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Research article

Enhancing control of cell-free metabolism through pH modulation

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Abstract

Engineering metabolism for the synthesis of bio-based products in non-model organisms can be challenging. One specific challenge is that biosynthetic pathways are often built from enzyme candidates sourced from diverse organisms, which can prove difficult to implement in recombinant hosts due to differences in their cellular environments (e.g. pH, cofactor balance). To address this problem, we report a cell-free synthetic biology approach for understanding metabolism in a range of environmental conditions, specifically under varied pH. The key idea is to control the pH of *Escherichia coli*-based cell-free systems for assessing pathway performance using enzymes sourced from organisms other than *E. coli*. As a model, we apply this approach to study the impact of pH on the *n*-butanol biosynthesis pathway derived from clostridia in *E. coli* lysates. Specifically, we exploit the open, cell-free reaction environment to explore pH outside the habitable range of *E. coli*, revealing insights into how chemical context impacts the interaction between native metabolism and heterologous enzymes. We find that the pH optimum for butanol production from acetyl-CoA is substantially lower than the optimal pH of glycolysis in *E. coli*-based crude lysates. In addition, pH is an essential factor to consider when activating metabolic pathways in the cell-free environment due to its effect on reaction yield or enzyme activity, the latter of which is demonstrated in this work for alcohol dehydrogenases from a range of extremophiles. Ultimately, altering metabolism through pH control will allow cell-free systems to be used in studying the metabolic state of organisms and identify suitable enzymes for pathway engineering.

Key words: cell-free systems; metabolic engineering; biosynthetic pathways; pH; butanol

1. Introduction

Organisms have evolved metabolic pathways to operate under a diverse range of environmental conditions. While some microorganisms thrive in extreme acidic or basic environments and some in high-salt or anaerobic environments (1), *Escherichia coli* and many model organisms have evolved in more neutral, laboratory environments. Even in acidic or alkaline environments, many organisms sustain cellular function by maintaining neutral intracellular pH. However, several fermentative acidophilic bacteria, such as *Lactococcus* (2) and *Clostridium* (3), adjust their intracellular pH, sometimes as low as pH 5, as a function of extracellular pH and environment. Under these harsh conditions, metabolic enzymes and organisms themselves have evolved to sustain life suggesting that their metabolic enzymes might be more robust over a wider range of pH values. Thus,

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some organisms are more or less amenable to metabolic engineering than others and some may have more beneficial enzymes for biosynthesis of desired chemicals (4, 5). Clostridia, industrially relevant organisms for large-scale fermentation, are an example of a class of organisms that have biosynthetic benefits over model laboratory organisms, but they can be challenging to engineer because they grow in anaerobic and slightly acidic environments (6, 7). Developing a deeper understanding of how metabolism and heterologous biosynthetic pathways interact under various environmental conditions will enable more informed selection of enzymes and pathways for metabolic engineering applications in many diverse organisms.

Often when expressing heterologous pathways, enzymes from evolutionary divergent organisms are chosen to catalyze biochemical reactions due to their perceived, enhanced activity (8). However, by selecting enzymes from diverse organisms, issues of expression incompatibility can arise (9). In fact, this can preclude the physiochemical environment for optimal enzyme activity because the reactions are confined inside a single host cell with highly regulated physiochemistry and metabolism that often differ from the sourced organism. Therefore, heterologous pathway expression requires metabolic rewiring and can result in suboptimal performance (10, 11). Cell-free systems, on the other hand, are beneficial platforms for rapid assembly of enzymatic pathways *in vitro* and regulation of the physiochemical environment to identify optimal conditions for pathway operation (12, 13). In addition, cell-free systems enable biological production of proteins and small molecules without the growth constraints of *in vivo* systems (12). Moreover, the open environment allows greater control over reaction conditions, no barriers to product access and direct access to complex reaction networks (13–17). These features suggest that cell-free systems offer an ideal setting to study metabolic and engineered biosynthetic pathways under many environmental conditions to inform traditional metabolic engineering efforts. However, while physiochemical conditions in crude lysates have been observed (13) identifying and manipulating pH as an engineering lever for control of metabolic systems has not been previously explored, to our knowledge.

In this study, we assess how pH can easily be modulated in the cell-free environment to: (i) control the rate of glycolysis in *E. coli* and a heterologous pathway for butanol production from clostridia and (ii) examine pH tolerance of alcohol dehydrogenases from a range of extremophiles. We find the pH optimum for butanol production from acetyl-CoA, a significant node in central carbon metabolism, is in opposition to central carbon metabolism itself. In addition, pH is an important factor to consider for each new cell-free pathway developed. In sum, this work sets the stage to apply cell-free systems for studying metabolic conditions of organisms that might grow under more acidic or basic conditions than the *E. coli* chassis most commonly used for heterologous expression of biosynthetic pathways.

