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Short communication: Conversion of lactose and whey into lactic acid by engineered yeast

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ABSTRACT

Lactose is often considered an unwanted and wasted byproduct, particularly lactose trapped in acid whey from yogurt production. But using specialized microbial fermentation, the surplus wasted acid whey could be converted into value-added chemicals. The baker's yeast *Saccharomyces cerevisiae,* which is commonly used for industrial fermentation, cannot natively ferment lactose. The present study describes how an engineered *S. cerevisiae* yeast was constructed to produce lactic acid from purified lactose, whey, or dairy milk. Lactic acid is an excellent proof-of-concept chemical to produce from lactose, because lactic acid has many food, pharmaceutical, and industrial uses, and over 250,000 t are produced for industrial use annually. To ferment the milk sugar lactose, a cellodextrin transporter (*CDT-1*, which also transports lactose) and a β-glucosidase (*GH1-1*, which also acts as a β-galactosidase) from *Neurospora crassa* were expressed in a *S. cerevisiae* strain. A heterologous lactate dehydrogenase (encoded by *ldhA*) from the fungus *Rhizopus oryzae* was integrated into the *CDT-1/GH1-1*–expressing strain of *S. cerevisiae.* As a result, the engineered strain was able to produce lactic acid from purified lactose, whey, and store-bought milk. A lactic acid yield of 0.358 g of lactic acid/g of lactose was achieved from whey fermentation, providing an initial proof of concept for the production of value-added chemicals from excess industrial whey using engineered yeast.
Key words: lactic acid, yeast, whey, lactose

Short Communication

Whey is a significant and troublesome byproduct of the dairy industry (Siso, 1996). In particular, cheese production can generate up to 9 kg of whey per 1 kg of cheese, and the disaccharide lactose serves as the major constituent of the permeate after whey filtration (González-Siso et al., 2015). Acid whey is particularly problematic, because it is produced in excess as a byproduct of Greek yogurt production and is difficult for the yogurt industry to efficiently reuse or discard, in part due to the relatively low pH of acid whey (pH ≤5.1) (Miller et al., 2006; Elliott, 2013). In response, one creative solution for the problem of whey as a dairy industry byproduct is to use engineered microbes to ferment the lactose in the whey to produce value-added fuels and chemicals (González-Siso et al., 2015). However, even this solution is not easy, because it is recommended that the whey concentration used should result in up to 120 g/L lactose to minimize downstream purification costs (Siso, 1996; Arif et al., 2008). Therefore, an engineered microbe must not only be able to withstand the acidity of acid whey and osmotic stress induced by a high concentration of lactose, it must also produce a target product at a high yield and productivity.

Recently, an engineered strain of *Saccharomyces cerevisiae* baker's yeast was developed that can efficiently produce ethanol at a yield of 0.361 g of ethanol/g of lactose from whey medium containing 150 g/L of lactose under anaerobic conditions (Liu et al., 2016). Most yeast, including *S. cerevisiae*, cannot natively ferment lactose. This lactose-fermenting strain was created by introducing and expressing 2 heterologous genes cloned from the fungus *Neurospora crassa*: a lactose transporter (*CDT-1*) to transport lactose into the yeast cell and a β-galactosidase (*GH1-1*) to cleave the lactose disaccharide into glucose and galactose (Galazka et al., 2010; Oh et al., 2016).

Here, we expand on the initial concept of producing an endogenous product of *S. cerevisiae* (ethanol) toward producing a heterologous value-added product (lactic acid). This study provides proof of concept for the conversion of purified lactose, milk, and whey into

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lactic acid using an engineered *S. cerevisiae* strain. Yeasts, especially *S. cerevisiae*, are particularly ideal microbes for industrial chemical production because of their tolerance of high external osmotic stress (such as high sugar concentrations) and low pH (essential for overproducing organic acids); because they are generally recognized as safe; and because a wide range of genetic perturbation tools are available to study, introduce, and delete genes in the yeast genome (Laluce et al., 2012). Furthermore, the theoretical maximum yield of lactic acid from lactose is 1.00 g lactic acid/g lactose due to the lack of carbon loss throughout the metabolic pathway. Perhaps most importantly, oxygen is theoretically unnecessary for this process to occur, so large-scale fermentation of lactose to lactic acid could be conducted entirely anaerobically, significantly reducing the costs accrued if oxygen were added (Porro et al., 1995).

