently and rapidly fermenting cellobiose into ethanol. To achieve this, we introduced the genes coding for a cellodextrin transporter (*cdt-1*) and a β -glucosidase (*gh1-1*) into an *S. cerevisiae* strain and evolved the resulting strain on cellobiose as the sole carbon source (Oh et al., 2016). As CDT-1 from *N. crassa* introduced as a cellodextrin transporter (Galazka et al., 2010) was originally annotated as a MFS (Major Facilitator Superfamily) lactose permease (<http://www.uniprot.org/uniprot/Q7SCU1>), we hypothesized that the EJ2 strain might be able to ferment lactose due to the similar structure of cellobiose and lactose. To test this hypothesis, lactose fermentations by the EJ2 strain were conducted in YP medium with 80 g/L of lactose under oxygen-limited conditions. As shown in Fig. 1, the EJ2 strain was able to grow on lactose and ferment lactose into ethanol. The EJ2 strain consumed 55 g/L of lactose in 48 hours, producing 18.37 g/L of ethanol with a volumetric productivity (P_{Ethanol})

of 0.38 g/L·h and ethanol yield (Y_{Ethanol}) of 0.334 g/g. This result suggested that the cellodextrin transporter, CDT-1, could also transport lactose and that the β -glucosidase, GH1-1, could hydrolyze lactose. However, the rate of lactose consumption by the EJ2 strain was significantly slower than the cellobiose consumption rate (Fig. 1).

3.2 Improvement of lactose utilization capability by the EJ2 strain through laboratory evolution on lactose

Adaptive evolution was then conducted to improve the lactose fermentation capacity of the EJ2 strain. YP medium containing 40 g/L of lactose was used for serial subcultures of the EJ2 strain. Initially, the cultures were transferred every 24 hours for 12 days. As only 3 g/L of lactose was consumed every 24 hours, the culture transfer time was then switched from 24 to 48 hours. Faster lactose consumption rates were observed after 20 days of subcultures and the rate became saturated after 29 days (Fig. S1). The culture from the 29th day was diluted and plated onto an agar plate containing lactose to isolate colonies exhibiting improved lactose fermentation capabilities. Nine randomly picked colonies (EJ2e1 to EJ2e9) were evaluated for their lactose fermentation capacities. Using YP medium containing 100 g/L of lactose as the sole carbon source, the EJ2e8 strain was the best performing strain among nine selected colonies regarding lactose consumption and ethanol production rates (Fig. 2a and Fig. S2a). The cellobiose fermentation capabilities of these evolved EJ2 strains (EJ2e1 to EJ2e9) were also examined using the YP medium containing 100 g/L of cellobiose. Interestingly, as shown in Fig. 2b and Fig. S2b, the cellobiose fermentation capacities of the evolved strains were better than the parental EJ2 strain. Previously, when the EJ2 strain was isolated after serial subcultures on cellobiose

conditions, no more improvement for fermenting cellobiose was observed after the seventh round of serial subcultures, suggesting a saturation of evolution was reached (Oh et al., 2016). However, the present study provides evidence that the cellobiose fermentation capabilities of the EJ2 strain can be further improved by serial subcultures on lactose. The best lactose-fermenting strain, EJ2e8, is also a better cellobiose-fermenting strain as compared with the parental EJ2 strain (Fig. 2b and Fig. S2b). In conclusion, lactose fermentation by the engineered yeast expressing *cdt-1* and *ghl-1* can be improved by serial subcultures on lactose and the resulting evolved strains were also able to ferment cellobiose faster than the parental strain EJ2.