2. Materials and methods

2.1 Crude cell extract preparation

Enzyme-enriched extracts were generated from five *E. coli* BL21 Star(DE3) strains individually harboring pET-based vectors with either Thl (*E. coli*), Hbd (*Clostridium beijerinckii*), Crt (*Clostridium acetobutylicum*), Ter (*Treponema denticola*), or AdhE2 (*C. acetobutylicum*) as previously utilized (13). Cells were cultured in 2.5 l Tunair shake flasks containing 1 l 2×YTPG at 37°C. Enzyme expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at OD_{600} 0.5, and the cells grew for another 4 h at 30°C prior to harvesting as previously described (18). Total protein concentration was estimated using a Bradford assay. Extracts for cell-free protein synthesis (CFPS) were generated from *E. coli* BL21 Star(DE3) during mid-exponential phase (OD_{600} 3.0) with induction of T7 RNA polymerase expression as previously described (18, 19).

2.2 Buffers

Reactions with set pH contained 100 mM or 500 mM Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis–Tris) for pH 5–7 or 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) for pH greater than 7. When testing buffers made from acetate salts, the reaction pH was set to pH ∼6 using 100 mM or 500 mM potassium acetate. All reactions were adjusted to the desired pH with glacial acetic acid or 5 N potassium hydroxide. All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

2.3 Cell-free metabolic engineering reactions

Cell-free metabolic engineering ('CFME')-style reactions, as previously described (12, 20), contained 8 mM magnesium glutamate, 10 mM ammonium glutamate, 134 mM potassium glutamate, 3 mM nicotinamide adenine dinucleotide, 1.5 mM CoA, 120 mM glucose, 0.5 mM kanamycin and 2 mg/ml each of the five enzyme-enriched extracts required for the butanol synthesis pathway. Reactions were incubated at 30°C for up to 30 h. Some CFME reactions were run independent of glycolysis by using 30 mM acetyl-CoA in place of glucose and supplemented with 60 mM reduced nicotinamide adenine dinucleotide (NADH).

2.4 Cell-free alcohol dehydrogenase assay reactions

To determine enzymatic reaction yields from alcohol dehydrogenases under different pH, nine additional alcohol dehydrogenases (ADHs) were selected from a variety of bacteria (Supplementary Table S2). CFPS reactions for *in vitro* production of these enzymes were assembled with 8.1 μM of template DNA, ∼10 mg/ml cell-free extract and the cofactors and crowding agents in 57 mM HEPES buffer. These reactions contained 8 mM magnesium glutamate; 10 mM ammonium glutamate; 130 mM potassium glutamate; 1.2 mM adenosine triphosphate; 0.85 mM each of guanosine, uridine and cytidine triphosphates; 0.034 mg/ml folinic acid; 0.171 mg/ml transfer RNAs; 33.33 mM phosphoenolpyruvate; 2 mM of all 20 canonical amino acids; 0.40 mM nicotinamide adenine dinucleotide; 0.27 mM cofactor A; 1 mM putrescine; 1.5 mM spermidine (19). The expression level of each enzyme was quantified using radioactive leucine incorporation assays as previously described (18) (Supplementary Figure S3). Then reactions were run for 10 min with 10 mM butyraldehyde, 20 mM NADH and 1 μM ADH enzyme produced via CFPS. Reaction yield is calculated as mM butanol produced per mM butyraldehyde consumed ($mol_{butanol}$/$mol_{butyraldehyde}$) for each dehydrogenase. These results are represented as percentages of the butanol yield on butyraldehyde for the alcohol dehydrogenase from *C. acetobutylicum*.

2.5 Metabolite analysis

All reactions were quenched with an equal volume of 10% trichloroacetic acid, and the precipitated proteins were separated from the aqueous solution via two centrifugations at 21 000 × g

for 10 min. The remaining supernatant was transferred to Agilent HPLC screw-cap vials with high-recovery inserts, which were loaded on an Agilent 1260 HPLC. Samples were run on a Bio-Rad Fast Acids column at 0.6 ml/min at 55°C with 5 mM sulfuric acid and analyzed with refractive index detection. Metabolite concentrations were calculated from chromatogram peak areas based on standards of known concentration.

2.6 pH measurements

Samples were analyzed with a Thermo Scientific™ Orion™ ROSS Ultra™ Refillable pH/ATC Triode™. Reactions for which a pH was set prior to reaction start were measured with a mixture of all components except the cell-free extract, CFPS reaction addition and NADH to avoid premature reaction initiation. Reaction pH was adjusted with glacial acetic acid or 5 N KOH as necessary. Measurements of reaction pH over time were taken from additional replicates of reactions run for metabolite analysis.

3. Results and discussion

3.1 pH corresponds to shifts in cell-free metabolic processes

CFME represents a powerful approach to produce biochemicals *in vitro* and to study both metabolic processes and the influence of the chemical environment on those processes (21–23). To investigate pH effects on metabolism, we probe cell-free reactions consisting of *E. coli* crude lysates which contain active glycolytic enzymes, entire modules of cellular metabolism (e.g. tricarboxylic acid cycle) and desired biosynthetic enzymes. Using cell-free butanol production as a model example, we modulate pH and observe an array of changes in metabolite profiles over the course of a reaction. In a typical cell-free butanol synthesis reaction, lactate and acetate are observed as major side-products (Figure 1A) and pH is known to decrease (13).

