Single cell measurements of enzyme level as a predictive tool for cellular fates during organic acid production

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Running title: Single cell measurements predict acidification fate
Abstract:

Organic acids derived from engineered microbes can replace fossil-derived chemicals in many applications. Fungal hosts are preferred for organic acid production because they tolerate lignocellulosic hydrolysates and low pH, allowing economic production and recovery of the free acid. However, cell death caused by cytosolic acidification constrains productivity. Cytosolic acidification affects cells asynchronously, suggesting that there is an underlying cell-to-cell heterogeneity in acid productivity and/or in resistance to toxicity. We used fluorescence microscopy to investigate the relationship between enzyme concentration, cytosolic pH and viability at the single cell level in \textit{S. cerevisiae} engineered to synthesize xylonic acid. We found that cultures producing xylonic acid accumulate cells with cytosolic pH below 5 (referred to here as “acidified”). Using live-cell time courses we found that the probability of acidification was related to the initial levels of xylose dehydrogenase, and sharply increased from 0.2 to 0.8 with just a 60% increase in enzyme abundance (Hill coefficient >6). This “switch-like” relationship likely results from an enzyme level threshold above which the produced acid overwhelsms the cell’s pH buffering capacity. Consistent with this hypothesis, we showed that expression of xylose dehydrogenase from a chromosomal locus yields ~20 times less acidified cells and ~2-fold more xylonic acid, relative to expression of the enzyme from a plasmid with variable copy number. These results suggest that strategies that further reduce cell-to-cell heterogeneity in enzyme levels could result in higher productivity.
in additional gains in xylonic acid productivity. Our results demonstrate a
generalizable approach that takes advantage of the cell-to-cell variation of a clonal
population to uncover causal relationships in the toxicity of engineered pathways.
Introduction

Replacing and/or supplementing fossil fuel-based production of chemicals and fuels with bio-based alternatives is a global challenge outlined in both an EU white paper “The European Bioeconomy in 2030” and the 2012 US “National Bioeconomy Blueprint”. To develop economically viable bulk production strategies (“biorefineries”) for bio-based chemicals and fuels, a suite of safe genetically tractable and robust microbes that are resistant to inhibitors in lignocellulosic hydrolysates and capable of high productivity, are needed.

Organic acids are the largest group of biomass-derived building blocks identified as priority targets by the US Department of Energy (DOE) (1) and the European Commission (2). Organic acids are cited as one of the top 30 high-value chemicals (DOE) because they have a wide range of potential applications – from platform chemicals to precursors for biomass-derived plastics (3, 4). Currently, gluconic acid is widely used in pharmaceuticals, food products, solvents, adhesives, dyes, paints and polishes (80 kilo-tons/year)(5). The rising price of glucose has focused attention on the potential of substituting bio-based xylonic acid for gluconic acid in the applications cited above.

Recently, engineered strains of E. coli (6), Saccharomyces cerevisiae (7) and Pichia kudriavzevii (7) were described that produce xylonic acid efficiently at a laboratory-scale using a xylose dehydrogenase from Caulobacter crescentus [39.2 g/l xylonic acid from 40 g/l xylose (E. coli); 43 g/l xylonic acid from 49 g/l xylose (S. cerevisiae); and 146 g/l xylonic acid from 153 g/l xylose (P. kudriavzevii)]. Fungal species are
preferred industrial production organisms because of their low nutrition requirements and tolerance to growth inhibitors in lignocellulosic hydrolysates. In
S. cerevisiae and P. kudriavzevii cultures xylonic acid production can occur at pH 3 (8), which is advantageous to the development of bulk production strategies for acids, because acid can be recovered directly from the spent medium and contamination by undesired microorganisms is minimized. S. cerevisiae is generally regarded as safe -it has been used for millennia in baking, brewing and large scale production of ethanol. It is anticipated that fungal laboratory-scale systems can be further developed and scaled to industrial-scale bio-based refineries that will require use of concentrated lignocellulosic hydrolysates as starting materials.

We used single cell methods to study the behavior of Saccharomyces cerevisiae cells engineered to synthesize xylonic acid (5). In this simple system the introduction of one enzyme, NAD+-dependent xylose dehydrogenase (encoded by the XylB gene from Caulobacter crescentus) directs synthesis of xylonic acid from xylose. XylB catalyzes the oxidation of xylose to xylonolactone coupled to the reduction of NAD+ to NADH+H+ (7). Xylonolactone is hydrolysed to xylonic acid either via a spontaneous reaction or catalyzed via a yeast lactonase that has not been identified (7). Xylonic acid production in S. cerevisiae causes a significant and progressive loss of metabolic activity (as assessed by methylene blue staining; 16 +/- 2% by 25 hr (CEN.PK strain) and 77 +/- 1% by 120 hr (B67002 strain)) and loss of cell viability (the percentage of viable colony forming units) over time (7, 9). A similar but less drastic effect on metabolic activity and cell viability was seen in P. kudriaevii cultures engineered to produce xylonic acid (8).
Here we explored the basis for heterogeneity in the sensitivity of cells to xylonic acid-induced acidification. We hypothesized that by applying single-cell analytical approaches we would be able to define cell states that are predictive of the differential sensitivity to acidification. Previous studies using a similar rationale uncovered fundamental regulatory mechanisms in yeast, bacteria and worms (10-17). When applied to a bio-based production system, such understanding could inspire innovative genetic modifications that are useful to improve production strategies.

To achieve our goals we needed to measure cytosolic pH non-intrusively, which can readily be achieved by expressing a fluorescent protein-based pH reporter. We used ratiometric pHluorin (herein "pHluorin"), a mutant of *Aequorea victoria* GFP (18). The ratio of pHluorin 510 nm fluorescence emitted under excitation at two different wavelengths (410 nm and 470 nm) can be used to measure intracellular pH between pH 5 and pH 9. Using pHluorin, Smits and collaborators showed that the pH of the yeast cytosol progressively acidifies during batch growth (19) from pH ~7.5 when inoculated to pH ~5.5 in stationary phase. However, no fluorescent protein-based pH reporter has been shown to perform at pH’s lower than 5. Such low pH’s induce the unfolding and loss of fluorescence of GFP and many of its derivatives (20).

Here we relied on a combination of pHluorin fluorescence (for pH’s above 5) and fluorescence from cellular metabolites (for pH’s below 5) to show that individual *S. cerevisiae* cells producing xylonic acid enter a path of cytosolic acidification at different times during culturing. The probability of early acidification depended on
the level of xylose dehydrogenase in the individual cell. Strains that generate a large
fraction of cells with xylose dehydrogenase levels above the threshold exhibit a
larger subpopulation of cells with acidified cytosols and a lower yield of xylonic acid.
These findings suggest that strain design strategies that maintain the single-cell
level of xylose dehydrogenase within a narrow range of high expression (just below
the acidification threshold) may lead to substantial gains in the yield of xylonic acid
in production cultures. Our work exemplifies a generalizable analytical approach
that uses naturally occurring population heterogeneity to determine cause and
effect relationships and provides an example of how such understanding can inform
the design of new approaches to improve metabolic engineering strategies.

Materials and Methods

Strains and DNA constructs:
All strains and plasmids used are listed in Tables 1 and 2. The construction of yeast
strains and plasmids are described in the Supplemental Materials.