We expressed a heterologous lactate dehydrogenase gene (*ldhA*), cloned from the fungus *Rhizopus oryzae,* into the *cdt-1* and *gh1-1* expressing *S. cerevisiae* as previously reported (Turner et al., 2016). Using the fungal-sourced *ldhA* instead of a bacterial lactate dehydrogenase in yeast was advantageous, because most bacterial lactate dehydrogenase enzymes perform optimally at an internal cellular pH range of ~5, but eukaryotic lactate dehydrogenase performs optimally in the neutral pH range (Skory, 2003). Because *S. cerevisiae* intracellular pH is maintained near neutral even in the presence of a low extracellular pH (Breeuwer and Abee, 2000), the fungal *R. oryzae ldhA* is preferential for integration in the *cdt-1*/*gh1-1*–expressing strain. The *ldhA* was integrated into the *S. cerevisiae* chromosome using the pITy3-*ldhA*-G418 plasmid to integrate at Ty δ loci (Parekh et al., 1996) and selected using G418 (geneticin) as an antibiotic selection pressure. For all experiments, 2 engineered *S. cerevisiae* yeast strains were used, both expressing the *cdt-1* and *gh1-1* lactose-fermenting pathway: EJ4 (no *ldhA*; Wei et al., 2015) and EJ4L (expressing *ldhA*; Turner et al., 2016). The EJ4 strain served as the control, which in all fermentations was unable to produce lactic acid. Importantly, the native ethanol production pathway consisting of pyruvate decarboxylase and alcohol dehydrogenase was not disrupted in either the EJ4 or EJ4L strains, allowing the yeast cells to produce ethanol as necessary (Turner et al., 2016).

Stock cultures of *S. cerevisiae* were maintained on yeast extract-peptone dextrose (**YPD**) agar plates (2% agar, 1% yeast extra, 2% peptone, and 2% glucose) at 4°C. Yeast precultures were grown in YPD40 medium at 300 rpm and harvested at mid-log phase and then washed twice with sterilized water to prepare inocula for fermentation. All flask fermentations were conducted using 25 mL of medium in a 125-mL Erlenmeyer flask maintained at 30°C on a 100 rpm MaxQ4000 orbital shaker (Thermo Fisher Scientific Inc., Waltham, MA). Flask fermentations in Figure 1 were performed using yeast-peptone medium containing 40 g/L of purified lactose (Sigma-Aldrich, St. Louis, MO) with an initial cell concentration of ~1 (0.47 dry cell weight/mL, optical density at 600 nm). The YP medium and lactose were sterilized separately via autoclave. Flask fermentations in Figure 2 were performed using Horizon Organic brand, shelf-stable, 1% fat, organic cow milk (Whitewave Services Inc., Denver, CO; lot 36-4016 03:37), which was used before the printed "best before" date. Because the milk was shelf-stable, we did not autoclave it before use. Flask fermentations in Figure 3 contained YP medium with ~80 g/L whey from bovine milk (Sigma-Aldrich), which resulted in ~50 g/L of free lactose. Due to suspected microbial contamination when using non-autoclaved whey (data not shown), all results in Figure 3 are from autoclaved whey medium. Approximately 35 g/L of calcium carbonate ($CaCO_3$) was used as a neutralizing agent for experiments in Figure 1 and Figure 3. All experiments were repeated independently in duplicate, with variations indicated with error bars.

Yeast cell concentration was monitored by optical density at 600 nm using a Biomate 3 UV-visible spectrophotometer (Thermo Fisher Scientific Inc.). Lactose,

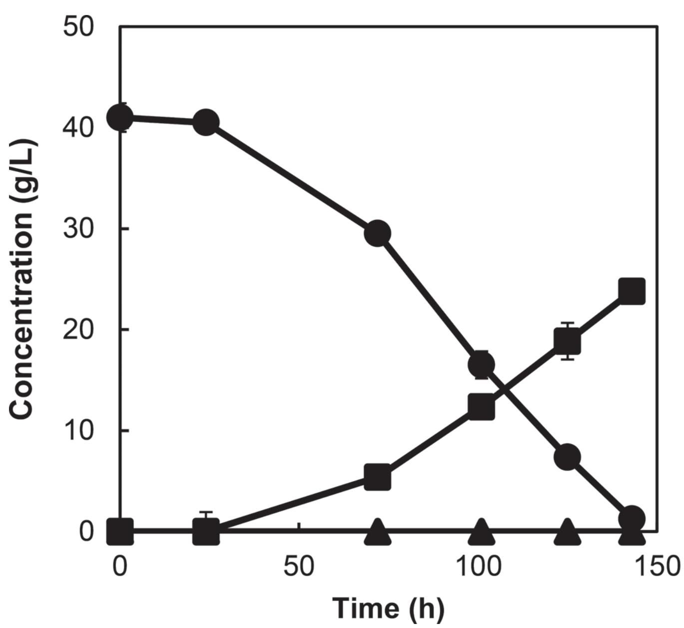

Figure 1. Fermentation profile of the EJ4L strain grown in yeast peptone medium containing 40 g/L of lactose as the sole carbon source and 35 g/L of $CaCO_3$, as shown by concentrations of lactose (●), lactic acid (■), and ethanol (▲). Results are the averages of duplicate experiments; error bars indicate standard deviations.