3.3 The evolved lactose-consuming yeast strain EJ2e8 has increased copy numbers of *cdt-1* and *ghl-1*

As reported previously (Oh et al., 2016), the EJ2 strain was evolved from the EJ1 strain which contains a single copy of *cdt-1* and *ghl-1*. The copy numbers of *cdt-1* and *ghl-1* in the EJ2 strain were 9 and 23, respectively, suggesting that the copy number increase is responsible for the improved cellobiose fermentation. Therefore, we hypothesized that a similar copy number increase of *cdt-1* and *ghl-1* in the EJ2 strain might be occurring during the serial subcultures on lactose. To check if the copy numbers increased in the lactose-evolved EJ2e8 strain, quantitative-PCR for determining the copy numbers of *cdt-1* and *ghl-1* was conducted. As expected, the copy numbers of *cdt-1* increased from 9 to 17 and those of *ghl-1* increased from 23 to 35 (Fig. 3a). The increased copy numbers of *cdt-1* and *ghl-1* were most likely responsible for the improved lactose and cellobiose fermentation capability of the evolved strain EJ2e8.

3.4 GH1-1 exhibits a β -galactosidase activity

Our results suggested that GH1-1 might exhibit β -galactosidase activity even though it is considered to be a β -glucosidase. To confirm the biochemical activities of β -galactosidase in the evolved EJ2e8 strain, β -glucosidase and β -galactosidase activities of the EJ2 and EJ2e8 strains were measured. Specifically, crude extracts from the cells harvested at 12 hours from the lactose fermentations were used for the enzymatic assay (Fig. 3b). The β -glucosidase activity in the EJ2e8 strain was 2.74 times higher than that in the EJ2 strain, which correlated well with the increased *ghl-1* copy numbers (from 23 to 35). β -galactosidase is a hydrolase that attacks the O-glucosyl group of lactose and is required for lactose fermentation (Sheetz and Dickson, 1981). While β -glucosidase encoded by *ghl-1* was reported as a cellobiose hydrolase (Galazka et al., 2010), the function of *ghl-1* as a β -galactosidase has not been documented. In this study, the β -galactosidase activity was measured using the same samples for the β -glucosidase activity measurement. As shown in Fig. 3b, *ghl-1* has a β -galactosidase activity of 12.5% as compared with β -glucosidase activity. This finding clarified the ability of the EJ2 strain for fermenting lactose and the slower fermentation rate of lactose as compared to cellobiose by the same strain.

3.5 The evolved EJ2e8 strain ferments lactose better than the parental EJ2 strain under oxygen-limited and anaerobic conditions

The lactose fermentation capabilities of the EJ2 and EJ2e8 strains were further evaluated in YP medium containing 80 g/L of lactose. As shown in Fig. 4, under the

oxygen-limited conditions, the EJ2e8 strain depleted 80 g/L of the lactose within 60 hours with a lactose consumption rate of 1.52 g/L·h, which is 45% higher than that (1.05 g/L·h) of the parental strain EJ2. The volumetric ethanol productivity and ethanol yield of the EJ2e8 strain are 0.55 g/L·h and 0.370 g/g, respectively, which are higher than those of the EJ2 strain (Table 2). The biomass yield of the EJ2e8 strain is 7.14% lower than that of the EJ2 strain, which might be caused by the increased ethanol yield.

Under anaerobic conditions, the lactose consumption rates of both strains were slightly slower than those under oxygen-limited conditions (Fig. 4). However, the EJ2e8 strain exhibited a higher ethanol yield (0.383 g/g vs. 0.361 g/g), lactose consumption rate (1.15 g/L·h vs. 0.87 g/L·h), and ethanol production rate (0.45 g/L·h vs. 0.33 g/L·h) than the EJ2 strain (Table 2). These results indicated that the evolved EJ2e8 strain is a better lactose-consuming strain than the parental EJ2 strain regardless of aeration.

3.6 The EJ2e8 strain is capable of fermenting whey efficiently under oxygen-limited and anaerobic conditions

Concentrated whey containing up to 120 g/L of lactose is preferable for the production of ethanol from lactose to reduce the distillation costs (Siso, 1996). To test the fermentation capability of the EJ2 and EJ2e8 strains in concentrated whey, a whey fermentation medium containing 150 g/L of lactose was used. Under oxygen-limited conditions, the EJ2e8 strain consumed all lactose within 48 hours and produced 48.78 g/L of ethanol with an ethanol yield of 0.336 g/g, while the EJ2 strain only consumed 94 g/L of lactose with an ethanol yield of 0.322 g/g at the same time (Fig. 5a and Table 2). The volumetric ethanol productivity of the EJ2e8 strain is 1.02 g/L·h, which is higher than that of the EJ2 strain

