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pH selectively regulates citric acid and lipid production in *Yarrowia lipolytica* W29 during nitrogen-limited growth on glucose

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ABSTRACT

Yarrowia lipolytica has been used to produce both citric acid and lipid-based bioproducts at high titers. In this study, we found that pH differentially affects citric acid and lipid production in *Y. lipolytica* W29, with citric acid production enhanced at more neutral pH's and lipid production enhanced at more acid pH's. To determine the mechanism governing this pH-dependent switch between citric acid and lipid production, we profiled gene expression at different pH's and found that the relative expression of multiple transporters is increased at neutral pH. These results suggest that this pH-dependent switch is mediated at the level of citric acid transport rather than changes in the expression of the enzymes involved in citric acid and lipid metabolism. In further support of this mechanism, thermodynamic calculations suggest that citric acid secretion is more energetically favorable at neutral pH's, assuming the fully protonated acid is the substrate for secretion. Collectively, these results provide new insights regarding citric acid and lipid production in *Y. lipolytica* and may offer new strategies for metabolic engineering and process design.

1. Introduction

Yarrowia lipolytica is a promising host for the production of a variety of compounds, including lipids, organic acids, and enzymes (Barth and Gaillardin, 1997; Beopoulos et al., 2009; Finogenova et al., 2005; Liu et al., 2015). In the case of lipids and citric acid, *Y. lipolytica* naturally produces these compounds in excess when some essential nutrient other than carbon, typically nitrogen, is growth limiting (Beopoulos et al., 2009). In the case of nitrogen limitation, citrate is thought to accumulate in the mitochondria due to the inhibition of isocitrate dehydrogenase (Ratledge, 2002). Citrate is then exported from the mitochondria to the cytoplasm via the citrate/malate shuttle. It can then be exported from the cell. Alternatively, the citrate is converted to acetyl-CoA by the enzyme ATP-citrate lyase for the subsequent production of lipids. Numerous studies have shown how the production of lipids or citric acid can be improved using metabolic engineering or optimized culture conditions (Ledesma-Amaro and Nicaud, 2016; Timoumi et al., 2018; Zhu and Jackson, 2015). However, less is known about how *Y. lipolytica* selectively regulates the flow of carbon into citric acid or lipids during nitrogen-limited growth.

During preliminary experiments investigating the production of lipids in *Y. lipolytica* W29 during shake-flask growth on glucose, we found that lipid production increased when the medium was not buffered (Fig. 1). Based on these results, we hypothesized that the pH of the growth medium might be a factor affecting lipid and citric acid production. Indeed, multiple studies have shown that citric acid production in *Y. lipolytica* is maximized at more neutral pH's (Egermeier et al., 2017; Timoumi et al., 2018; Tomaszewska et al., 2014). However, these studies did not explore the concomitant production of lipids. In this study, we investigated how pH selectively affects the flow of carbon into lipids or citric acid during nitrogen-limited growth of *Y. lipolytica* W29 on glucose.

2. Materials and methods

2.1. Strains, media and growth condition

Yarrowia lipolytica W29 strain was used for all experiments in this work. Seeding cultures were grown in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). Nitrogen-limiting medium (50 g/L glucose, 1.7 g/L yeast nitrogen base without amino acid and ammonium sulfate, yeast extract 1.5 g/L and 1 g/L ammonium sulfate) with or without 0.1 M potassium phosphate (71.7 mM K_2HPO_4 and 28.3 mM KH_2PO_4) was used for shake flask cultures. The initial pH was

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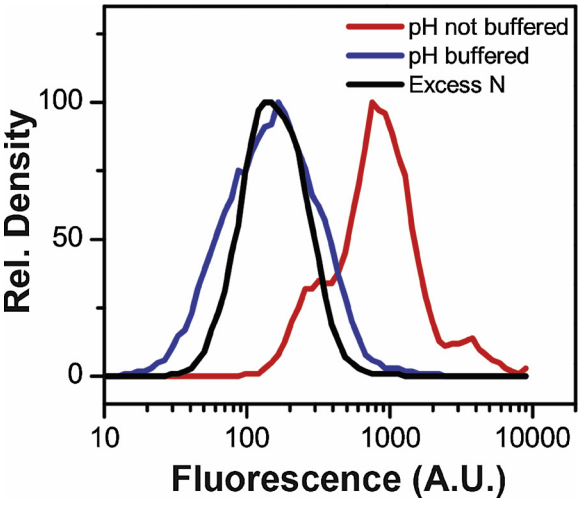