To explore the role of pH in biosynthetic processes, we first varied the initial pH of the cell-free butanol synthesis reactions and observed how metabolites form over time. We adjusted the starting pH to pH 5.8, 6.6 (unadjusted from our previously reported conditions (12)), 7.1 and 7.8 in separate butanol-producing reactions and observed that, when unbuffered, the pH dramatically drops to pH ~5.6 by 9 h in all cases (Figure 1B). In the case where pH remains ~5.6 for the reaction duration, glucose consumption and butanol synthesis are slow (Figure 1C). Focusing on changes in glucose and butanol concentrations over time, it is clear that increasing initial pH increases glucose consumption rates and butanol production rates (Figure 1C). At high initial pH, glycolysis can generate more carbon flux toward acidic side-products before reaching a pH where metabolism becomes less active (Figure 1D). Specifically,

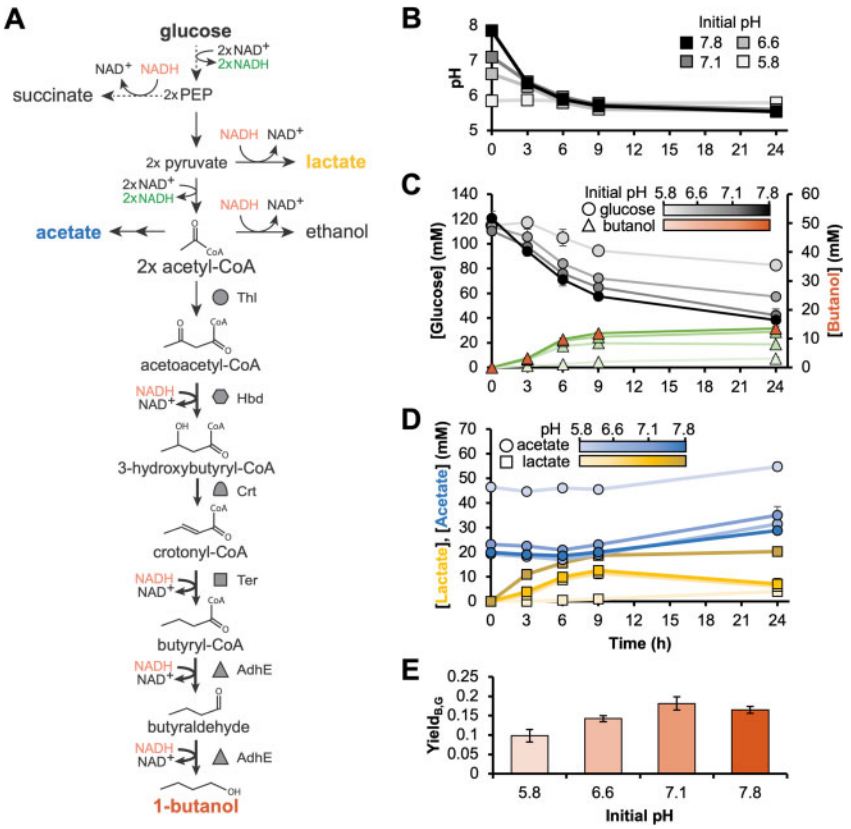

Figure 1. Increasing the initial pH of unbuffered cell-free reactions leads to increases butanol yield. Cell-free reactions containing a mixture of five lysates, individually pre-enriched with one pathway enzyme for the production of butanol, were supplemented with glucose and incubated for 24 h after pH adjustment at the start of the reaction to pH 5.8, 6.6, 7.1 and 7.8. The pH was monitored over the course of the butanol synthesis reactions and metabolite measurements were taken at 0, 3, 6, 9 and 24 h. (**A**) Schematic representation of key cell-free metabolic reactions taking place during cell-free butanol synthesis. (**B**) pH for each reaction condition is shaded from light to dark (low initial pH to high initial pH; gray squares). (**C**) Glucose (left-axis; gray circles) and butanol (right-axis; orange triangles) are plotted over time. (**D**) Lactate (yellow squares) and acetate (blue circles) are plotted over time. Acetate values are initially greater than zero due to the prevalence of acetate salts in CFPS reactions and the use of glacial acetic acid to adjust the pH of each reaction. Measurements are mean values with error bars representing standard deviation for triplicate reactions. (**E**) Yield of butanol on glucose consumed was calculated for each reaction. Error bars here represent propagated error from $n = 3$ independent reactions.

lactate is produced during the highest rate of glucose consumption while acetate increases when glucose consumption slows, with acetate levels starting at non-zero values due to acetate salts used during extract preparation. Additionally, acetic acid was used to set some of the pH conditions. Due to the greater rate of glucose consumption prior to the reaction reaching this minimum pH, increasing the initial reaction pH increased butanol yield on glucose from 0.10 ± 0.02 to 0.16 ± 0.01 $mol_{butanol}/mol_{glucose}$ (Figure 1E). This suggests that pH plays a key role in these cell-free metabolic processes, leading to the hypothesis that buffering reactions to control the pH would enable more active butanol metabolism while maintaining sufficient glucose consumption.