(0.78 g/L·h) (Table 2). Under anaerobic conditions, very similar trends were observed. Specifically, the EJ2e8 strain exhibited a faster lactose consumption rate (2.16 g/L·h vs. 1.75 g/L·h), and ethanol production rate (0.80 g/L·h vs. 0.66 g/L·h) than the EJ2 strain (Fig. 5b and Table 2). Overall, the lactose fermentation rates by the EJ2 and EJ2e8 strains are slower under anaerobic conditions than oxygen-limited conditions. However, the ethanol yields are higher under anaerobic conditions (0.361 g/g vs. 0.336 g/g for the EJ2e8 strain).

4. Conclusions

In this study, we demonstrated that an engineered cellobiose-fermenting yeast strain, EJ2, expressing *cdt-1* and *gh1-1* from *N. crassa* is also capable of fermenting lactose. This result suggests that the cellobiose transporter, CDT-1, can transport lactose and the β -glucosidase, GH1-1, has β -galactosidase activity. Additionally, a faster lactose-fermenting strain, EJ2e8, was obtained through laboratory evolution of the EJ2 strain on lactose. The improved lactose fermentation capacity is ascribed to the further increased copy numbers of *cdt-1* and *gh1-1*, which in turn resulted in improved cellobiose fermentations. This study provides evidence that our engineered EJ2e8 yeast strain can be used for the production of ethanol from lactose. The EJ2e8 strain could also be exploited for the production of other non-ethanol fuels and chemicals from lactose through further metabolic engineering.

Acknowledgements

This project was supported by funding from the Energy Biosciences Institute (EBI) and by the Agriculture and Food Research Initiative Competitive Grant No. 2015-67011-22806

289 from the USDA National Institute of Food and Agriculture.

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291 **References**

292 Bellaver, L.H., de Carvalho, N.M., Abrahao-Neto, J., Gombert, A.K., (2004) Ethanol

293 formation and enzyme activities around glucose-6-phosphate in *Kluyveromyces*

294 *marxianus* CBS 6556 exposed to glucose or lactose excess. *FEMS Yeast Res* 4, 691-698.

295 Bettiga, M., Bengtsson, O., Hahn-Hagerdal, B., Gorwa-Grauslund, M.F., (2009)

296 Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing

297 a fungal pentose utilization pathway. *Microb Cell Fact* 8, 40.

298 Christensen, A.D., Kadar, Z., Oleskowicz-Popiel, P., Thomsen, M.H., (2011) Production

299 of bioethanol from organic whey using *Kluyveromyces marxianus*. *J Ind Microbiol*

300 *Biotechnol* 38, 283-289.

301 Domingues, L., Lima, N., Teixeira, J.A., (2001) Alcohol production from cheese whey

302 permeate using genetically modified flocculent yeast cells. *Biotechnol Bioeng* 72,

303 507-514.

304 Fonseca, G.G., Heinzle, E., Wittmann, C., Gombert, A.K., (2008) The yeast

305 Kluyveromyces marxianus and its biotechnological potential. Appl Microbiol Biotechnol
 306 79, 339-354.

307 Galazka, J.M., Tian, C., Beeson, W.T., Martinez, B., Glass, N.L., Cate, J.H., (2010)
 308 Cellodextrin transport in yeast for improved biofuel production. Science 330, 84-86.

309 Gonzalez-Siso, M.I., Tourino, A., Vizoso, A., Pereira-Rodriguez, A., Rodriguez-Belmonte,
 310 E., Becerra, M., Cerdan, M.E., (2015) Improved bioethanol production in an engineered
 311 Kluyveromyces lactis strain shifted from respiratory to fermentative metabolism by
 312 deletion of NDI1. Microb Biotechnol 8, 319-330.

