Enhancing control of cell-free metabolism through pH modulation

Ashty S. Karim, Blake J. Rasor, and Michael C. Jewett

Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Tech E-136, Evanston, IL, 60208, USA, Chemistry of Life Processes Institute, Northwestern University, 2145 Sheridan Road, Tech E-136, Evanston, IL, 60208, USA, Center for Synthetic Biology, Northwestern University, 2145 Sheridan Road, Tech E-136, Evanston, IL, 60208, USA, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL, 60611, USA and Simpson Querrey Institute, Northwestern University, Chicago, IL, 60611, USA

Corresponding authors: E-mails: m-jewett@northwestern.edu and ashty.karim@northwestern.edu

These authors contributed equally to this work.

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,
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 physicochemical 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 Thi (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 lYTPG at 37°C. Enzyme expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at OD600 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 (OD600 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 5N 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 folic 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 μmol butanol produced per mM butyraldehyde consumed (molbutanol/molbutyraldehyde) 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](https://academic.oup.com/synbio/article-fig/5/1/ysz027/5640505)
lactate is produced per 2 glucose molecules consumed (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 variations.
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 burladoranaria sp. 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.

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 molbutanol/mol butyraldehyde) from butyraldehyde (Table 1), which is consistent with the use of this homolog for butanol biosynthesis in recombinant hosts (39, 40).

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| 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) [molbutanol/mol butyraldehyde] | 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% ± 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 burladoranaria (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 the 'ref. yield' in mol of butanol produced per mol of butyraldehyde consumed (mol butanol/mol butyraldehyde).
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 pathway 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.

Funding

We would like to thank the Department of Energy for funding (DE-SC0018249). A.S.K. was partially supported under a National Science Foundation's Graduate Research Fellowship (DGE-1324585-008). B.J.R. is funded by a National Defense Science and Engineering Graduate Fellowship (ND-CEN-017-095). M.C.J. also acknowledges the David and Lucile Packard Foundation and the Dreyfus Teacher-Scholar program.

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.

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