Fig. 1. Comparison of lipid production in *Y. lipolytica* W29 following 48 h of shake flask growth in buffered (initial of pH 7.2) and not buffered (initial pH of 5.6) low-nitrogen medium as determined using flow cytometry with the lipid-soluble, fluorescent dye BODIPY. Increased fluorescence correlates here with increased lipid production. Data were smoothed and normalized to a peak value of 100 to facilitate interpretation. As a control, the cells were also grown in high-nitrogen medium containing 5 g/L ammonium sulfate (excess N). Growth data are provided in **Figure S1**.

7.2 when the growth medium was buffered with potassium phosphate and 5.6 when the growth medium was not buffered. In the bioreactor experiments, the same nitrogen-limiting medium was used without potassium phosphate buffer. The pH-controlled fermentations were carried in Multifors 2 bioreactors (INFORS HT). The culture volume was 450 mL in a 500 mL tank. Temperature was kept at 30 °C. The dissolved oxygen concentration was kept at 50% by controlling the stir rate. Air was sparged into the bioreactor at 1.4 vvm. The pH was fixed at 5 for the first 17 h of growth and then switched to the specified value for remainder of the experiment. The pH was adjusted using 2 M H_2SO_4 and 4 M NaOH.

2.2. Analytical methods

Glucose and citric acid concentrations were measured using a Shimadzu high-performance liquid chromatography system equipped with a RID-10 A refractive index detector, an Aminex HPX-87H carbohydrate analysis column, and a Bio-Rad cation H micro-guard cartridge. The column and guard cartridge were maintained at 65 °C, and 5 mM sulfuric acid was used as the mobile phase at a flow rate of 0.6 mL/min.. Peaks were identified and quantified by retention time comparison to authentic glucose and citric acid standards.

Lipids were extracted using the Folch method (Folch et al., 1957) and measured as follows. Briefly, 1 mL of liquid culture was harvested and immediately frozen at -80 °C and then freeze-dried in Labconco FreeZone 6 freeze dryer overnight. 1 mL of chloroform/methanol (2:1 volumetric) was added to the dried sample, and the mixture was then homogenized three times for 30 s in a FastPrep-24 homogenizer (MP Biomedicals) with a beating speed at 5 m/s. The samples were then mixed with 0.2 mL water and vortexed for 15 s. The organic layer was harvested with a Hamilton syringe and washed with 0.1 mL of a 0.1% (w/v) NaCl water solution, extracted again, and then the lower organic layer was harvested and dried in a hood at room temperature overnight in a pre-weighed tube. The tube was further dried in an 80 °C oven for 1 h and then weighed to determine lipid quantity.

Dry cell weight was determined as follows. 2 mL of liquid culture was harvested in a pre-weighed tube, pelleted, and then washed with 0.5 mL water twice. The tubes with the washed cell pellets were then dried in an oven at 80 °C overnight before weighing. Dry cell weight measurements were performed in triplicates.

2.3. BODIPY strain

Cells were pelleted and resuspended in PBS to OD_{600} ~2. 1 µl of BODIPY 505/515 (Life Technology) (1 mg/mL in ethanol) was added to the 500 µl cell suspension. The mixture was then incubated in the dark for 15 min at room temperature, pelleted, and washed with PBS twice. The suspension was then diluted in PBS to OD_{600} ~0.1 and analyzed with a BD LSR II flow cytometer. The FITC channel (excitation, 488 nm; emission, 530/30 nm) was used and around 100,000 cells were recorded.

2.4. RNASeq experimental procedure

Total RNA was extracted with an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Next, the extracted total RNA was treated with Turbo RNase-free DNase kit (ThermoFisher) according to the manufacturer's protocol and purified again with the RNeasy mini kit according to the manufacturer's protocol. The stranded RNAseq libraries were prepared with Illumina's TruSeq Stranded RNA Sample Prep kit. The libraries were pooled in equimolar concentration, and sequenced for 101 cycles from each single end of the fragments on a HiSeq2500 (Illumina). Fastq files were generated and demultiplexed with the bcl2fastq v1.8.4 Conversion Software.

