Final report for project grant 19-505 - Biotechnological production of lactic acid - a raw material for bioplastics

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The project was set to start with recruitment of a postdoctoral fellow, which ended up being a lengthy process, as the selected candidates of two recruitment rounds declined the offer due to pandemic related issues. Meanwhile, the work was started by the PI and a PhD student and postdoc in the lab, whose research is related to the project. After some Covid and visa related delay, Dr BoHyun Choi started at the end of May 2021. As the work still continues (with funding from FORMAS) and is expected to result in in at least one high impact publication, experimental detail is kept to a minimum when the results are summarized in relation to the project plan.

Background and state-of-the-art

Lactic acid is an organic acid with dual functional groups, making it suitable for use in a variety of chemical transformations and products. It is used for applications in food, pharmaceutical, textile, cosmetics, and chemical industries (Abedi and Hashemi, 2020). The demand for lactic acid has recently grown dramatically due to its use for production of PLA materials, a type of bioplastic. Lactic acid bacteria that are commonly used for production of lactic acid from starch are quite sensitive to stress, grow at near-neutral pH and require complex nutrients that increase production costs.

Lactic acid production at low pH is one way to solve the problems of toxicity, without need for added neutralizing agents (Abedi and Hashemi, 2020). Yeasts, such as *Saccharomyces cerevisiae* can tolerate low pH and grow in stressful conditions such as on lignocellulosic hydrolysate, a raw material made from residual plan biomass. A production of > 80 g/l lactic acid by *S. cerevisiae*, when using a defined media has already been achieved (Baek et al., 2017). A high titre is needed for commercially feasible purification and to the best of our knowledge lactic acid production from lignocellulosic hydrolysates has not yet been demonstrated with *S. cerevisiae*. The use of residual biomass instead of starch that competes with use for food and feed is crucial for a sustainable production process.

The purpose of the project was to develop cell factory concepts for microbial production of lactic acid, from lignocellulosic (plant-derived) biomass.

Results and discussion

WP1: Host selection and development

The host for the production of high optical purity isomers of lactic acid needs to display tolerance to low pH and reasonable robustness. A thorough review of existing scientific literature was done in order to select a yeast strains for lactic acid production at low pH, using hydrolysates as raw material. A set of potential strains were obtained from manuscript authors and evaluated for production of lactic acid in synthetic growth medium. The strains tested included laboratory strains expressing the lactate dehydrogenase from a plasmid as well as strains with the lactate dehydrogenase integrated to the genome. We also received and tested laboratory strains of different

origin and strains developed through adaptive laboratory evolution, targeting an increase lactic acid tolerance (Baek et al., 2016a; Baek et al., 2016b; Totaro et al., 2020). Despite advantages such as high expression and relatively easy genetic manipulation, the use of plasmids selected through auxotrophic markers is not feasible in complex hydrolysates and adopting received plasmid strains for our project would have demanded quite some work. Moreover, initial tests of received strains revealed much lower or even no lactic acid production under our preferred conditions. Therefore, we decided to construct our own lactic acid production strain.

The growth of various *S. cerevisiae* strains in lignocellulosic hydrolysate was assessed, demonstrating great differences among the strains. The laboratory strain typically performed much worse compared to strains of industrial background or even wild type strains (Figure 1). Notably, the strains displayed little difference in performance when grown on glucose alone (Figure 1a, b), whereas growth in medium containing 5 g/l acetic acid was severely challenged for many strains (Figure 1c, d). Acetic acid is the most common inhibitor found in lignocellulosic hydrolysates and severely impacts microbes growing on hydrolysates (Guaragnella and Bettiga, 2021) and thus the economy of biorefinery processes. When the strains were screened in medium containing 70% wheat straw hydrolysate (containing ~5 g/l acetic acid), the differences between the strains were more pronounced (Figure 1e) and very little growth was seen in anaerobic conditions (Figure 1f). Previously semi-aerobic or even anaerobic conditions have been suggested as optimal for lactic acid production (Novy et al., 2018). The screening was done using a methodology we developed earlier, for screening strains directly in lignocellulosic hydrolysates (van Dijk et al., 2020).