Laboratory scale cultures:
Yeast were cultured in glass tubes (height, 20 cm; diameter, 4 cm) at 30°C, rotated
at ~30° angle. All strains were first inoculated in synthetic yeast media with 2%
glucose (SDC) and grown overnight to saturation. Xylinic acid-producing and
control cultures were inoculated at OD₆₀₀ 4 in synthetic yeast media with or without
2% xylose (and no other carbon source). *Saccharomyces cerevisiae* yeast are unable
to use xylose as a carbon source for cell growth. In these conditions xylonic acid-
producing cultures produced ~1.5 g/L Xylonic acid in 24 h and up to 7 g/L maximum, after 4-5 days. In both control and xylonic acid producing cultures the media acidified from pH ~5 to pH ~3 in the first 5-10 hours of culture due to the consumption of ammonium sulfate.

**Media and culture conditions**

Yeasts were grown in synthetic complete medium (SC, Sherman 1983), lacking specific amino acids or uracil, as needed to select unstable constructs in the strains used in each experiment (MP Biomedicals, #4550-412). When indicated G418 Sulfate (Fisher, BP673-1) was added at 200 μg/L. Carbon source was 2% glucose (SDC) (ACROS, #41095-0010) or 2% xylose (SXC) (SIGMA X1500).

For xylonic acid producing cultures we first grew an SDC overnight inoculum from a frozen stock. Cells from the inoculum were harvested by centrifugation and inoculated in SXC (xylonic acid production) or SC (control) at OD 4. For experiments not involving estradiol the inoculum was grown for approximately 24 h. For estradiol experiments a 24 h culture was used as a seed culture for cultures containing different doses of estradiol, which were themselves grown for 24 h. The volume of the culture was between 5 and 15 mL.

1 mM estradiol stocks (SIGMA E8875-1g) were prepared in ethanol and stored at -20 °C for less than 1 week. Estradiol was diluted for each experiment in ethanol to 200x the final concentration, which was then added to the media at the required dose.
Xylonic acid measurements

Xylonic acid concentrations were measured using the hydroxamate method (21), as described (9). First, the sample is heated in acid, which converts xylonic acid to xylonolactone. Second, a hydroxylamine reaction with the lactone esters at neutral pH is used to obtain quantitative amounts of hydroxamate, which in a third step binds iron(III) in acidic conditions yielding a colored complex. This method determines the combined amounts of xylonolactone and xylonic acid in the sample.

Microscopy methods

For all imaging we attached cells to glass-bottom 96- or 384-well plates (BD Falcon, #357312) using concanavalin, as described (22). The microscope room was maintained at 30 °C. Cultures were loaded into the wells, allowed to settle and then washed repeatedly to remove unbound cells. When imaging cells in the same environment that they were being cultured in, the washes were done with the supernatant of a centrifuged culture sample ("spent media"). For the XylB-mCherry time courses, the washes were in fresh SXC or SC media at 30°C. We imaged cells in a Nikon TE2000 inverted fluorescence microscope with a Plan Apo oil-immersion 60x objective (NA=1.4). We controlled bright field and fluorescent lamp shutters (Uniblitz, Rochester, NY), and a 6-position rotating filter cube turret with Metamorph v7.1 (Molecular Devices, Sunnyvale, CA). Filters were from Chroma Technology Corp (Bellows Falls, VT). For mCherry and propidium iodide we used the #49910 set. For pHluorin we used the following custom filters: 410 nm excitation: D410/30x, 500dcxr, HQ535/50m, 470 nm excitation: D470/20x,
500dcxr, HQ535/50m. Live cell time courses were imaged manually. We processed images using custom-made software CellID (22, 23). We analyzed the CellID output using Physics Analysis Workstation (PAW) (24) and R (25). To calculate the relationship between initial mCherry fluorescence and the probability of acidification we used Generalized Linear Models in R (26). Standard errors were estimated using 1,000 bootstrapping iterations (27). We transformed the logistic equation coefficients into their Hill equation equivalents for easier interpretation (details available upon request). To calculate the percent of cells predicted correctly we counted how many cells with a probability smaller than 0.5 did not acidify and how many cells with probability larger or equal than 0.5 did acidify, and expressed the sum of the two values as percent of total cells.

**Vitality staining with propidium iodide**

For vitality staining, 100 ng/mL propidium iodide (Invitrogen, #P3556) was added to the wash media and incubated for 10 min.

**pHluorin calibration**

We calibrated pHluorin fluorescence at various cytosolic pH’s using cells fixed with a brief incubation with paraformaldehyde and in the presence of 20 μM nigericin. To fix the cells we added 1 volume of 8% m/v paraformaldehyde 2x PBS to the culture media, incubated on ice for 5 min, then centrifuged and resuspended the supernatant in ice-cold PBS buffer, then repeated the last 2 steps. We compared this treatment with adding 0.2% azide or no treatment. PFA fixation was the only treatment that yielded pHluorin readings in buffers at pH 7-7.5 that matched the
readings obtained in cells growing exponentially in SDC. To change the cytosolic pH’s to desired values we used buffered solutions as described (28) containing 150 mM KCl, 20 μM nigericin and the following buffering agents at 50 mM concentration: for pH’s 5.5 and lower: sodium acetate, for pH’s 6 to 6.75, morpholine-ethane-sulfonic acid (MES), for pH’s 7 to 7.8, morpholine-propane-sulfonic acid (MOPS), for pH’s 8 and above, Tris. 1 N HCl and 1 N NaOH were used as appropriate to bring the buffered solutions to the desired pH. To image the cells we bound them to concanavalin-coated glass as above but instead of using media we washed them and incubated them in the corresponding buffered solution. We first imaged time courses on the fixed cells to determine when the signal had stabilized (after 5 min) and how long it remained stable (1 h or more)(data not shown). We then imaged cells within those time limits, incubated at the pH’s indicated in Figure 2B.

Results

System to study cell death in acid producing cells

We used a small-scale culture system to investigate cytosol acidification and loss of viability in xylonic acid-producing yeast cells expressing ratiometric pHfluorin (18). pHfluorin was driven by the TPI1 gene promoter and expressed from constructs integrated in a yeast chromosome. To increase the signal-to-noise ratio in the pHfluorin measurements we constructed strains with 15-20 integrated copies of the pHfluorin expression construct (see Materials and Methods).

We cultured a xylonate producing strain and a control strain in 2% xylose and followed the cultures for about 70 hr (note that S. cerevisiae yeasts are unable to use
xylose as a carbon source for cell growth). We noticed that in the xylonic acid producing culture a fraction of the cells lost pHluorin fluorescence while in the control culture the pHluorin fluorescence was maintained (Figure 1A, compare control vs XylB, pHluorin panels). We refer to the cells that lost pHluorin fluorescence as “dark cells”, defined as having less than 17 fluorescent units per volume, F.U./vol, at 410 nm excitation. The fraction of dark cells increased over the time of incubation; at 68 hours 85% of the cells in the xylonate producing strain were dark (Figure 1C). We used propidium iodide (PI) staining to test the metabolic activity of cells. PI fluoresces red when bound to nucleic acids and is actively pumped out of metabolically active cells. Only a subset of the cells stained with PI; all cells that stained with PI were dark cells, but not all dark cells stained with PI (Figure 1B). The fraction of dark cells that stained with PI increased over time, with 25% of dark cells being positive for PI at 52 h and 77% at 68 h (Figure 1.B.ii).

Xylonic acid and xylonolactone accumulated to ~4 g/L in the culture media during the initial 24 h of culture in xylose (Figure 1.C). After 24 h, coinciding with a rise in the accumulation of dark cells, xylonic acid and xylonolactone accumulation was slower, reaching ~ 7 g/L by 72 h. These results suggest that yeast cells producing xylonic acid undergo a process of cell death with variable start times. During this process the cells first lose pHluorin fluorescence, but remain metabolically active and, later, lose metabolic activity and presumably die which compromises the xylonic acid productivity of the culture.