3.2 pH controls the rate of glycolysis and butanol synthesis

Buffering cell-free reactions neutralize small amounts of produced acids (e.g. lactate, acetate), thereby maintaining a relatively stable reaction pH. The concentration of the buffer and pH chosen to be maintained represent new levers to control cell-free metabolism. Using strong (500 mM) buffers, the reactions maintained three separate pH conditions at pH 6.6, 7.1 and 7.8. We observed that as the held pH decreased, butanol titer increased to nearly 40 mM while the rate of glucose consumption remained comparable (Figure 2A). This suggested that allowing for moderate fluctuation in pH over time by reducing the buffer concentration to 100 mM—creating an intermediate case between rapidly decreasing pH (Figure 1) and stable pH (Figure 2)—could balance glycolysis with butanol synthesis (Supplementary Figure S1). These moderately buffered reactions gave higher butanol titers with yields approaching 1 butanol molecule produced per 2 glucose molecules consumed (Supplementary Figure S1; Figure 2B). The best condition was at neutral pH with 100 mM buffer, which displayed a 6-fold improvement in butanol production over the unbuffered initial condition. Interestingly, low pH acetate buffers inhibit glucose consumption and yet result in higher butanol yields per glucose consumed (Supplementary Figure S2). These data are not directly comparable to the conditions in Figure 1 due to the high concentration of acetate (>100 mM), which suppresses glycolytic activity in *E. coli* and contributes to butanol synthesis in clostridia (24, 25). Taken together, these results suggest that pH significantly impacts pathway performance for cell-free metabolism and highlight the importance of the buffer capacity.

Comparing reactions across a biologically relevant pH range (pH ~6–8) with varying rates of acidification, it became clear that the effect of pH on glucose consumption could confound pH effects on butanol synthesis. Therefore, to separate the catabolic and anabolic branches of this reaction, we ran cell-free reactions from glucose with no heterologous pathway to analyze glycolysis and reactions from acetyl-CoA with the butanol pathway enzymes present to analyze butanol synthesis alone. Glycolysis in cell-free reactions without the butanol pathway occurs most rapidly at pH 8 (as measured by glucose consumption), reaching a maximum of 17.07 ± 0.75 mM glucose consumed per hour, and slows as reaction pH decreases (Figure 3A). In contrast, when glycolysis is bypassed through the addition of acetyl-CoA, butanol synthesis is enhanced by lower pH, reaching a maximum of 10.40 ± 0.38 mM butanol produced per hour at pH 5.75 (Figure 3B). Because *E. coli* grows best in environments slightly above neutral pH and clostridia, the source of many of the butanol pathway enzymes (26), thrive in slightly acidic environments, it is logical that glycolytic enzymes from *E. coli* are more active at higher pH than the biosynthetic enzymes that evolved in clostridia. The optimal pH for clostridial butanol synthesis is 6.2, and the intracellular pH can vary considerably from 4 to 7 depending on environmental conditions and metabolic states (3, 27–29). It may be possible to construct pathways that operate better together by selecting enzymes with more similar pH optima.

3.3 Examining pH tolerance of enzymes

Identifying enzymes that perform well at low and high pH can aid in selecting the best or most tolerant enzymes for a given biosynthetic pathway. Utilizing the ability to control cell-free reaction pH, we examined enzyme performance under different pH values to identify how robust an enzyme might be to pH