313 Guimaraes, P.M., Francois, J., Parrou, J.L., Teixeira, J.A., Domingues, L., (2008a)
 314 Adaptive evolution of a lactose-consuming Saccharomyces cerevisiae recombinant. Appl
 315 Environ Microbiol 74, 1748-1756.

316 Guimaraes, P.M., Teixeira, J.A., Domingues, L., (2008b) Fermentation of high
 317 concentrations of lactose to ethanol by engineered flocculent Saccharomyces cerevisiae.
 318 Biotechnol Lett 30, 1953-1958.

319 Guimaraes, P.M., Teixeira, J.A., Domingues, L., (2010) Fermentation of lactose to

320 bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey.
 321 Biotechnol Adv 28, 375-384.
 322 Ha, S.J., Galazka, J.M., Kim, S.R., Choi, J.H., Yang, X., Seo, J.H., Glass, N.L., Cate, J.H.,
 323 Jin, Y.S., (2011) Engineered *Saccharomyces cerevisiae* capable of simultaneous
 324 cellobiose and xylose fermentation. Proc Natl Acad Sci U S A 108, 504-509.
 325 Ha, S.J., Kim, S.R., Kim, H., Du, J., Cate, J.H., Jin, Y.S., (2013) Continuous
 326 co-fermentation of cellobiose and xylose by engineered *Saccharomyces cerevisiae*.
 327 Bioresour Technol 149, 525-531.
 328 Kim, S.R., Skerker, J.M., Kang, W., Lesmana, A., Wei, N., Arkin, A.P., Jin, Y.S., (2013)
 329 Rational and evolutionary engineering approaches uncover a small set of genetic changes
 330 efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. PloS one 8, e57048.
 331 Lane, M.M., Morrissey, J.P., (2010) *Kluyveromyces marxianus*: A yeast emerging from
 332 its sister's shadow. Fungal Biology Reviews 24, 17-26.
 333 Maullu, C., Lampis, G., Desogus, A., Ingianni, A., Rossolini, G.M., Pompei, R., (1999)
 334 High-level production of heterologous protein by engineered yeasts grown in cottage

335 cheese whey. *Appl Environ Microbiol* 65, 2745-2747.

336 Nielsen, J., Larsson, C., van Maris, A., Pronk, J., (2013) Metabolic engineering of yeast
 337 for production of fuels and chemicals. *Curr Opin Biotechnol* 24, 398-404.

338 Oh, E.J., Skerker, J.M., Kim, S.R., Wei, N., Turner, T.L., Maurer, M.J., Arkin, A.P., Jin,
 339 Y.S., (2016) Gene Amplification on Demand Accelerates Cellobiose Utilization in
 340 Engineered *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 82, 3631-3639.

341 Ornelas, A.P., Silveira, W.B., Sampaio, F.C., Passos, F.M., (2008) The activity of
 342 beta-galactosidase and lactose metabolism in *Kluyveromyces lactis* cultured in cheese
 343 whey as a function of growth rate. *J Appl Microbiol* 104, 1008-1013.

344 Ozmihci, S., Kargi, F., (2007) Kinetics of batch ethanol fermentation of cheese-whey
 345 powder (CWP) solution as function of substrate and yeast concentrations. *Bioresour*
 346 *Technol* 98, 2978-2984.

347 Ribeiro, O., Gombert, A.K., Teixeira, J.A., Domingues, L., (2007) Application of the
 348 Cre-loxP system for multiple gene disruption in the yeast *Kluyveromyces marxianus*. *J*
 349 *Biotechnol* 131, 20-26.