Loss of pHluorin fluorescence and pH-dependent autofluorescence at acid pH's
Previous studies used pHluorin to measure intracellular pH in yeast and other systems in the pH range 5-9 (18, 28, 29). We interpreted the appearance of dark cells in our xylonic acid producing cultures as meaning that their cytosols had acidified below pH 5.5, because at such acid pH’s wild type GFP unfolds and loses fluorescence (30). We tested this interpretation experimentally using permeabilized cells in buffered solutions (pH’s 3-9) (Figure 2). Below pH 5 the population average of fluorescence from pHluorin in single cells was substantially reduced at both excitation wavelengths (410 and 470 nm); at pH 4.3 and below the fluorescence signal matched the average signal of untransformed cells (Figure 2.i-ii, the level of fluorescence of untransformed cells is marked with dotted lines). This decrease in fluorescence resulted in dark cells reminiscent of those in xylonic acid producing cultures. The $R_{410/470}$ increased with increasing pH above pH 5 (Figure 2.iii). From pH 5 to pH 4.3, the fluorescence from pHluorin dimmed and the $R_{410/470}$ was relatively stable. Below pH 4.3 the $R_{410/470}$ of pHluorin-expressing cells and of untransformed reference cells was similar (see Figure S1 in the Supplemental Materials). Below pH 4.3, the $R_{410/470}$, arising from autofluorescence, showed a dependence on pH that is the inverse of the $R_{410/470}$ above pH 5. This causes the $R_{410/470}$ from pH 3-9 to have a biphasic relationship to pH (Figure 2.ii). A given $R_{410/470}$ value in pHluorin-expressing cells can correspond to two different pH’s: one pH in the 5-9 range (pHluorin signal) and a second pH below 4.3 (due to cellular autofluorescence). Bright cells that have cytosols at pH > 5 can clearly be distinguished from dark cells with cytosols at pH 4.5 or lower when both 410-nm fluorescence and $R_{410/470}$ values are plotted for single cells (Figure 2.iv).
studies below we exploited these properties of the 410-nm fluorescence and R_{410/470 vs pH relationships to monitor the cytosolic pH of single cells as they acidify below pH 5 during xyloic acid synthesis.

**Cytosol acidification in xyloic acid producing cultures is cell autonomous**

We measured R_{410/470 to monitor cytosol acidification in xyloic acid producing cultures. At the start of the experiment both the xyloic acid producing culture and the reference culture had a cytosolic pH ~6 (data not shown), which is in agreement with published values for yeast cultures in stationary phase (19). 6 hr after the addition of 2% xylose, the cytosol of the xyloic acid producing cells shifted to pH ~5.5 while in the reference strain the ratio still reflected pH ~6 (Figure 3.i). By 32 hr the cytosolic pH in the reference cells acidified uniformly to pH ~5.5 (Figure 3.ii), which was maintained through 52 hr (Figure 3.iii). In contrast, at both 32 and 52 hr xyloic acid-producing cells showed clear evidence of cytosolic acidification below pH 5. The 410 nm-excitation fluorescence in xyloic acid-producing cells progressively decreased as compared to reference cells (Figure 3. i-iii) in a fraction of xyloic acid-producing cells. These dimming cells had R_{410/470 that corresponded to cytosolic pH’s lower than 5. Thus, production of xyloic acid caused the cytosolic pH of some cells to drop below pH 5. In the remainder of this article, unless stated otherwise, we will use the term “acidify” (and its derivatives) only in reference to this extreme type of cytosol acidification. To capture the temporal evolution of acidification we imaged single cells over time (22). The single cells time courses suggest that, once acidification starts, the xyloic-acid producing
cells follow a stereotypical path to terminal acidification that takes ~8-hours (See Figure S2 in Supplemental Materials).

We considered two possible explanations for the cytosolic acidification we observed in the xylonic acid-producing cultures. In one view, acidification is a direct consequence of the synthesis of xylonic acid from xylose within each individual cell. In this view acidification is a cell-autonomous process (in the sense that it does not result from interactions with other cells). Alternatively, and not exclusively, the combination of chemicals secreted by the xylonic acid-producing cells, including xylonolactone and xylonic acid, could be toxic (note that we have previously shown that high levels of xylonic acid added to yeast cultures are not toxic (9)). In this alternative, acidification is a cell non-autonomous process.

To distinguish between these two possibilities we co-cultured xylose-producing with non-producing cells (Figure 4). We used a XylB-expressing strain marked with resistance to G418 (XylB cells) and a reference strain that express a red fluorescent protein (mRFP) driven by a constitutive promoter and carries a distinct selectable marker (resistance to hygromycin B, control cells). We analyzed co-cultures of these two strains or isolated cultures of each strain (Figure 4.A.i). We measured cell viability in two modes of culture: high density cultures with 2% xylose or exponential cultures grown with 2% glucose (Figure 4.B). We used antibiotic selection and a plating strategy to distinguish XylB and control cells. We also quantified cytosolic acidification by means of 410-nm fluorescence and R_{410/470} values, using a fluorescence microscope strategy that used mRFP fluorescence to distinguish XylB and control cells in the mixed cultures (Figure 4.C.i-ii). The table in
Figure 4.A.ii summarizes predicted results predicted for the mixed cultures in 2% xylose. If cytosol acidification was cell autonomous, we would expect both the XylB and control cells to lose viability and to show acidified cytosols. If, instead, cytosolic acidification were cell non-autonomous, only the XylB cells would be affected. Our results support the hypothesis that acidification is a cell-autonomous process (Figure 4). XylB cells show an approximate 90% reduction in viability (Figure 4.B, compare “Glucose XylB” row with “Xylose XylB” row), while control cells show almost no loss of viability. In 2% xylose cultures, the frequency of acidified XylB cells was similar in the presence or absence of control cells (Figure 4.C.ii). In contrast, very few control cells in the mixed culture had acidified cytosols (Figure 4.C.i). Thus, cytosolic acidification in xylonic acid-producing cultures is largely a cell-autonomous process. However, the occurrence of a small number of dark cells among the control cells in the mixed culture (and not in the single-strain culture of control cells) indicates that changes in the media can drive some naïve, non-producing cells to acidification. Also, the population of control cells in the mixed culture showed a small but coherent drop in pH (in average from pH 5.5 to pH 5; Figure 4.C.i). This is further evidence that non-producing cells, while not being driven to acidification, are affected by the changes in the media that result from the xylonic acid producing cells. Our results suggest that cytosol acidification in xylonic acid-producing cells is primarily a consequence of the accumulation of xylonic acid or xylonolactone (or some metabolite) within the cells. XylB expression levels correlate with the probability of acidification.
We hypothesized that differential susceptibility to acidification was determined by pre-existing cellular parameters. To test whether the abundance of xylose dehydrogenase was the determining parameter we constructed strains expressing XylB fused to the fluorescent protein mCherry at its C-terminus (XylB-mCherry) and expressed it at different levels using the GEV/ \( P_{GAL1} \) inducible gene expression system (31). In this system the yeast \( GAL1 \) gene promoter is induced by estradiol, which in yeast is a nearly gratuitous inducer (32). The abundance of XylB-mCherry in saturated cultures as a function of estradiol dose followed a first-order non-cooperative curve (nH=0.9±0.04, E.C.50=9.6±0.3 nM) (Figure 5.A.i). XylB levels showed substantial cell-to-cell variability (note the size of the standard deviation error bars in Figure 5.A, the coefficient of variation is ~0.35 near the E.C.50 dose and ~0.31 at saturating doses). We compared the xylonic acid productivity of strains expressing either mCherry-tagged or untagged XylB controlled by the GEV/ \( P_{GAL1} \) system. At estradiol doses equal or lower than the EC50 dose and after 24 h in 2% xylose media, both strains had similar productivity (Figure 5.A.ii) showing that the XylB-mCherry fusion resulted in a functional enzyme. However, at higher estradiol doses the productivity of the XylB-mCherry strain was markedly lower than the productivity of the XylB strain (Figure 5.A.ii). We hypothesized that the mCherry tag had changed the activity or abundance of the tagged enzyme, such that the XylB-mCherry cells produced xylonic acid at a faster initial rate, which more rapidly overwhelmed the ability of a cell to prevent cytosolic acidification. To test this hypothesis we measured cytosolic acidification in the strains expressing tagged and untagged XylB controlled by the GEV/ \( P_{GAL1} \) system and compared them with a
strain where untagged XylB is controlled by the \textit{PGK1} promoter (Figure 5.B). At a saturating estradiol dose (81 nM) after 22 h in 2% xylose media, the two strains showed a similar fraction of acidified cells (Figure 5.B.i-ii). In the same conditions, the \textit{GEV/ P}_{\textit{PGK1}} \textit{XylB} strain (Figure 5.B.iii) showed a $\sim$2-fold higher accumulation of acidified cells (72% vs 39%) than the \textit{GEV/P}_{\textit{GAL1} XylB} strain. These results support the notion that the strain expressing the mCherry tagged enzyme accumulates more xylonic acid and thus acidification is visible in a higher fraction of the cells.