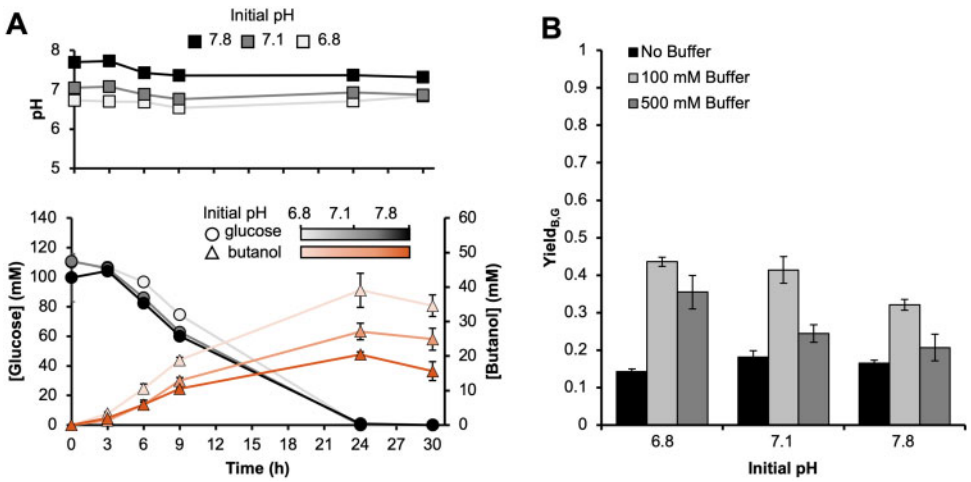

Figure 2. Buffering allows for constant pH, which results in increased butanol titers with similar glucose consumption rates. Cell-free reactions were run with three different buffers at 100 mM and 500 mM final concentrations. (**A**) pH is measured over the course of the 500 mM buffered reactions shaded from light to dark (top; low initial pH to high initial pH; gray squares) with corresponding glucose (left-axis; gray circles) and butanol (right-axis; orange triangles) concentrations at 0, 3, 6, 9 and 24 h (bottom). Error bars are representative of one standard deviation for $n = 3$. (**B**) Overall butanol yield on glucose consumed was calculated for reactions run at starting pH 6.6, 7.1 and 7.8 in 100 mM buffer (light grey), 500 mM buffer (dark grey) and no buffer (black). Error bars here represent propagated error from $n = 3$ independent reactions.

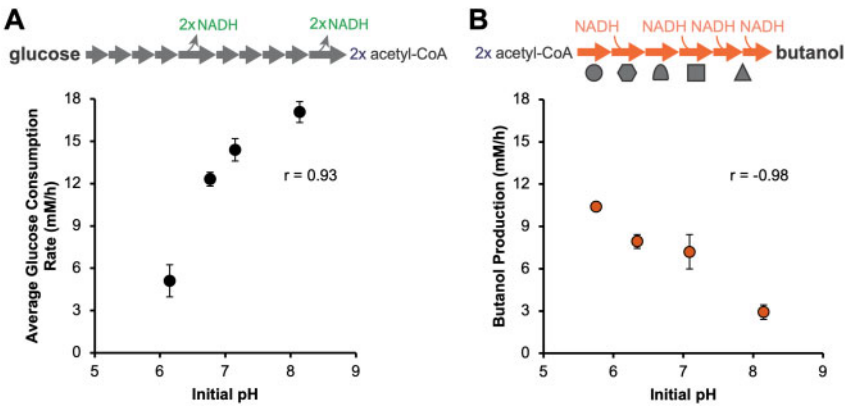

Figure 3. Pathway segment performance depends on pH. Cell-free reactions were run in two separate setups: (1) extract with no butanol enzymes present were used to assemble a CFME reaction where glucose is supplemented and (2) mixed extracts containing all butanol enzymes were used to assemble reactions with acetyl-CoA supplemented (schematics are represented for each setup). The pH of each setup was then adjusted and buffered to range from pH 5.8 to 8.1. Glucose and butanol were measured at 0, 3, 6, 9 and 24 h for both reaction setups. (**A**) The average glucose consumption rate was calculated from glucose measurements over the 24 h time course for setup 1 with no butanol enzymes present. (**B**) Butanol production rates were measured for setup 2 with all butanol enzymes present and acetyl-CoA supplementation. Error bars are the propagated error from metabolite measurements in triplicate ($n = 3$).

Table 1. Enzyme performance can be modulated by tuning pH in crude lysates

ADH source (classification)	% ADH reaction yield compared to that of *C. acetobutylicum*					
Initial pH (ref. yield) [$mol_{butanol}/mol_{butyrald}$]	5 (0.61 ± 0.05)	6 (0.50 ± 0.03)	7 (0.46 ± 0.02)	8 (0.41 ± 0.04)	9 (0.46 ± 0.07)	10 (0.45 ± 0.06)
Clostridium acetobutylicum (acidophile)	100% ± 11%	100% ± 8%	100% ± 8%	100% ± 13%	100% ± 22%	100% ± 18%
Helicobacter pylori (acidophile)	75% ± 7%	83% ± 9%	76% ± 9%	79% ± 11%	95% ± 15%	96% ± 16%
Acetobacter aceti (acidophile)	77% ± 9%	80% ± 5%	83% ± 11%	86% ± 10%	95% ± 21%	99% ± 20%
Zymomonas mobilis: zinc-dependent (acidophile)	84% ± 7%	72% ± 4%	76% ± 8%	85% ± 23%	88% ± 17%	95% ± 19%
Zymomonas mobilis: iron-dependent (acidophile)	59% ± 14%	77% ± 8%	62% ± 9%	60% ± 9%	78% ± 14%	92% ± 16%
Thermosynechococcus sp. *NK55a* (mesophile)	98% ± 12%	94% ± 7%	106% ± 12%	111% ± 15%	110% ± 23%	111% ± 15%
Providencia burhodogranariea (mesophile)	69% ± 11%	98% ± 6%	99% ± 13%	107% ± 14%	118% ± 26%	120% ± 19%
Natronomonas pharaonis (alkaliphile)	65% ± 12%	79% ± 6%	70% ± 15%	76% ± 12%	82% ± 14%	94% ± 14%
Bacillus pseudofirmus (alkaliphile)	70% ± 12%	88% ± 8%	82% ± 9%	92% ± 11%	77% ± 12%	90% ± 15%