350 Sansonetti, S., Hobley, T.J., Calabro, V., Villadsen, J., Sin, G., (2011) A biochemically
 351 structured model for ethanol fermentation by *Kluyveromyces marxianus*: A batch
 352 fermentation and kinetic study. *Bioresour Technol* 102, 7513-7520.
 353 Sansonetti, S., Hobley, T.J., Curcio, S., Villadsen, J., Sin, G., (2013) Use of continuous
 354 lactose fermentation for ethanol production by *Kluyveromyces marxianus* for verification
 355 and extension of a biochemically structured model. *Bioresour Technol* 130, 703-709.
 356 Sheetz, R.M., Dickson, R.C., (1981) LAC4 Is the Structural Gene for β -Galactosidase in
 357 *Kluyveromyces lactis*. *Genetics* 98, 729-745.
 358 Silveira, W.B., Passos, F.J.V., Mantovani, H.C., Passos, F.M.L., (2005) Ethanol
 359 production from cheese whey permeate by *Kluyveromyces marxianus* UFV-3: A flux
 360 analysis of oxido-reductive metabolism as a function of lactose concentration and oxygen
 361 levels. *Enzyme Microb Tech* 36, 930-936.
 362 Siso, M.I.G., (1996) The biotechnological utilization of cheese whey: A review.
 363 *Bioresour Technol* 57, 1-11.
 364 Sreekrishna, K., Dickson, R.C., (1985) Construction of strains of *Saccharomyces*

365 cerevisiae that grow on lactose. Proceedings of the National Academy of Sciences of the
 366 United States of America 82, 7909-7913.

367 Wei, N., Oh, E.J., Million, G., Cate, J.H., Jin, Y.S., (2015) Simultaneous Utilization of
 368 Cellobiose, Xylose, and Acetic Acid from Lignocellulosic Biomass for Biofuel
 369 Production by an Engineered Yeast Platform. ACS synthetic biology.

370 Wisselink, H.W., Toirkens, M.J., del Rosario Franco Berriel, M., Winkler, A.A., van
 371 Dijken, J.P., Pronk, J.T., van Maris, A.J., (2007) Engineering of *Saccharomyces*
 372 *cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Appl Environ
 373 Microbiol 73, 4881-4891.

374 Zeeman, A.M., Kuyper, M., Pronk, J.T., van Dijken, J.P., Steensma, H.Y., (2000)
 375 Regulation of pyruvate metabolism in chemostat cultures of *Kluyveromyces lactis* CBS
 376 2359. Yeast 16, 611-620.

377 Zhou, H., Cheng, J.S., Wang, B.L., Fink, G.R., Stephanopoulos, G., (2012) Xylose
 378 isomerase overexpression along with engineering of the pentose phosphate pathway and
 379 evolutionary engineering enable rapid xylose utilization and ethanol production by

380 *Saccharomyces cerevisiae*. *Metab Eng* 14, 611-622.

381 Zou, J., Guo, X., Shen, T., Dong, J., Zhang, C., Xiao, D., (2013) Construction of

382 lactose-consuming *Saccharomyces cerevisiae* for lactose fermentation into ethanol fuel. *J*

383 *Ind Microbiol Biotechnol* 40, 353-363.

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Figure legends

Graphical abstract: Diagram of lactose-consuming strain carrying multi-copies of lactose transporter, CDT-1 and β -glucosidase/ β -galactosidase, GH1-1.

Fig. 1 The cellobiose-fermenting strain EJ2 can ferment lactose. (a): The fermentation profiles of the EJ2 strain on YP medium containing 80 g/L lactose under oxygen-limited conditions; (b): The fermentation profiles of the EJ2 strain on YP medium containing 80 g/L cellobiose under oxygen-limited conditions. Data are presented as mean value and standard deviations of three independent biological replicates.

Fig. 2 The colonies from serial subcultures of the EJ2 strain exhibited better lactose (a) and cellobiose (b) fermentation phenotypes than the parental EJ2 strain. Symbols: Dark bar, consumed lactose or cellobiose; Grey bar, ethanol produced after 48 hours (lactose as carbon source) or 24 hours (cellobiose as carbon source) under the oxygen-limited conditions.

Fig. 3 (a): The copy numbers of *cdt-1* and *ghl-1* in the EJ2 and EJ2e8 strains; (b): β -galactosidase activities of the EJ2 and EJ2e8 strains.

Fig. 4 Lactose fermentation profiles of the EJ2 and EJ2e8 strains in YP medium containing 80 g/L lactose. (a) Lactose consumption and ethanol production and (b) cell growth under the oxygen-limited conditions; (c) Lactose consumption and ethanol production and (d) cell growth under the anaerobic conditions. Symbols: square, lactose consumption; circle,

ethanol production; triangle, OD600. Filled symbols, the EJ2e8 strain; open symbols, the EJ2 strain.