These results suggest that the strain expressing XylB-mCherry might show a faster time course of acidification than what we observed in the \textit{P}_{\textit{PGK1} XylB} strain (Figure 1). To test this possibility we measured cytosolic pH in the \textit{GEV/ P}_{\textit{GAL1} XylB-mCherry} strain during the initial period of incubation with xylose. We imaged cells expressing pHluorin and XylB-mCherry for 6 hours using cultures exposed to different doses of estradiol (Figure 5.C). We found rapid cytosolic acidification in a subset of the cells (Figure 5.C.i). During the 6 hour-time course the cells largely followed two distinct paths, which we refer to as “acidifying” and “non-acidifying.” The cytosolic pH of the non-acidifying cells started at $\sim$7.4 and dropped to $\sim$5.5 (Figure 5.C.ii-iii). The cytosolic pH of acidifying cells started at $\sim$7.4 but dropped to below pH 5. This drop causes a complete loss of pHluorin fluorescence and an increase in R\textsubscript{410/470}. The two types of cells (“acidifying” and “non-acidifying”) also showed characteristic R\textsubscript{410/470} vs. time curves (Figure 5.C.iii).

We tested the effect of modulating XylB levels on the ratio of acidifying to non-acidifying cells. We used concentrations of estradiol that induce to $\sim$50% (10
nM), ~90% (30 nM) and ~99% (150 nM) of maximal levels (Figure 5.C.iv). The increasing levels of XylB are correlated with an increase in the fraction of acidifying cells (6%, 20% and 43% respectively). Is the abundance of xylose dehydrogenase in an individual cell a major determinant of its fate? (Figure 6). We marked acidifying cells (black) on the distribution of XylB-mCherry fluorescence for all cells (red) from the time-course experiments (Figure 6.i-iii). At all doses, the acidifying cells correspond to cells with higher initial levels of XylB-mCherry expression than the majority of the population. To quantify the extent to which XylB levels influenced the acidification fate we used logistic regression (33) (Figure 6.iv-viii). Our analysis shows a strong correlation between initial XylB levels and the probability of acidification (Hill coefficients >6; Figure 6.viii). For 150 nM estradiol the probability of acidification increased from 20% to 80% when XylB-mCherry fluorescence increased from 1550±327 to 2450±683 F.U./vol. This narrow transition indicates that the fluorescence level of the tagged enzyme is a strong predictor of acidification. In fact, for 150 nM estradiol the calculated probability of acidification correctly predicts the acidification fate for 78.5±4% of the cells (Figure 6.viii).

Cultures with lower cell-to-cell variation in XylB levels have higher productivity

Cultures with high cell-to-cell variation in expression levels of xylose dehydrogenase might yield lower-than-expected amounts of xylonic acid due to acidification of cells with an enzyme content above a distinct threshold. To test this hypothesis we generated two strains expressing XylB-mCherry under the control of
the same constitutive promoter (from the PGK1 gene), but from different loci. In one strain (the “single copy” strain) one copy of the XylB construct was integrated in the genome. In the second strain (the “variable copy number” strain) the construct was carried in an episomal plasmid with a centromere/autonomous replication sequence. Such plasmids are present in one copy in most cells but do occur in two or more copies in some cells. In cells with two or more copies of the plasmid, XylB levels should be increased in proportion to the gene dosage. The measured gene expression results corresponded to these expectations (Figure 7A.i-ii). The mean XylB-mCherry level in the variable copy number strain was higher (1.74 fold) than the single copy strain (Figure 7A.i-ii). Also as expected, variability in XylB-mCherry levels was noticeably larger for the variable copy number strain (coefficients of variation (CoV) of mCherry fluorescence were 0.78 for the variable copy number strain and 0.2 for the single copy strain (a 3.7-fold increase; Figure 7A.i-ii). In the single copy strain XylB-mCherry levels were tightly distributed around the mean: 97% of the cells fell within a 20% interval around the mean (dotted gray line in Figure 7A.i-ii,v-vi corresponds to ±2 standard deviations.). In the variable copy number strain, only 46% of the cells fell within the same interval. Importantly, 36% of cells in the variable copy number strain had XylB-mCherry levels higher than 98% of the cells in the single copy strain (Figure 7A.ii). This group of cells likely corresponds to cells that have two or more copies of the plasmid. Despite having a higher mean enzyme content and one third of the population of cells with higher XylB-mCherry enzyme levels than the single copy strain culture, the variable copy

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number strain culture produced half the amount of xylonolactone and xylonic acid in 24 hr (1.8±0.2 vs. 4±0.3 g/l) (Figure 7B).

We used live-cell time courses to correlate initial XylB-mCherry levels with acidification fate. In the variable copy number strain 31% of the cells acidified, during the 5-hour-time course (Figure 7A.iv, black traces are acidifying cells and red traces are non-acidifying cells). The cells that acidified corresponded to cells with high enzyme content (Figure 7A.vi, red bars correspond to the total cell distribution and black bars correspond to the acidifying cells). In the variable copy number strain the enzyme levels of the acidifying cells of were higher than 98% of the cells in the single copy strain. In contrast, in the single copy strain only ~2% of the cells acidified (Figure 7A.iii), and those cells were scattered throughout the range of XylB-mCherry levels (Figure 7A.v). The low level of acidification in the single copy strain was similar to that seen in a control culture that was not incubated with xylose (data not shown). Thus, cultures containing a large subset of cells expressing levels of xylose dehydrogenase that cause acidification, can yield lower amounts of xylonic acid because of faster cytosolic acidification in these cells. In the strain in which the P_{PGK1} promoter-XylB-mCherry construct is integrated in a chromosome the XylB-mCherry levels are below the threshold level at which cells acidify, in this time-frame.