2.5. RNASeq data analysis

Adaptor sequences and low quality reads were trimmed by Trimmomatic (Bolger et al., 2014). The trimmed reads were analyzed by FastQC (Andrews, 2010). Genome alignments were carried by Tophat2 (Kim et al., 2013). RNA reads were counted by HTSeq (Anders et al., 2015). Statistical analysis was performed in R using the packages: EdgeR, DESeq, and GOstats (Anders and Huber, 2010; Falcon and Gentleman, 2007; Robinson et al., 2010). *Yarrowia lipolytica* CLIB122 genome ASM252v1 was used as the reference genome (Dujon et al., 2004). Gene ontology enrichments were performed using GOstat with a hypergeometric test (Subramanian et al., 2005). Gene ontology (GO) information for *Yarrowia lipolytica* was obtained from the DOE Joint Genome Institute website.

2.6. Equilibrium and thermodynamic calculations

The equilibrium and thermodynamic calculations for citric acid were performed using the parameters values provided by Burgstaller (Burgstaller, 2006). Following Burgstaller, we assumed that the intracellular concentrations of citric acid and magnesium, free to bind citrate, were 6 mM. Increasing or decreasing these concentrations did not substantively changes the results from the calculations. We also assumed that the extracellular concentration of magnesium was 4.4 mM, which is the concentration of magnesium in the growth medium. The intracellular pH was assumed to be 7.6. For the secretion of neutral species, we assumed that the intracellular and extracellular concentrations were the same at equilibrium (i.e. $[H_3Cit^{\circ}]_{in} = [H_3Cit^{\circ}]_{out}$) and then calculated the corresponding amount of total acid at the specified pH's. For the secretion of the anionic species, H_2Cit^{-1} or $MgCit^{-1}$, we used the Nernst equation to calculate the intracellular and extracellular concentrations at equilibrium and then determined the corresponding amount of total acid at the specified pH's. Following Burgstaller (Burgstaller, 2006), we assumed that only one ion was secreted. In the case of energy-dependent transport, we assumed that the energy results from ATP hydrolysis. Here, $\log_{10}([X]_{in}/[X]_{out}) = -\Delta G_{ATP}$, where $\Delta G_{ATP} = -414$ mV, and X is the ion concentration of interest. In the case of transport involving anionic species, we used the equation $\log_{10}([X]_{in}/[X]_{out}) = -\Delta\psi/Z$, where $\Delta\psi$ denotes the membrane potential and Z = 60 mV. All calculations were performed using