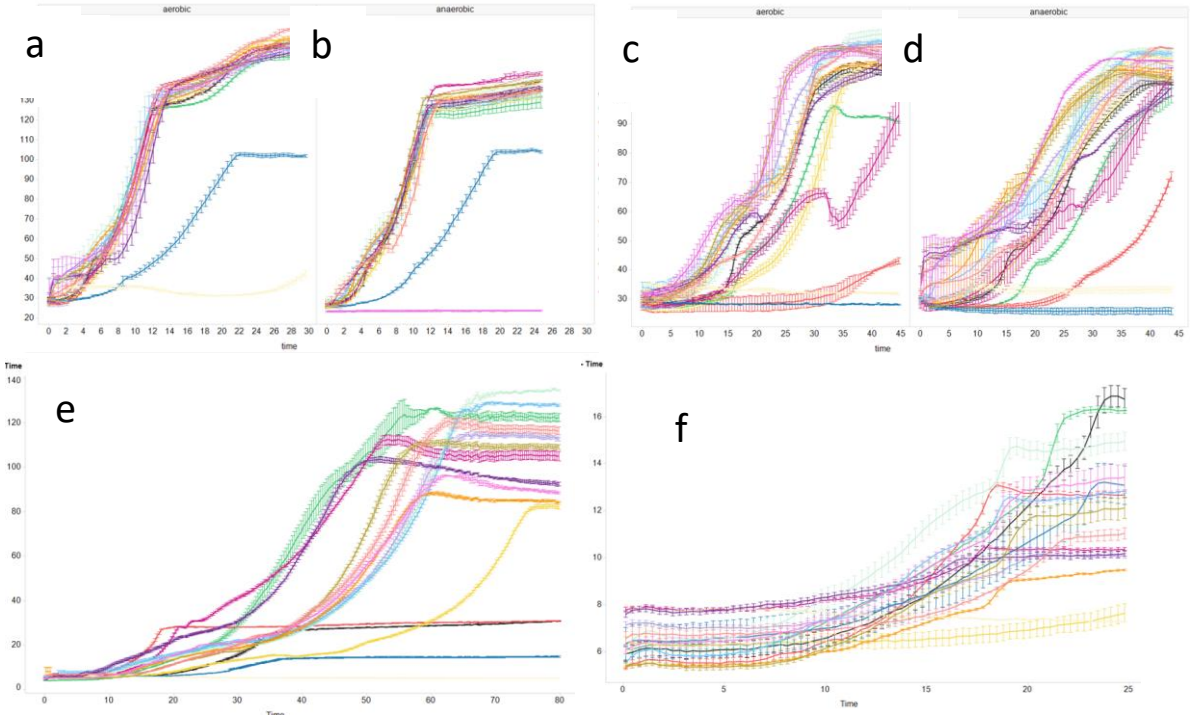

Figure 1. Cultivation of different *S. cerevisiae* strains in microbioreactors under aerobic (a, c, e) or anaerobic (b, d, f) conditions. The growth of the strains was analysed in a, b) in synthetic medium with glucose as a carbon source, c, d) in synthetic medium with glucose, supplemented with 5 g/l acetic acid or e, f) in medium containing 70% wheat straw hydrolysate (WSH). The reference strain (CEN.PK-113-7A, a commonly used laboratory strain) is shown in black.

A novel lactic acid production strain was constructed, based on an industrial, polyploid strain that we have worked with in the laboratory and know that performs well in lignocellulosic hydrolysates. An additional benefit of this strain is that is already made capable of fermenting xylose (insertion of *xyl1* and *xyl2* from *Scheffersomyces stipitis,* rewiring native metabolism to increase flux). We envisioned that our strain would be able to convert not only glucose (like typically is the case) but also xylose into lactic acid, therefore significantly increasing the lactic acid yield. The lactic acid production strain developed expresses multiple copies of a codon-optimized lactate dehydrogenase originating from *Bos taurus*. The strain was constructed using CRISPR/Cas9 based tools previously developed in the lab (Cámara et al., 2020).

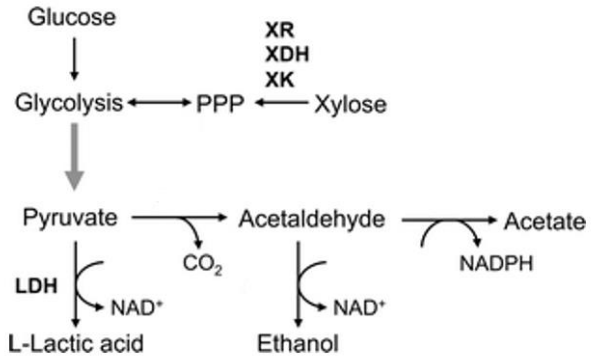

Figure 2: Conversion of lactic acid from glucose, through pyruvate demands expression of a lactate dehydrogenase (LDH) encoding gene. When conversion of xylose is desired also a xylose reductase (XR) and a xylose dehydrogenase (XDH) encoding gene needs to be expressed. Overexpression of the endogenous xylose kinase (XK) encoding gene can increase the xylose consumption. Figure adapted from (Turner et al., 2015).