Discussion

Heterogeneity in the behavior of genetically identical cells that share an environment provides a way to determine cause and effect relationships (34). Here
we used single cell analytical tools to show that the cytosol of *S. cerevisiae* cells producing xylonic acid acidifies below pH 5 with a timing that is highly variable from cell to cell and which can be predicted by the level of xylose dehydrogenase (XylB) in individual cells. Cells with XylB levels above a threshold were more likely to undergo early cytosolic acidification. Cultures of strains with a lower fraction of such cells produced higher yields of xylonic acid. These results show that by controlling XylB levels within a narrower range of values (below the toxicity threshold) the productivity of xylonic acid is increased. Strategies that achieve an even narrower distribution of XylB levels may achieve a further increase in productivity (35-39). More broadly, our results show that single cell measurements can reveal useful causal relationships that can be used to enhance the design of metabolic engineering projects. This highlights the importance of studying cell-to-cell heterogeneity to optimize production strategies.

Our single cell measurements show that XylB levels above a narrow range trigger rapid cytosol acidification. These results suggest that cellular mechanisms that monitor and control cytosolic pH can effectively counter the accumulation of acid products up to a certain level, above which they are overwhelmed. During cell growth the metabolic oxidation of carbon sources generates large amounts of organic and inorganic acids (40, 41). Cells maintain cytosolic pH homeostasis by controlling the rate at which protons are transported out of the cytosol (to the extracellular environment or into the vacuole) and also by controlling metabolism, which can consume acids, as well as generate them (40). Interestingly, of these two mechanisms, metabolism control is the major source of cytosolic pH control in
Neurospora crassa cells grown in sucrose (41). The fact that the XylB gene is of bacterial origin, and thus encodes an enzyme that is not subject to any known yeast metabolic regulation, may be an important determinant of the failure of pH homeostasis in xylonate synthesis conditions.

Our results also imply that the rate of xylose oxidation is limited by the amount of XylB, suggesting that in these conditions there is, in most cells, an unlimited supply of cytosolic xylose and oxidized NAD+. This may not be the case in other culture conditions, for example at other temperatures or when raw lignocellulosic feedstock is used as carbon source. As conditions are changed, new measurements will need to be made to determine the impact that XylB abundance has on cell fate. In agreement with this view, our own results show an apparent increase in the XylB level threshold for acidification of individual cells in which the XylB-mCherry construct is induced using the highest concentration of inducer (150 nM estradiol) as compared to the lowest concentration of inducer (10 nM estradiol) (Figure 6.C.X, note the rightward shift of the 10 nM estradiol (green) curve relative to the 150 nM estradiol (orange) curve). The increased sensitivity to XylB levels at the higher estradiol concentrations could be due to changes in the media (including increased accumulation of xylonate and/or xylonoactone) caused by the higher fraction of cells with high XylB levels in those wells. Consistent with this suggestion, we observed increased acidification of non-xylonic acid producing cells when co-cultured with cells that produce xylonic acid (Figure 4.i-ii).
Our findings depend on being able to measure cytosol acidification in single cells using fluorescence microscopy. We used the established ratiometric pHluorin reporter but were constrained by the reporter’s loss of fluorescence below pH 5. For future studies it would be advantageous to have a fluorescent pH-reporter that is resistant to acid-induced unfolding. Our studies suggest that the R410A417 of cellular autofluorescence below pH 5 is an approximate pH indicator. By combining the loss of pHluorin fluorescence with the decrease in pH as measured by the autofluorescence signal we were able to effectively identify and track cells undergoing cytosol acidification during time course experiments. This approach is useful to determine the timing of cytosol acidification in individual cells. Because cellular autofluorescence is subject to changes that depend upon cellular metabolism, the composition of the growth media, the strain genetic background and other unknown factors, the generality of this approach may be limited.

The functional unit of any bioprocess is the individual cell. The application of single cell analytical methods that we describe here to other metabolic engineering projects will allow further understanding of the role of cell-to-cell heterogeneity to production. That understanding can then be used to design strategies for increased productivity of bio-based chemicals, including fuels. The logistic regression analysis that we applied in this work (Figure 6C.vii-xi) can be readily extended to incorporate and combine multiple variables in a model using L1-regularized multivariate logistic regression (42). The approach allows researchers to identify cell states that are the most relevant correlates of the measured outcome. For example, fluorescent protein-based transcriptional reporters can be used to
measure specific stress pathways and other genetic programs in single cells during production. As single-cell methods to probe cellular metabolism become available, readouts other than toxicity and cytosolic pH can be used, such as ATP and/or NADH levels (43-45). A multiplex strategy that monitors a combination of cell states and behaviors assorted uniquely within each cell in a culture can reveal cause-and-effect relationships that would not be noticed using methods that rely on population measurements and/or cell lysates (34). The ability to establish cause and effect relationships in single cells is especially important when optimizing genetic interventions that aim to impose homeostatic control on the activity of an introduced metabolic pathway (38, 46).

Acknowledgements

Authors contributions

Project was formulated by CGP, OR, LR, MP and JJ. Experiments were designed by CGP and SZ and carried out by SZ, DW and KC. All authors participated in discussions of the findings. The manuscript was written by CGP and OR who guarantee the integrity of the results.

Acknowledgements

Authors thank Alan Bush (IFIByNE-CONICET and University of Buenos Aires, Buenos Aires, Argentina) for help with image analysis with Cell-ID and for implementing logistic analysis and calculating the relevant parameters, Marilyn Wiebe (VTT Technical Research Centre of Finland (VTT)) for advice on culturing
yeast cells for xylonic acid production and for pioneering work in this field, Mervi Toivari (VTT) for plasmids and strains used for expression of $XylB$ in yeast, Mari Valkonen (VTT) for reagents, technical advice and preliminary work with pHluorin in yeast cells producing xylonic acid, Yvonne Nygard (VTT) and Dominik Mojzita (VTT) for discussions, Peter Pryciak (University of Massachusetts) for reagents and advice on the GEV/$GAL1$ system and Yvonne Nygard (VTT) and Richard Yu (MSI) for comments on the manuscript. Research was supported by VTT Technical Research Centre of Finland.
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Figure Legends

**Figure 1** – Cultures that produce xylonic acid accumulate cells that lose pHluorin fluorescence and metabolic activity. GPY3016 (P<sub>PGK1</sub>-XylB, P<sub>TPI1</sub>-pHluorin) and GPY3020 (control, P<sub>TPI1</sub>-pHluorin) cells were cultured with 2% xylose. At the times indicated cells were attached to glass-bottom wells, imaged and stained with propidium iodide (PI). **A.** Brightfield or pHluorin channel images at 52 h. Note many “dark cells” (low signal) in the GPY3016 (XylB) culture as compared to the relatively uniform pHluorin fluorescence (“bright cells”) in the GPY3020 (control) culture. **B.i.** Overlay of 410 nm-excitation fluorescence, PI fluorescence and brightfield images at 32, 52 and 68 hr. **B.ii.** Quantification of fluorescence intensity of PI vs 410-nm excitation (times are as in Bi). Dotted lines mark threshold values for dark cells (F.U./vol=18) and PI positive cells (log (F.U./vol=2.7). Red circles represent cells lacking pHluorin fluorescence (dark cells) that stain with PI. **C.** Time course of the loss of 410 nm-ex. fluorescence relative to the production of extracellular xylonic acid + xylonolactone in GPY3016 (P<sub>PGK1</sub>-XylB, P<sub>TPI1</sub>-pHluorin) and GPY3098 (control, P<sub>TPI1</sub>-pHluorin) cells.