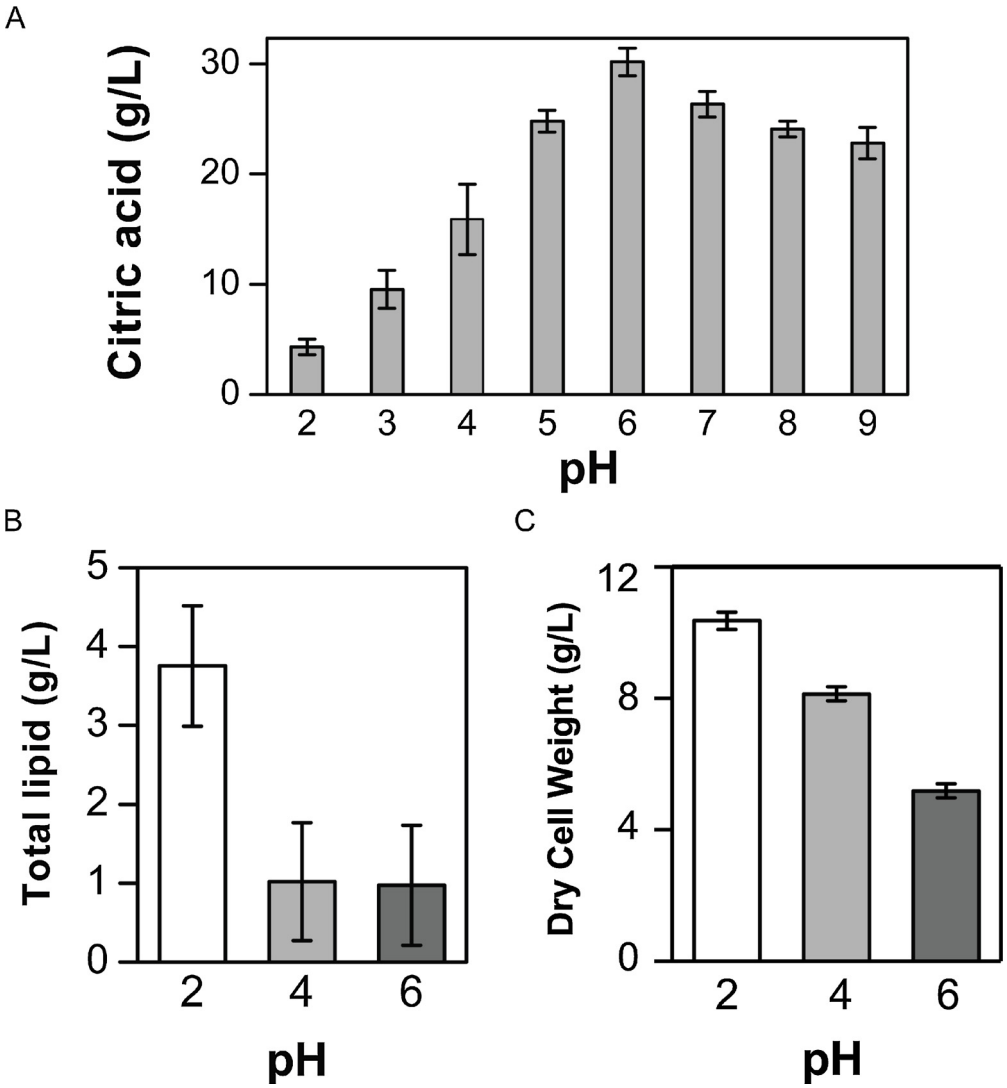

Fig. 2. Citric acid and lipid production by *Y. lipolytica* during growth in pH-controlled bioreactors: (a) citric acid production, (b) total lipid, and (c) dry cell weight at different pH's after 96 h of growth in low-nitrogen medium with an initial glucose concentration of 50 g/L. Results are the average of three bioreactors replicates with the standard deviations reported.

custom Python scripts and are available from the authors on request.

3. Results and discussion

3.1. pH selectively controls citric acid and lipid production in Y. Lipolytica

To test the hypothesis that pH selectively controls citric acid and lipid production, we grew *Y. lipolytica* W29 in bioreactors to precisely determine the effect of pH. In these experiments, we first grew the cells in nitrogen-limiting medium at pH 5 for 17 h to maximize cell growth. We then changed the pH and continued to grow the cells for a total of 96 h, sufficient for the glucose in the medium to be consumed. As shown in Fig. 2A, citric acid production increased with pH, reaching a maximum at pH 6 and then dropping slightly at pH's greater than 6. We also measured lipid production at three pH's: 2, 4, and 6. As shown in Figs. 2B, a different trend was observed, where lipid production increased only at pH 2. In fact, lipid titers were generally low for the other two pH's. The final dry cell weight also decreased as the pH increased (Fig. 2C). The reduction in cell mass at the two higher pH's explains some of the reduction in lipid production; however, the decrease is lipid production is still greater than the decrease in cell mass. These results demonstrate that pH affects citric acid and lipid production in *Y. lipolytica* W29 under the growth conditions investigated.

We also monitored the time course of growth, glucose consumption, and citric acid production at the three pH's (Fig. 3). During the first 17 h, when the pH was fixed at 5, the cultures were identical, as expected. After the pH switch, the cultures at pH 6 ceased to grow whereas those at pH's 2 and 4 continued to grow. Also, the culture grown at pH 6 started to produce significantly more citric acid than the other cultures. In addition, the culture grown at pH 6 consumed the glucose more rapidly than the other cultures grown under more acidic conditions.