The source organism for each alcohol dehydrogenase is listed in the first column. Across are the initial pH of the enzyme assay from pH 5 to pH 10 in reactions containing 1 μM ADH and 10 mM butyraldehyde that ran for 10 min. Enzymatic reaction yield for each ADH is represented as a percent of the yield of butanol for the dehydrogenase compared to the butanol yield from that of *C. acetobutylicum*. The absolute values for the yield of this reference ADH are provided underneath the initial pH as 'ref. yield' in mol of butanol produced per mol of butyraldehyde consumed ($mol_{butanol}/mol_{butyrald}$).

change. To do this, we selected nine alcohol dehydrogenases (ADH) from a diverse range of organisms—one from clostridia (30), four from other acidophiles (31–33), two from mesophiles (34, 35) and two from alkaliphiles (36, 37) (Supplementary Table S2), with the hypothesis that acidophiles and alkaliphiles might contain enzymes with pH optima at low and high pH, respectively (38). The ADH variants were produced by CFPS, and *in vitro* enzyme synthesis was quantified by radioactive C14-leucine incorporation (Supplementary Figure S3). Then 1 μM of CFPS-produced ADH was added to reactions to convert butyraldehyde (10 mM supplemented) to butanol, the final step of the butanol biosynthetic pathway, measured at 10 min highlighting the ease of using cell-free systems for prototyping pathway performance. It is important to note that these assays can be performed without purification, which can limit the ability to characterize specific activities of enzymes but highlights changes in overall reaction yields. In 500 mM buffer, these reactions maintained constant acidic (pH 5) to alkaline (pH 10) conditions to probe enzyme performance determined by reaction yields. The ADH enzyme from *C. acetobutylicum* shows the highest yield of butanol (0.61 $mol_{butanol}/mol_{butyraldehyde}$) from butyraldehyde (Table 1), which is consistent with the use of this homolog for butanol biosynthesis in recombinant hosts (39, 40). Generally, ADH homologs derived from acidophiles perform near 80% (based on butanol yield) of the *C. acetobutylicum* homolog while the alkaliphile-derived ADHs perform at ~65% of the *C. acetobutylicum* homolog at low pH (pH 5) (Table 1). This is consistent with the initial hypothesis that acidophile-derived enzymes perform better at low pH while alkaliphile-derived enzymes perform worse. We also observed that mesophile-derived enzymes from *Thermosynechococcus* sp. *NK55a* and *Providencia burhodogranariea* had similar enzymatic reaction yields to that of *C. acetobutylicum*. Furthermore, a majority of the acidophile and alkaliphile homologs performed at 80% of the *C. acetobutylicum* homolog at high pH (pH 9–10) (Table 1). While surprising, it is not entirely clear whether this result is due to an enzyme's pH tolerance or a generally poor enzyme homolog for butanol production. In a full biosynthetic pathway, these effects are likely compounded by the pH tolerance of each pathway enzyme for the given biosynthetic pathway. Finding sets of enzymes that are either robust under varying pH or with common pH optima will aid in designing biosynthetic pathways, both *in vitro* and *in vivo*.

4. Summary

In this work, we demonstrate that pH can easily be modulated in the cell-free environment to (i) evaluate pH tolerance of glycolysis and of the heterologous butanol production pathway module and (ii) examine pH tolerance of enzymes from a range of extremophiles. This is important for a few reasons. First, we anticipate that this will aid in understanding how to better utilize the cell-free environment to prototype biosynthetic pathways and to move toward cell-free biomanufacturing. For example, the cell-free platform can be used to routinely and rapidly test enzymes for pH tolerance and for selecting pathway enzymes based on 'pH preference'. This can also facilitate the selection of sets of pathway enzymes with pH optima that match the optimal intracellular pH of a desired organism for biomanufacturing. Second, a key finding in this study was that the pH optimum for the clostridia-derived butanol production pathway from acetyl-CoA is pH ∼6 while central carbon metabolism, or glycolysis, has a pH optimum of pH ∼8. While using native metabolism (i.e. glycolysis) in cell-free reactions can be highly beneficial, it can be problematic when the downstream pathway module (i.e. butanol production pathway) has a conflicting pH optimum. Third, these results suggest the possibility of establishing biphasic *in vitro* metabolism, where one pathway operating at higher pH generates a pool of precursor compounds prior to the initiation of a secondary pathway operating at low pH to generate a product. The transient acetyl-CoA intermediate seen in this study would present a challenge for this approach, but the natural acidification that occurs during glucose catabolism in unbuffered solution would provide a suitable environment for modules from clostridial metabolism.

Looking forward, cell-free systems will be useful to study metabolic conditions of organisms that might grow under more acidic or basic conditions than the *E. coli* chassis most commonly used for heterologous expression of biosynthetic pathways. Specifically, controlling pH to alter metabolism will allow cell-free systems to assess the metabolic state of organisms and identify suitable enzymes for pathway engineering.

Materials and data availability

Any data or unique materials (e.g. DNA sequences) presented in the manuscript may be available from the authors upon reasonable request and through a materials transfer agreement. This includes DNA sequences.

Supplementary data

Supplementary data are available at SYNBIO online.