Fig. 5 Lactose fermentation profiles of the EJ2 and EJ2e8 strains in a whey medium containing 150 g/L lactose. (a): Lactose consumption and ethanol production under the oxygen-limited conditions; (b): Lactose consumption and ethanol production under the anaerobic conditions. Symbols: square, lactose consumption; circle, ethanol production. Filled symbols, the EJ2e8 strain; open symbols, the EJ2 strain.

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422 Table 1. Primers used for the determination of *cdt-1* and *gh1-1* copy numbers.

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Primer name	Sequences	References
CDT1 qPCR-F	TCCAATATCAAGCCCTGGAG	(Oh et al., 2016)
CDT1 qPCR-R	GGACCAGTGTGTCACCAGTGTG	(Oh et al., 2016)
GH1-1 qPCR-F	CAAGCACTGGATCACCTTCA	(Oh et al., 2016)
GH1-1 qPCR-R	TGAGCGATGAGCAGGTTATG	(Oh et al., 2016)

424 Table 2. Fermentation parameters of the EJ2 and EJ2e8 strain under different fermentation conditions.

	YP medium containing 80 g/L lactose				Whey medium containing 150 g/L lactose			
	Oxygen-limited		Anaerobic		Oxygen-limited		Anaerobic	
	EJ2	EJ2e8	EJ2	EJ2e8	EJ2	EJ2e8	EJ2	EJ2e8
Y_{ethanol}	0.339 ±0.002	0.370±0.001	0.361±0.003	0.383±0.002	0.322±0.001	0.336±0.002	0.345±0.003	0.361±0.001
Y_{biomass}	0.084±0.001	0.078±0.002	0.080±0.000	0.061±0.001	ND	ND	ND	ND
R_{lactose}	1.05±0.06	1.52±0.04	0.87±0.01	1.15±0.02	2.46±0.02	3.07±0.04	1.75±0.03	2.16±0.08
R_{lactose}^*	0.19±0.01	0.27±0.01	0.20±0.02	0.28±0.00	ND	ND	ND	ND
P_{ethanol}	0.36±0.03	0.55±0.01	0.33±0.02	0.45±0.01	0.78±0.01	1.02±0.01	0.66±0.01	0.80±0.01
P_{ethanol}^*	0.065±0.001	0.100±0.001	0.072±0.000	0.107±0.002	ND	ND	ND	ND

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426 1. Results are presented as the mean value and standard deviation of three independent biological replicates. The parameters listed here were the maximum
 427 parameters for each strain during the lactose fermentation under different conditions.

428 2. ND: Not Determined. The cell density cannot be measured during lactose fermentation in whey medium **due to the presence of insoluble proteins**.

429 3. For YP medium containing 80 g/L lactose, an initial cell density was adjusted to 0.29 g/L ($OD_{600} = 1$). For whey medium containing 150 g/L lactose, an initial cell
 430 density was adjusted to 5.8 g/L ($OD_{600} = 20$).

431 4. Parameters: Y_{ethanol} , ethanol yield (g/g consumed lactose); Y_{biomass} , biomass yield (g/g consumed lactose); R_{lactose} , lactose consumption rate (g/L·h); R_{lactose}^* , specific
 432 lactose consumption rate (g/g cell/h); P_{ethanol} , volumetric ethanol productivity (g/L·h); P_{ethanol}^* , specific ethanol productivity (g/g cell/h).

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Fig. 5

Fig. 5 Lactose fermentation profiles of the EJ2 and EJ2e8 strains in a whey medium containing 150 g/L lactose. (a): Lactose consumption and ethanol production under the oxygen-limited conditions; (b): Lactose consumption and ethanol production under the anaerobic conditions. Symbols: square, lactose consumption; circle, ethanol production. Filled symbols, the EJ2e8 strain; open symbols, the EJ2 strain.