3.2. Differentially expressed genes at different pH's

To better understand the mechanism governing how pH affects carbon flow into citric acid or lipids, we profiled gene expression at the three pH's using RNAseq. Briefly, the cells were grown for 50 h in the bioreactors at the specified pH. For each pH, we measured expression from three biological replicates using RNAseq. We aligned our reads to the genomes of *Y. lipolytica* CIB122 (Dujon et al., 2004) and PO1f (Liu and Alper, 2014). We chose to use CLIB122 as the reference genome, because it has more detailed gene annotations and ontology information. The percentage of aligned reads versus total trimmed reads were

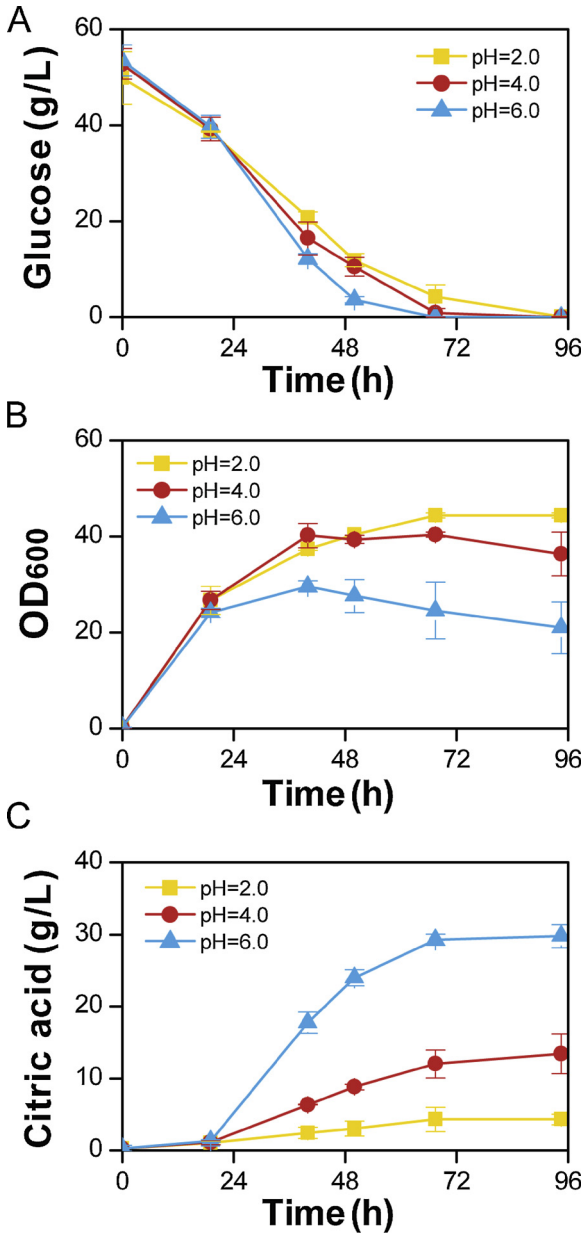

Fig. 3. Comparison of glucose utilization, cell growth, and citric acid production as a function of time in the fermenter experiments. Results were the average of three bioreactors replicates with the standard deviations reported.

very similar for CLIB122 and PO1f (**Table S1**). The results from the RNAseq experiments are summarized in Fig. 4, **Figure S2,** and tabulated in **Table S2**.

Not surprisingly, we observed significant changes in gene expression during growth at the three different pH's (fold change > 2; P < 0.05). However, we found that the expression of the key enzymes involved in lipid synthesis, such as ATP-citrate lyase *ACL1* (YALI0E34793 g and YALI0D24431 g), acetyl-CoA carboxylase (YALI0C11407 g), and diacylglycerol acyltransferase (YALI0E32769gand YALI0D07986 g), were not significantly altered at the three different pH's (FC > 2, P value < 0.05). We also did not find any significant changes for the TCA cycle genes. In addition, we did not find any significant changes in the expression of pyruvate carboxylase (YALI0C24101 g) or phosphenolpyruvate carboxylase (YALI0C16995 g) involved in citric acid production. These results suggest that increased lipid production at pH 2 is not due to changes in gene expression.