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Conflict of interest statement. M.C.J. has a financial interest in SwiftScale Biologics and Design Pharmaceuticals Inc. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict of interest policies. All other authors declare no conflicts of interest.

References

1. Stan-Lotter,H. and Fendrihan,S. (2017) *Adaption of Microbial Life to Environmental Extremes*. Novel Research Results and Application, Springer.
2. Cook,G.M. and Russell,J.B. (1994) The effect of extracellular pH and lactic acid on pH homeostasis in *Lactococcus lactis* and *Streptococcus bovis*. *Curr. Microbiol.*, 28, 165–168.
3. Huang,L., Forsberg,C.W. and Gibbins,L.N. (1986) Influence of external pH and fermentation products on *Clostridium acetobutylicum* intracellular pH and cellular distribution of fermentation products. *Appl. Environ. Microbiol.*, 51, 1230–1234.
4. Rathinam,N.K. and Sani RK. (2018) Bioprospecting of extremophiles for biotechnology applications. In: Sani,R.K. and Rathinam,N.K. (eds). Extremophilic Microbial Processing of Lignocellulosic Feedstocks to Biofuels, Value-Added Products, and Usable Power). Springer, pp 1–23.
5. Podar,M. and Reysenbach,A.L. (2006) New opportunities revealed by biotechnological explorations of extremophiles. *Curr. Opin. Biotechnol.*, 17, 250–255.
6. Abubackar,H.N., Fernández-Naveira,Á., Veiga,M.C. and Kennes,C. (2016) Impact of cyclic pH shifts on carbon monoxide fermentation to ethanol by *Clostridium autoethanogenum*. *Fuel*, 178, 56–62.
7. Charubin,K., Bennett,R.K., Fast,A.G. and Papoutsakis,E.T. (2018) Engineering clostridium organisms as microbial cell-factories: challenges & opportunities. *Metab. Eng.*, 50, 173–191.
8. Galanie,S., Thodey,K., Trenchard,I.J., Filsinger Interrante,M. and Smolke,C.D. (2015) Complete biosynthesis of opioids in yeast. *Science*, 349, 1095–1100.
9. Gustafsson,C., Govindarajan,S. and Minshull,J. (2004) Codon bias and heterologous protein expression. *Trends Biotechnol.*, 22, 346–353.
10. Wu,G., Yan,Q., Jones,J.A., Tang,Y.J., Fong,S.S. and Koffas,M.A.G. (2016) Metabolic burden: cornerstones in synthetic biology and metabolic engineering applications. *Trends Biotechnol.*, 34, 652–664.
11. Yu,T., Dabirian,Y., Liu,Q., Siewers,V. and Nielsen,J. (2019) Strategies and challenges for metabolic rewiring. *Curr. Opin. Syst. Biol.*, 15, 30–38.
12. Karim,A.S. and Jewett,M.C. (2016) A cell-free framework for rapid biosynthetic pathway prototyping and enzyme discovery. *Metab. Eng.*, 36, 116–126.
13. Karim,A.S., Heggestad,J.T., Crowe,S.A. and Jewett,M.C. (2018) Controlling cell-free metabolism through physiochemical perturbations. *Metab. Eng.*, 45, 86–94.
14. Martin,R.W., Des Soye,B.J., Kwon,Y.-C., Kay,J., Davis,R.G., Thomas,P.M., Majewska,N.I., Chen,C.X., Marcum,R.D. and Weiss,M.G. (2018) Cell-free protein synthesis from genomically recoded bacteria enables multisite incorporation of noncanonical amino acids. *Nat. Commun.*, 9, 1203.
15. O'Kane,P.T., Dudley,Q.M., McMillan,A.K., Jewett,M.C. and Mrksich,M. (2019) High-throughput mapping of CoA metabolites by SAMDI-MS to optimize the cell-free biosynthesis of HMG-CoA. *Sci. Adv.*, 5, eaaw9180.
16. Jaroentomeechai,T., Stark,J.C., Natarajan,A., Glasscock,C.J., Yates,L.E., Hsu,K.J., Mrksich,M., Jewett,M.C. and DeLisa,M.P. (2018) Single-pot glycoprotein biosynthesis using a cell-free transcription-translation system enriched with glycosylation machinery. *Nat. Commun.*, 9, 2686.