**Figure 2** – Permeabilized cells incubated in buffers pH 3-9 show a biphasic R<sub>410/470</sub> dependence on pH.

GPY3016 (P<sub>PGK1</sub>-XylB, P<sub>TPI1</sub>-pHluorin) cells were grown exponentially in 2% glucose. Cells were fixed, attached to glass, incubated with nigericin-containing buffers at the
indicated pH’s and imaged. i-iii. Circles indicate population averages of fluorescence in single cells, gray shading illustrates standard deviation from the mean. Solid blue circles: pH’s at which fluorescence is higher than in untransformed cells. Black outline circles: pH’s at which fluorescence is the same as in untransformed cells. i-ii. Fluorescence (per volume) at 470 nm-excitation (i) and 410 nm-excitation (ii), vs pH. Dotted lines mark the fluorescence levels of untransformed cells. iii. Population average of R_{410/470} in single cells vs pH. To the right of the dotted line, cells are bright and the R_{410/470} of pFluorin fluorescence increases with pH. To the left of the dotted line the R_{410/470} signal primarily reflects cellular autofluorescence. iv. Single-cell 410 nm-exc. fluorescence (per volume) vs R_{410/470}, for cells incubated in nigericin buffers at 5 pH’s (color coded in the inset). pH axes (for bright cells and dark cells) relate R_{410/470} values to pH’s inferred from iii. Note that acidification to pH 4.5 or below, causes a coherent shift of the populations to low 410 nm-exc. fluorescence and higher R_{410/470} values.

**Figure 3: The cytosol of cells producing xylonic acid acidifies below pH 5**

Cells producing xylonic acid progressively lose pFluorin fluorescence and show increasingly high R_{410/470} values that correspond to a pH below 5. GPY3016 (P_{PKI}^XylB, P_{TPPI}^XylB-pFluorin, red) and GPY3098 (control, P_{TPPI}^XylB-pFluorin, blue) were cultured with 2% xylose and analyzed as described in Figure 1 at 6 (i), 32 (ii) and 52 (iii) hours. **Top plots:** Distribution of R_{410/470} ratios in GPY3016 (red, control) and GPY3098 (blue, XylB). Note that the histograms for the cells producing xylonic acid (red) become progressively broader at 32 h and 52 h, while those for the control...
cells (blue) are compact at all time points. **Bottom plots:** Single-cell 410 nm-exc. fluorescence (per volume) vs R_{410/470} (same R_{410/470} axis as in top plot). pH axes as in Figure 2.iv. Note that cells producing xysonic acid progressively accumulate a distinct subpopulation of cells with low 410 nm-exc. fluorescence (“dark”) and high R_{410/470} at 32 h and 52 h. The dotted line marks the 410 nm-exc. fluorescence boundary between bright and dark cells.

**Figure 4 - Xylic acid production causes cell-autonomous cytosolic acidification.**

In co-cultures of xyonic acid-producing and non-producing cells, only producing cells lose viability and show cytosol acidification. A.i. GPY3016 (XylB cells: P_{PGK1-}

\textit{XylB}, P_{TP11-}pfluorin, G418-resistant, non-mRFP-expressing) and GPY3098 (Control cells: No XylB, P_{TP11-}pfluorin, hygromycin B resistant, mRFP-expressing) were cultured separately or together (at a 1:1 ratio) in two conditions: 1) 2% xylose, high density (as in previous experiments) and 2) 2% glucose, exponential growth. A.ii.

Predicted results for a cell autonomous and a cell non-autonomous response in the mixed (XylB + Control), 2% xylose, high-density culture. **Top table (Plating):** “*”

signs reflect the number of viable colonies relative to the mixed culture grown in 2% glucose, plated in the indicated media. **Bottom table (Imaging):** B. Plating assay showing viability of the cultures at 52 h. 3-fold serial dilutions of the indicated cultures were spotted on YPD plates with no additions (no antibiotic) or with either G418 or hygromycin B. Labels: Glucose: Cultures growing exponentially in 2% glucose, Xylose: Cultures incubated with 2% xylose at high density. Note the ~9-fold
reduction in viability in GPY3016 (XylB) cells cultured in xylose (no antibiotic and
G418 plates) or mixed with control cells GPY3098 (G418 plate). In contrast, the
viability of GPY3098 (control) was not reduced in the 2% xylose high density
culture relative to the 2% glucose, exponential phase culture (no antibiotic and
hygromycin B plates), in the absence or presence of GPY3016 (XylB)(Hygromycin B
plate). C. 410 nm-excitation fluorescence (per volume) and pH (inferred from
R410/470, not shown) at 52 h. Single-cell data from images of cells in the mixed
culture were manually separated based on their mRFP signal into producing and
non-producing cells, and compared to images of the same strain grown in isolation.
C.i. GPY3098 (control) cells from single-strain culture (blue) and from mixed culture
(red). The population of control cells in the mixed culture has a lower pH and
include some “dark” cells with cytosol acidification, both of which do not happen
when control cells are grown independently. C.ii. GPY3016 (XylB) cells from single-
strain culture (blue) and from the mixed culture (red). The distributions are similar
to one another and include a large fraction of cells with acidified cytosols.

FIGURE 5: Expression of a XylB-mCherry fusion protein leads to faster
cytosolic acidification than expression of untagged XylB.

A-B. GPY3045 (GEV/PGAL1-XylB) and GPY3039 (GEV/PGAL1-XylB-mCherry) were
grown in 2% glucose at the indicated estradiol concentrations for 24 h. GPY3016
(PPGK1-XylB) was grown as in Figure 2. For xylonic acid production (A.ii and B.iii)
cells were additionally incubated in 2% xylose for 24 h. A.i. Population averages of
XylB-mCherry fluorescence (per volume) in single cells for GPY3039 (2% glucose,
saturated, no xylose). Bars show standard deviation. Standard deviations are large, reflecting high cell-to-cell variation in XylB-mCherry levels. The EC$_{50}$ dose is indicated. **A.ii.** Extracellular yield of xylonic acid + xylonolactone vs estradiol concentration, as described in Materials and Methods. For GPY3045 the product yield increased with increasing estradiol concentrations, while in GPY3039 it was maximal at $\sim$10 nM estradiol (the EC$_{50}$ dose in **A.i**). **B.** Single-cell 410 nm-exc. fluorescence (per volume) vs pH. pH axes as in Figure 2.iv. Cells were sampled after 24 hr of incubation in 2% xylose, attached to glass-bottom wells and imaged. For **B.ii-iii**, cells were cultured with 81 nM estradiol. Cultures expressing XylB-mCherry controlled by the GEV/P$_{GAL1}$ system (GPY3039) accumulate 72% dark cells (**B.iii**), while those expressing XylB controlled by the same promoter (GPY3045, **B.ii**) or by P$_{PGK1}$ (GPY3016, **B.i**) accumulate 39% and 27% dark cells, respectively. **C.** Single cell time courses illustrate the dependence of acidification on the level of expression of a XylB-mCherry fusion protein during xylonic acid production. **C.i.** Time-lapse, 410 nm-exc. (pHluorin) images. GPY3039 cells was grown to saturation in 2% glucose with 150 nM estradiol, then adhered to glass-bottom wells, incubated with 2% xylose and imaged for 7 hr. Red arrow: a non-acidifying cell. Gray arrow: an acidifying cell. Fluorescence values from cells in **C.i.** marked with arrows are plotted in **C.ii-iii**. 410 nm-exc. fluorescence (per volume, relative to initial value) (**C.ii.**) and R$_{410/470}$ (**C.iii.**) vs time. Dotted line in **C.ii.** marks the relative (to initial value) 410 nm-excitation fluorescence at which cells were considered dark (0.1 fraction of initial value). The red curve reflects a cell that does not acidify. The black curve reflects a cell that acidifies. Gray arrow marks the point at which the fluorescence...
from pHluorin in the acidifying cell is not detectable above the autofluorescence background. Note the biphasic shape of the black R_{410/470} curve in C.iii, reflecting first a drop in R_{410/470}, caused by a decreasing cytosolic pH, and then an increase in R_{410/470} (at ~5 hr), caused by acidification below pH 5. C.iv. GPY3039 cells were induced with 10, 30 and 150 nM estradiol, incubated in 2% xylose and imaged, as above. Cells were marked as acidifying if their relative 410 nm-exc. fluorescence was smaller than 0.1 at the end of the time course. Acidifying cells are shown as black traces and non-acidifying cells as red traces. The subset of cells that acidify increases with increasing concentrations of estradiol (10 nM estradiol, 6% acidification; 30 nM estradiol, 20% acidification; 150 nM estradiol, 43% acidification). Increasing concentrations of estradiol lead to increased levels of XylB-mCherry.