We next used statistical gene ontology (GO) enrichment analysis to

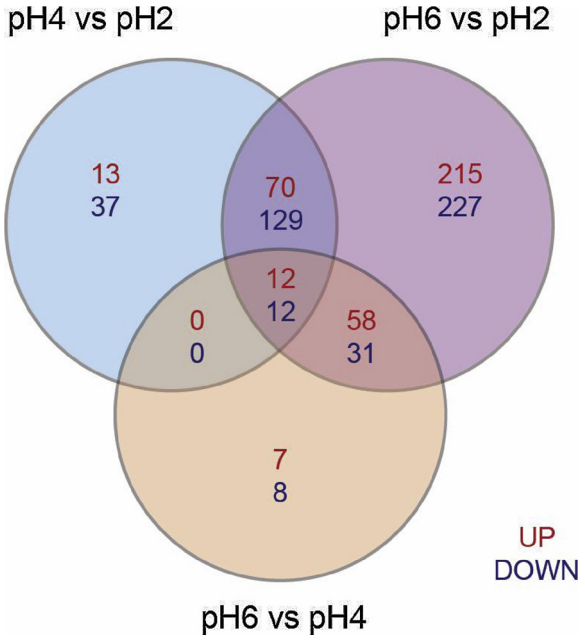

Fig. 4. Venn diagram showing differentially expressed genes (FC > 2, P < 0.05) at the three different pH's from the bioreactor experiments. The changes were determined from three biological replicates.

Table 1
Enriched genes between pH 2 and pH 6 groups.

GO ID	P value	Term
Stronger molecular functions in pH 6		
GO:0005215	2.43E-07	transporter activity
GO:0022857	4.46E-06	transmembrane transporter activity
GO:0015075	5.15E-05	ion transmembrane transporter activity
GO:0022891	1.46E-04	substrate-specific transmembrane transporter activity
GO:0008509	1.73E-04	anion transmembrane transporter activity
GO:0015291	3.17E-04	secondary active transmembrane transporter activity
Stronger molecular functions in pH 2		
GO:0008863	7.23E-05	formate dehydrogenase (NAD +) activity
GO:0016860	8.73E-04	intramolecular oxidoreductase activity
GO:0016620	9.44E-04	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor
GO:0004190	2.33E-03	aspartic-type endopeptidase activity
GO:0070001	2.33E-03	aspartic-type peptidase activity
GO:0016903	2.64E-03	oxidoreductase activity, acting on the aldehyde or oxo group of donors

Table 2
Enriched genes between pH 2 and pH 4 groups.

GO ID	P value	Term
Stronger molecular functions in pH 4		
GO:0046873	5.16E-05	metal ion transmembrane transporter activity
GO:0,005,215	7.64E-05	transporter activity
GO:0015171	3.22E-04	amino acid transmembrane transporter activity
GO:0046943	5.05E-04	carboxylic acid transmembrane transporter activity
GO:0005342	5.05E-04	organic acid transmembrane transporter activity
GO:0008514	7.53E-04	organic anion transmembrane transporter activity
Stronger molecular functions in pH 2		
GO:0016813	0.005203	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amidines
GO:0042972	0.005203	licheninase activity
GO:0030414	0.005203	peptidase inhibitor activity
GO:0061134	0.005203	peptidase regulator activity
GO:0004553	0.00686	hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0016798	0.00912	hydrolase activity, acting on glycosyl bonds

Table 3
Enriched genes between pH 4 and pH 6 groups.

GO ID	P value	Term
Stronger molecular functions in pH 6		
GO:,005,	1.99E-10	transporter activity
GO:0,022,857	9.13E-08	transmembrane transporter activity
GO:,015,	3.99E-07	secondary active transmembrane transporter activity
GO:0008324	3.06E-06	cation transmembrane transporter activity
GO:0,015,075	3.36E-06	ion transmembrane transporter activity
GO:0,022,891	6.33E-06	substrate-specific transmembrane transporter activity
Stronger molecular functions in pH 4		
GO:0015293	0.000291	symporter activity
GO:0015294	0.000291	solute:cation symporter activity
GO:0,015,291	0.001022	secondary active transmembrane transporter activity
GO:0008324	0.003879	cation transmembrane transporter activity
GO:0008733	0.00454	L-arabinose isomerase activity
GO:0,005,215	0.00459	transporter activity