17. Kightlinger,W., Lin,L., Rosztoczy,M., Li,W., DeLisa,M.P., Mrksich,M. and Jewett,M.C. (2018) Design of glycosylation sites by rapid synthesis and analysis of glycosyltransferases. *Nat. Chem. Biol.*, 14, 627–635.
18. Karim,A.S. and Jewett,M.C. (2018) Cell-free synthetic biology for pathway prototyping. *Methods Enzymol.*, 608, 31–57.
19. Kwon,Y.C. and Jewett,M.C. (2015) High-throughput preparation methods of crude extract for robust cell-free protein synthesis. *Sci. Rep.*, 5, 8663.
20. Dudley,Q.M., Nash,C.J. and Jewett,M.C. (2019) Cell-free biosynthesis of limonene using enzyme-enriched *Escherichia coli* lysates. *Synth. Biol.*, 4,
21. Karim,A.S., Dudley,Q.M. and Jewett,M.C. (2016) Cell-free synthetic systems for metabolic engineering and biosynthetic pathway prototyping. In: Wittmann,C. and Liao,J.C. (eds) Industrial Biotechnology. Wiley, pp. 125–148.
22. Lim,H.J. and Kim,D.-M. (2019) Cell-free metabolic engineering: recent developments and future prospects. *Methods Protoc.*, 2, 33.
23. Rollin,J.A., Tam,T.K. and Zhang,Y.H.P. (2013) New biotechnology paradigm: cell-free biosystems for biomanufacturing. *Green Chem.*, 15, 1708.
24. Jones,D.T. and Wood,D.R. (1986) Acetone-butanol fermentation revisited. *Microbiol. Rev.*, 50, 484–524.
25. Wolfe,A.J. (2005) The acetate switch. *Microbiol. Mol. Biol. Rev.*, 69, 12–50.
26. Nielsen,D.R., Leonard,E., Yoon,S.-H., Tseng,H.-C., Yuan,C. and Prather,K.L.J. (2009) Engineering alternative butanol production platforms in heterologous bacteria. *Metab. Eng.*, 11, 262–273.
27. Al-Shorgani,N.K.N., Kalil,M.S., Yusoff,W.M.W. and Hamid,A.A. (2018) Impact of pH and butyric acid on butanol production during batch fermentation using a new local isolate of *Clostridium acetobutylicum* YM1. *Saudi J. Biol. Sci.*, 25, 339–348.
28. Baronovsky,J.J., Schreurs,W.J.A. and Kashket,E.R. (1984) Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. *Appl. Environ. Microbiol.*, 48, 1134–1139.
29. Gottwald,M. and Gottschalk,G. (1985) The internal pH of *Clostridium acetobutylicum* and its effect on the shift from acid to solvent formation. *Arch. Microbiol.* 143, 42–46.
30. Nolling,J. (2001) Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. *J. Bacteriol.*, 183, 4823–4838.
31. Tomb,J.-F., White,O., Kerlavage,A.R., Clayton,R.A., Sutton,G.G., Fleischmann,R.D., Ketchum,K.A., Klenk,H.P., Gill,S., Dougherty,B.A. et al. (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature*, 388, 539–547.
32. Brandt,J.U., Jakob,F., Geissler,A.J., Behr,J. and Vogel,R.F. (2017) Multiple genome sequences of heteropolysaccharide-forming acetic acid bacteria. *Genome Announc.*, 5, 1–3.
33. Yang,S., Fei,Q., Zhang,Y., Contreras,L.M., Utturkar,S.M., Brown,S.D., Himmel,M.E. and Zhang,M. (2016) *Zymomonas mobilis* as a model system for production of biofuels and biochemicals. *Microb. Biotechnol.*, 9, 699–717.
34. Sergey,S., Liu,Z., Thiel,V., Tomsho,L.P., Pinel,N., Nelson,W.C., Lindemann,S.R., Romine,M.F., Haruta,S., Schuster,S.C., et al. (2014) Genome sequence of the thermophilic cyanobacterium *Thermosynechococcus* sp. strain NK55a. *Genome Announc.*, 2, 1–2.
35. Juneja,P. and Lazzaro,B.P. (2009) *Providencia sneebia* sp. nov. and *Providencia burhodogranariea* sp. nov., isolated from wild *Drosophila melanogaster*. *Int. J. Syst. Evol. Microbiol.*, 59(Pt 5), 1108–1111.
36. Falb,M., Pfeiffer,F., Palm,P., Rodewald,K., Hickmann,V., Tittor,J. and Oesterhelt,D. (2005) Living with two extremes: conclusions from the genome sequence of *Natronomonas pharaonis*. *Genome Res.*, 15, 1336–1343.
37. Janto,B., Ahmed,A., Ito,M., Liu,J., Hicks,D.B., Pagni,S., Fackelmayer,O.J., Smith,T.-A., Earl,J., Elbourne,L.D.H. et al. (2011) Genome of alkaliphilic *Bacillus pseudofirmus* OF4 reveals adaptations that support the ability to grow in an external pH range from 7.5 to 11.4. *Environ. Microbiol.*, 13, 3289–3309.
38. Niehaus,F., Bertoldo,C., Kähler,M. and Antranikian,G. (1999) Extremophiles as a source of novel enzymes for industrial application. *Appl. Microbiol. Biotechnol.*, 51, 711–729.
39. Shen,C.R., Lan,E.I., Dekishima,Y., Baez,A., Cho,K.M. and Liao,J.C. (2011) Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl. Environ. Microbiol.*, 77, 2905–2915.
40. Atsumi,S., Cann,A.F., Connor,M.R., Shen,C.R., Smith,K.M., Brynildsen,M.P., Chou,K.J.Y., Hanai,T. and Liao,J.C. (2008) Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab. Eng.*, 10, 305–311.