**FIGURE 6 - The initial levels of XylB-mCherry predict the probability of acidification during xylonic acid production.**

GPY3039 (GEV/P_{GAL1}-XylB-mCherry) cells were grown and imaged as described in Figure 5.C.i-iii. Histograms show the distribution of XylB-mCherry fluorescence (cell counts per bin). Red histogram: total cells; black histogram: acidifying cells. Note that black histograms are right-shifted, reflecting higher XylB-mCherry levels/cell. iv-vi. Histograms show the distribution of XylB-mCherry fluorescence within acidifying (black) and non-acidifying (red) cells (fraction-of-total per bin, within each class). Superimposed logistic regression curves (green, blue and orange) show the dependence of the probability of acidification on XylB-mCherry
levels and were calculated as described in Materials and Methods. The inflection points in the 30 and 150 nM estradiol curves correspond to a sharp increase in the probability of acidification. Curve shading illustrates the standard error of the estimated probability curves. vii. Logistic curves from iv-vi, overlaid. Note that the 10 nM estradiol curve is shifted to the right. viii. Table of values derived from the logistic curves. mCherry fluorescence values that correspond to a 0.5 probability of acidification illustrate the sensitivity to levels of XylB-mCherry in individual cells. To calculate the percent predicted correctly we counted the number of cells with probability smaller than 0.5 that did not acidify and the number of cells with probability larger or equal than 0.5 that did acidify. We expressed the sum of the two values as the percent of total cells.

FIGURE 7 - Cultures with a higher fraction of cells with high XylB levels have lower xylonic acid productivity

GPY3177 (Δgre3::PGK1-XylB-mCherry, single copy) and GPY3126 (CEN/ARS PGK1-XylB-mCherry, variable copy number) were grown in 2% glucose under selection (G418) for 24 h, and either attached to glass and incubated with 2% xylose and imaged, as described in Figure 5.C (A), or incubated with 2% xylose for 24 h (B). i. Distribution of XylB-mCherry fluorescence (at time zero) for each strain. To prevent saturation in the brightest cells mCherry imaging was performed using a shorter exposure than in Figure 5.C (0.02 s vs 0.1 s). Gray dotted lines mark the fluorescence values for the mean ± 2 standard deviations in GPY3177 cells (single copy). The percent of cells in each fluorescence region of the distribution is shown
above each plot, 36% of the GPY3126 (variable copy number) cells have XylB-mCherry levels higher than 97% of GPY3177 cells (single copy). It is likely that these cells contain 2, 3 or more copies of the CEN/ARS plasmid. Note that 18% of GPY3126 cells (variable copy number) have fluorescence values below 97% of GPY3177 cells (single copy). It is likely that at least some of these cells had lost the CEN/ARS plasmid (we measured a 7% percent plasmid loss in plating assays of this culture, data not shown). iii-iv. R_{410/470} vs time. iii. GPY3177 (single copy). iv.

Red traces: non-acidifying cells; black traces: acidifying cells. 31% of GPY3126 (variable copy number) cells acidify vs 2% of GPY3177 cells (single copy). v-vi. Initial XylB-mCherry values for total cells (red) vs acidifying cells (black). Dotted lines are as in A.i-ii. Almost all acidifying cells in GPY3126 (variable copy number) have XylB-mCherry levels greater than 2 standard deviations from the mean in GPY3177 (single copy) cells. B. Measurement of xylonic acid and xylonolactone in culture supernatants from GPY3177 and GPY3126 cultures incubated with 2% xylose in tubes for 24 h. GPY3177 cells (single copy) yield >2-fold more xylonic acid and xylonolactone than GPY3126 cells (variable copy number).
**Table 1: Yeast strains used.** All strains were derived in this work from GPY3015, a CENPK 2-1C derivative constructed in this work. Strain construction is detailed in the Supplemental Materials. The composition of the integrated DNAs is shown schematically, followed by a parenthesis with the estimated copy number (1 copy if not specified) and the plasmids from which they were derived. Plasmids are described in Table 2 and, in more detail, in the Supplemental Materials.

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### Table 2

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**Table 2: Plasmids used.** Main features are listed. For a detailed description see Supplemental Materials.
Figure 1 – Cultures that produce xylonic acid accumulate cells that lose pHluorin fluorescence and metabolic activity.

GPY3016 (PPGK1-XylB, PTPI1-pHluorin) and GPY3020 (control, PTPI1-pHluorin) cells were cultured with 2% xylose. At the times indicated cells were attached to glass-bottom wells, imaged and stained with propidium iodide (PI). A. Brightfield or pHluorin channel images at 52 h. Note many “dark cells” (low signal) in the GPY3016 (XylB) culture as compared to the relatively uniform pHluorin fluorescence (“bright cells”) in the GPY3020 (control) culture. B.i. Overlay of 410 nm-excitation fluorescence, PI fluorescence and brightfield images at 32, 52 and 68 hr. B.ii. Quantification of fluorescence intensity of PI vs 410-nm excitation (times are as in Bi). Dotted lines mark threshold values for dark cells (F.U./vol=18) and PI positive cells (log (F.U./vol=2.7). Red circles represent cells lacking pHluorin fluorescence (dark cells) that stain with PI. The percent that such cells represent relative to all dark cells is shown above each plot. C. Time course of the loss of 410 nm-ex. fluorescence relative to the production of extracellular xylonic acid + xylonolactone in GPY3016 (PPGK1-XylB, PTPI1-pHluorin) and GPY3098 (control, PTPI1-pHluorin) cells.
GPY3016 (PPGK1-XylB, PTP11-pHluorin) cells were grown exponentially in 2% glucose. Cells were fixed, attached to glass, incubated with nigericin-containing buffers at the indicated pH’s and imaged. i-iii. Circles indicate population averages of fluorescence in single cells, gray shading illustrates standard deviation from the mean. Solid blue circles: pH’s at which fluorescence is higher than in untransformed cells. Black outline circles: pH’s at which fluorescence is the same as in untransformed cells. i-ii. Fluorescence (per volume) at 470 nm-excitation (i) and 410 nm-excitation (ii), vs pH. Dotted lines mark the fluorescence levels of untransformed cells. iii. Population average of R410/470 in single cells vs pH. To the right of the dotted line, cells are bright and the R410/470 signal primarily reflects cellular autofluorescence. To the left of the dotted line the R410/470 signal primarily reflects pHluorin fluorescence increases with pH. iv. Single-cell 410 nm-exc. fluorescence (per volume) vs R410/470, for cells incubated in nigericin buffers at 5 pH’s (color coded in the inset). pH axes (for bright cells and dark cells) relate R410/470 values to pH’s inferred from iii. Note that acidification to pH 4.5 or below, causes a coherent shift of the populations to low 410 nm-exc. fluorescence and higher R410/470 values.