WP2. Process optimization

As hydrolysates commonly contain various amounts of both xylose and glucose, co-consumption of these carbon sources is crucial. Our engineered strain performs very well in medium containing xylose and glucose, or even in medium with xylose as sole carbon source (Figure 3a). Moreover, our strain shows a great tolerance to low pH; similar growth was seen at pH 3-6.8 (Figure 3b). Lactic acid production at low pH is beneficial for down-stream purification and less prone to contamination in industrial settings (Abedi and Hashemi, 2020). Our strain showed good tolerance to acetic acid (Figure 3c), the major inhibitor in lignocellulosic hydrolysates (Guaragnella and Bettiga, 2021) and to lactic acid (Figure 3d). Th growth was impaired at concentrations of >100 mM acetic acid (~6 g/l, below concentrations typically found in lignocellulosic hydrolysates). Similarly, the growth was stable until 250 mM (~22 g/L) and inhibited above 500 mM (~ 45 g/L) of lactic acid. Thus, for very high production titres lactic acid tolerance may need to be engineered. Adaptive laboratory evolution is another approach, that has previously been used for successfully increasing lactic acid tolerance in *S. cerevisiae* (Baek et al., 2017).

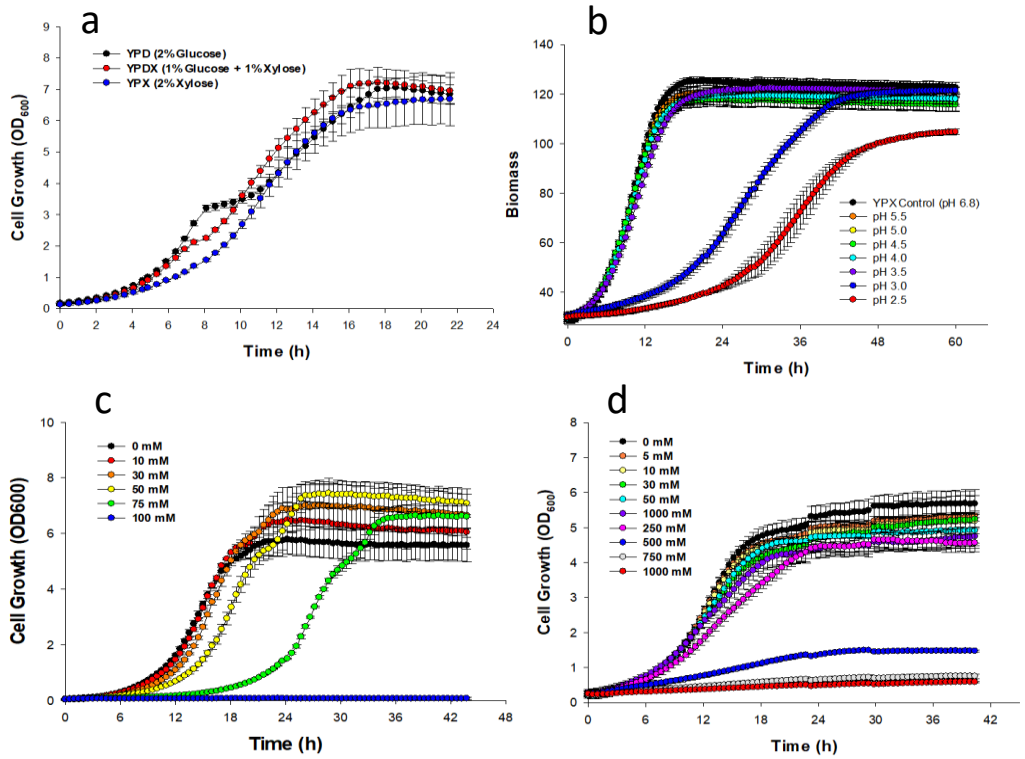

Figure 3. Cultivation of the host strain in synthetic medium with different carbon sources (a), at different pH (b) and with addition of varying amounts of acetic (c) and lactic acid (d). In b-d xylose was used as sole carbon source.

Finally, the production of lactic acid production from xylose was measured from cultures supplemented with varying amounts of acetic acid (Figure 4 a, b). Our lactic acid producing strain produced up to 10 g/l of lactic acid from 20 g/l of xylose (yield of ~0.5 g/g), when up to 3 g/l of acetic acid was present in the medium. When the acetic acid concentration was increased, less or no lactic acid was produced (Figure 4b). In line with this, when no acetic acid was added, the production was increased, to up to 12 g/l at pH 3.5 (Figure 4c). The performance of our lactic acid producing strain was in comparison to previously reported lactic acid titres and yield, at a very decent level, especially considering that the experiments were conducted in non-controlled conditions. We further tested lactic acid production at different pH and in medium containing glucose instead of xylose (Figure 4 c, d). With our strain (two different variants, with different genetic modifications tested), the lactic acid production was significantly higher at low pH (Figure 4c) and when xylose was used as a carbon source instead of glucose (Figure 4d).