examine differentially expressed genes (FC > 2, P value < 0.05) in all pair-wise pH groups and found transport activity was overrepresented in the higher pH group as compared to lower pH groups (Tables 1–3). This means genes expressed at higher levels, for example, in pH 6 than in pH 4 or pH 2 are mainly associated with transport. To rule out mitochondrial transporters, we identified signal peptides using TargetP 1.1 (Emanuelsson et al., 2000). We only found 2 transporters with mitochondria localization signals, out of the forty transporters that were more strongly expressed at pH 6 than pH 2. Based on the GO enrichment analysis, efflux of citrate at higher/neutral pH is associated with the higher transcription of transporters. Whether any of these transporter actually transport citric acid is not known, because they have not yet been definitively identified. We also performed a homology search based on known citrate transporters, but did not find significant matches within this group (FC > 2, P value < 0.05).

Collectively, these results suggest that reduced citric acid efflux at pH 2 may be the reason why lipid production increases. In other words, at pH 2, citrate accumulates in cell, due to reduced efflux, which in turn leads to more lipid production. This model suggests that these two processes, efflux and lipid synthesis, are competitive, with the former more dominant at more neutral pH's and the latter more dominant at more acidic pH's. That said, while the expression data are consistent with the observed production of citric acid (Fig. 2), the identity of the transporters involved citric acid efflux are not known. In addition, there is not a one-to-one correlation between reduced citric acid production and increased lipid production, as the latter is observed only at pH 2. This would suggest that other mechanisms are involved as well.

3.3. Energetics of citric acid efflux may be more favorable at neutral pH's

Why would *Y. lipolytica* secrete more citric acid at neutral pH's than acidic ones? One possibility is that pH alters the energetics of citrate transport. If we assume that the intracellular pH is 7.6 and the total intracellular concentration of citrate is 6 mM with an equal amount of free magnesium (Burgstaller, 2006), then the intracellular citrate will be predominantly (> 98%) in the anionic forms: Cit^{-3} and $MgCit^{-1}$. Only a tiny fraction (< 0.1%) will be in the neutral forms: H_3Cit° or $MgHCit^{\circ}$. Conversely, at pH 2, extracellular citrate will be predominantly in the neutral form H_3Cit° (> 85%). If only the neutral form is secreted, H_3Cit°, then it would have to overcome a significant concentration gradient in order for extracellular citrate to accumulate at the lower pH's. In particular, the total amount of intracellular citrate would need to be significantly greater than the total amount of extracellular citrate to overcome concentration differences ($\Delta C = [H_3Cit^{\circ}]_{out} - [H_3Cit^{\circ}]_{in}$), in the absence of an active transport mechanism. The reason is that there is very little H_3Cit° inside the cell and a lot outside over a wide range of total citrate concentrations (Fig. 2).

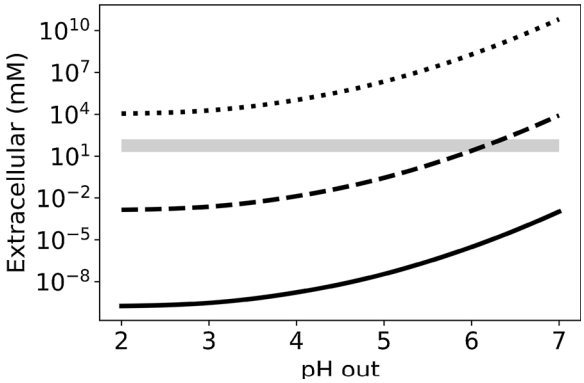

Fig. 5. Effect of extracellular pH on the production of extracellular citric acid (total) based on equilibrium calculations. The black solid line shows the equilibrium distribution when secreted ion is H_3Cit° and the transport mechanism is passive. The dashed and dotted lines show the equilibrium distribution when transport mechanism employs active mechanisms using ATP as the energy source where the dashed line denotes 1 ATP per molecule transported and the dotted line 2 ATP per molecule transported. These ratios have been reported to vary between 1 and 50 (Patzlaff et al., 2003; van Maris et al., 2004). The gray bar corresponds to the range of extracellular concentrations observed in our experiments. Any value below the black lines corresponds to a thermodynamically favorable situation for the different scenarios investigated.