Figure 2 – Permeabilized cells incubated in buffers pH 3-9 show a biphasic R410/470 dependence on pH.
Figure 3: The cytosol of cells producing xylonic acid acidifies below pH 5

Cells producing xylonic acid progressively lose pHluorin fluorescence and show increasingly high R410/470 values that correspond to a pH below 5. GPY3016 (PPGK1-XylB, PTPI1-pHluorin, red) and GPY3098 (control, PTPI1-pHluorin, blue) were cultured with 2% xylose and analyzed as described in Figure 1 at 6 (i), 32 (ii) and 52 (iii) hours. Top plots: Distribution of R410/470 ratios in GPY3016 (red, control) and GPY3098 (blue, XylB). Note that the histograms for the cells producing xylonic acid (red) become progressively broader at 32 h and 52 h, while those for the control cells (blue) are compact at all time points. Bottom plots: Single-cell 410 nm-exc. fluorescence (per volume) vs R410/470 (same R410/470 axis as in top plot). pH axes as in Figure 2.iv. Note that cells producing xylonic acid progressively accumulate a distinct subpopulation of cells with low 410 nm-exc. fluorescence (“dark”) and high R410/470, at 32 h and 52 h. The dotted line marks the 410 nm-exc. fluorescence boundary between bright and dark cells.
Figure 4 - Xyloonic acid production causes cell-autonomous cytosolic acidification.
FIGURE 5: Expression of a XylB-mCherry fusion protein leads to faster cytosolic acidification than expression of untagged XylB. (Panels A and B described here, panel C described in next page, under the panel C visuals)

A-B. GPY3045 (GEV/P\text{GAL1}-XylB) and GPY3039 (GEV/P\text{GAL1}-XylB-mCherry) were grown in 2% glucose at the indicated estradiol concentrations for 24 h. GPY3016 (PP\text{GK1}-XylB) was grown as in Figure 2. For xylonic acid production (A.ii and B.i-iii) cells were additionally incubated in 2% xylose for 24 h. A.i. Population averages of XylB-mCherry fluorescence (per volume) in single cells for GPY3039 (2% glucose, saturated, no xylose). Bars show standard deviation. Standard deviations are large, reflecting high cell-to-cell variation in XylB-mCherry levels. The EC50 dose is indicated. A.ii. Extracellular yield of xylonic acid + xylonolactone vs estradiol concentration, as described in Materials and Methods. For GPY3045 the product yield increased with increasing estradiol concentrations, while in GPY3039 it was maximal at ~10 nM estradiol (the EC50 dose in Ai). B. Single-cell 410 nm-exc. fluorescence (per volume) vs pH. pH axes as in Figure 2.iv. Cells were sampled after 24 hr of incubation in 2% xylose, attached to glass-bottom wells and imaged. For B.ii-iii, cells were cultured with 81 nM estradiol. Cultures expressing XylB-mCherry controlled by the GEV/P\text{GAL1} system (GPY3039) accumulate 72% dark cells (B.iii), while those expressing XylB controlled by the same promoter (GPY3045, B.ii) or by P\text{GK1} (GPY3016, B.i.) accumulate 39% and 27% dark cells, respectively.
FIGURE 5: Expression of a XylB-mCherry fusion protein leads to faster cytosolic acidification than expression of untagged XylB (continued from previous describing panels A and B)

C. Single cell time courses illustrate the dependence of acidification on the level of expression of a XylB-mCherry fusion protein during xylonic acid production. C.i. Time-lapse, 410 nm-exc. (pHluorin) images. GPY3039 cells was grown to saturation in 2% glucose with 150 nM estradiol, then adhered to glass-bottom wells, incubated with 2% xylose and imaged for 7 hr. Red arrow: a non-acidifying cell. Gray arrow: an acidifying cell. Fluorescence values from cells in C.i. marked with arrows are plotted in C.ii-iii. 410 nm-exc. fluorescence (per volume, relative to initial value) (C.ii.) and R410/470 (C.iii.) vs time. Dotted line in C.ii. marks the relative (to initial value) 410 nm-excitation fluorescence at which cells were considered dark (0.1 fraction of initial value). The red curve reflects a cell that does not acidify. The black curve reflects a cell that acidifies. Gray arrow marks the point at which the fluorescence from pHluorin in the acidifying cell is not detectable above the autofluorescence background. Note the biphasic shape of the black R410/470 curve in C.iii, reflecting first a drop in R410/470, caused by a decreasing cytosolic pH, and then an increase in R410/470 (at ~5 hr), caused by acidification below pH 5. C.iv. GPY3039 cells were induced with 10, 30 and 150 nM estradiol, incubated in 2% xylose and imaged, as above. Cells were marked as acidifying if their relative 410 nm-exc. fluorescence was smaller than 0.1 at the end of the time course. Acidifying cells are shown as black traces and non-acidifying cells as red traces. The subset of cells that acidify increases with increasing concentrations of estradiol (10 nM estradiol, 6% acidification; 30 nM estradiol, 20% acidification; 150 nM estradiol, 43% acidification). Increasing concentrations of estradiol lead to increased levels of XylB-mCherry.
FIGURE 6 - The initial levels of XylB-mCherry predict the probability of acidification during xylonic acid production.

GPY3039 (GEV/PGAL1-XylB-mCherry) cells were grown and imaged as described in Figure 5.C. i-iii. Histograms show the distribution of XylB-mCherry fluorescence (cell counts per bin). Red histogram: total cells; black histogram: acidifying cells. Note that black histograms are right-shifted, reflecting higher XylB-mCherry levels/cell. iv-vi. Histograms show the distribution of XylB-mCherry fluorescence within acidifying (black) and non-acidifying (red) cells (fraction-of-total per bin, within each class). Superimposed logistic regression curves (green, blue and orange) show the dependence of the probability of acidification on XylB-mCherry levels and were calculated as described in Materials and Methods. The inflection points in the 30 and 150 nM estradiol curves correspond to a sharp increase in the probability of acidification. (truncated, full version of legend in manuscript’s main text)
FIGURE 7 - Cultures with a higher fraction of cells with high XylB levels have lower xylonic acid productivity

GPY3177 (Δgre3::PPGK1-XylB-mCherry, single copy) and GPY3126 (CEN/ARS PPGK1-XylB-mCherry, variable copy number) were grown in 2% glucose under selection (G418) for 24 h, and either attached to glass and incubated with 2% xylose and imaged, as described in Figure 5.C (A), or incubated with 2% xylose for 24 h (B). A.i-ii. Distribution of XylB-mCherry fluorescence (at time zero) for each strain. To prevent saturation in the brightest cells mCherry imaging was performed using a shorter exposure than in Figure 5.C (0.02 s vs 0.1 s). Gray dotted lines mark the fluorescence values for the mean ± 2 standard deviations in GPY3177 cells (single copy). The percent of cells in each fluorescence region of the distribution is shown above each plot. 36% of the GPY3126 (variable copy number) cells have XylB-mCherry levels higher than 97% of GPY3177 cells (single copy). It is likely that these cells contain 2, 3 or more copies of the CEN/ARS plasmid. (truncated, full version of legend in manuscript’s main text)