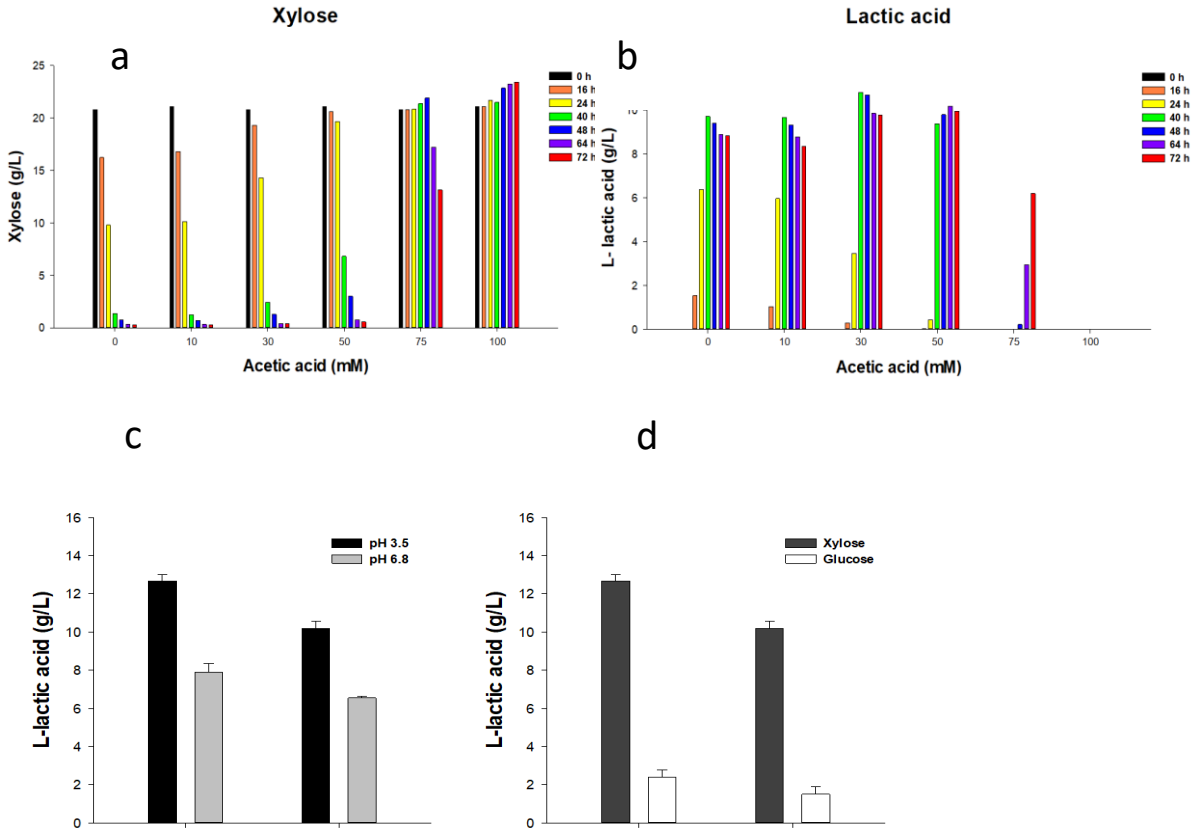

Figure 4. Xylose consumption (a) and lactic acid production at different concentration of acetic acid (b), or at different pH (c). In d) the effect of xylose vs. glucose as a carbon source was estimated. In c, d) two different variants of the production strain, with different genetic modifications were tested at a pH of 3.5.

Conclusions and future outlook

In this project, we successfully established lactic acid production from xylose at high titre and yield, using medium mimicking lignocellulosic hydrolysate composition, so-called synthetic hydrolysates. The physiology of the production host chosen was characterized, and the genetic improvements done to the strain increased its value as a future industrial lactic acid production host. In particular, the strain developed performs well at low pH and with xylose as a substrate. Future work will focus on improving the strain further, characterizing it in different lignocellulosic biomasses as well as optimizing the production process in bioreactors. The work showed great potential as a steppingstone towards commercial production of lactic acid from plant biomass. Financial support from the Åforsk foundation is greatly acknowledged.

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