Equilibrium calculations are provided in Fig. 5 based on the parameters and equations provided by Burgstaller (Burgstaller, 2006). These calculations suggest that if the secretion substrate is H_3Cit°, then citrate secretion is energetically unfavorable at lower pH's, consistent with our experimental observations. Furthermore, they indicate that an energy-dependent (ATP utilizing) transport mechanism is required to achieve the observed extracellular concentrations of citrate. Interestingly, the converse behavior is observed if $MgHCit^{\circ}$ is the substrate for transport, where citrate secretion is more favorable at lower pH's (**Figure S3**).

We also performed similar calculations assuming that secretion substrate was H_2Cit^{-1} or $MgCit^{-1}$ (**Figure S4**). These calculations employed the Nernst equation and were performed at different values for the membrane potential ($\Delta\psi$). In this case, citrate acid secretion was energetically favorable at both low and neutral pH's when H_2Cit^{-1} was the secretion substrate and the membrane potential was negative. However, the predicted extracellular concentrations were less than what we observed (Fig. 2). Interestingly, secretion was far more favorable at low pH's if $MgCit^{-1}$ was the substrate for transport, even in the absence of a negative membrane potential. If we assume that the citrate transporters are specific for just one anionic form, then these results would suggest *Y. lipolytica* secretes the neutral form H_3Cit°. They also suggest that other fungal species, which produce citric acid at lower pH's (Max et al., 2010; Show et al., 2015), may secrete the magnesium-bound form $MgHCit^{\circ}$ or some other anionic form such as $MgCit^{-1}$.

These equilibrium calculations show that the pH of the growth medium affects the energetics of citrate secretion, consistent with analogous arguments in the literature (Burgstaller, 2006; van Maris et al., 2004). Furthermore, they were also able to identify one scenario where citrate secretion is less energetically favorable at low pH's and more favorable at neutral pH's. In these regards, they provide one possible explanation for the pH-dependent secretion of citric acid, namely that it is energetically unfavorable at low pH's.

These energetic arguments, however, do not explain why lipid production is favored at pH 2. As we did not observe any significant changes in the expression of key lipid synthesis genes, one possibility is that the pH switch involves competition between two pathways, one involving citric acid efflux and the other lipid synthesis. According to this model, the demand for citrate by the lipid synthesis pathway is

constant, which is consistent with the gene expression data. In addition, the first enzyme in this pathway, ATP-citrate lyase, has a relatively high Km (3.6 mM) (Zhang et al., 2014), suggest that intracellular citrate needs to accumulate in order for the requisite acetyl-CoA to be generated. The demand for citrate by the efflux pathway, on the other hand, increases with extracellular pH, most likely due to increased transporter expression. The reason that transporters expression is increased at higher pH's is presumably because it provides the only scenario where citrate efflux is energetically favorable. Even if the transporters are expressed at lower pH, the energetics of efflux suggests that this pathway will be less active.

Of course, this begs the question as to why *Y. lipolytica* secretes citric acid rather than make lipid (as nitrogen is limiting, the cells are limited in their ability to use the carbon for biomass formation). One possibility is that citric acid efflux is a more energy efficient mechanism for carbon overflow metabolism, as lipid synthesis is more energetically expensive. Why the cells make more lipid at pH 2 may be that they have no other alternative – it simply reflects constraints arising from transporter specificity. Other organisms may not have these transporter constraints, due to different anion specificities, and thus have different responses to the pH of the growth medium. Clearly, these arguments are speculative at this stage but nonetheless provide one possible mechanism that explains the observed behavior of cells grown at different pH's.

4. Conclusion

We have shown that pH can be used to selectively control citric acid and lipid production in *Y. lipolytica* W29. To identify the mechanism involved in this pH switch, we profiled gene expression at three different pH's. While we were unable to identify any differentially expressed genes specifically known to be involved in citric acid and lipid metabolism, we nonetheless found that many transporters were more highly expressed at pH 6 versus pH 2 or 4. These results suggest that the switch between lipid and citric acid production is mediated at the level of transport, though further work is necessary to determine whether any of these transporters are indeed involved in citric acid secretion. In further support of this mechanism, we identified one scenario where the energetics of citric acid secretion are more favorable at more neutral pH's, which could possibly explain the observed phenomenon. Collectively, these results have uncovered an additional facet to lipid and citrate metabolism in *Y. lipolytica* and may provide additional targets for future metabolic engineering studies.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.10.012.

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