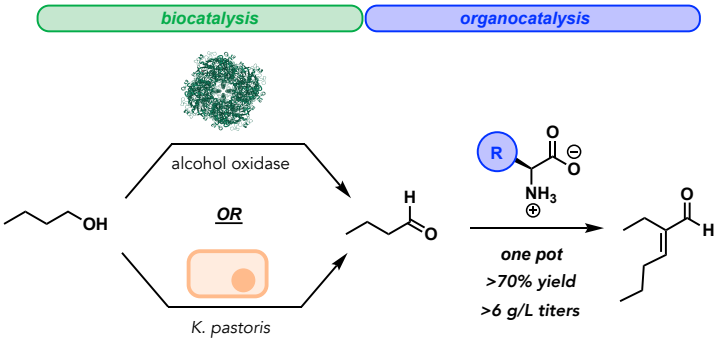
A One-Pot Biocatalytic and Organocatalytic Cascade Delivers High Titers of 2-Ethyl-2-Hexenal from n-Butanol

Kelsey N. Stewart and Dylan W. Domaille*

Department of Chemistry, Colorado School of Mines, Golden CO 80401

*To whom correspondence should be addressed: ddomaille@mines.edu



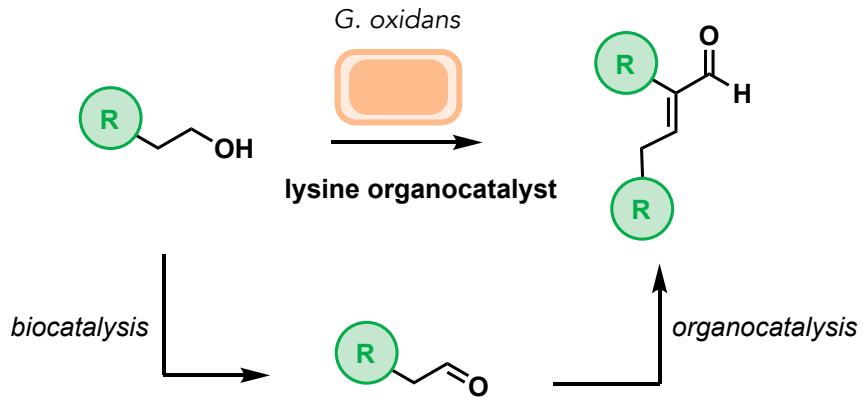
Abstract:

Biocatalysis provides facile access to selective chemical transformations and helps satisfy sustainable chemical production criteria. However, the reaction scope of biocatalysts is significantly narrower compared to synthetic chemical transformations. Hybrid biocatalytic-chemocatalytic cascades expand the scope of products while maintaining many of the benefits associated with biocatalysis. Here, we report that single-pot systems with whole cell *K. pastoris* (ATCC® 28485™) or isolated enzyme alcohol oxidase (E 1.1.3.13) as oxidative biocatalysts with a lysine organocatalyst yields the commercial target, 2-ethyl-2-hexenal (2-EH) from n-butanol in a two-step hybrid cascade. Peak yields for both biocatalysts were achieved with 100 mM n-butanol at pH 8 and 30°C. The isolated enzyme slightly outperformed whole cell *K. pastoris*, reaching 73% conversion (4.7 g/L titers) compared to 61% (3.9 g/L titers) in whole cells systems. Titers could be improved for both biocatalysts (5.7 – 6.7 g/L) at increased butanol loading; however, this came at the expense of decreased yields. Compared to our initial results with a *Gluconobactor oxidans* whole cell biocatalyst, the reported system improves upon 2-EH titers by 2.8–3.3-fold at maximal yields.

Introduction

Biocatalysis—the use of isolated enzymes, partially purified enzymes, and/or whole cells to execute a chemical transformation—plays an important role in sustainable chemical synthesis.[1,2] Biocatalytic transformations offer significant benefits over traditional methodology by obviating the need for expensive metal catalysts[3], organic solvents[4], and high temperatures[5], satisfying green chemistry criteria for hazard reduction and benign, non-toxic solvents and auxiliaries.[6] The high level of selectivity and stereocontrol afforded by enzymes decreases the need for intermediate protecting/deprotection steps that are often necessary to achieve the same stereospecific product with traditional synthetic techniques.[7,8] Recent efforts have yielded impressive improvements in the biocatalysis toolbox, with *de novo* designed and evolved biocatalysts mediating the Kemp elimination[9] and Diels-Alder[10] reactions and playing central roles in chemical syntheses, such as the production of S-adenosyl-l-methionine (SAM) analogs[11]. However, despite major advances in the fields of metabolic and protein engineering, biocatalysts catalyze comparatively few transformations compared to the portfolio of chemical catalyzed reactions.[12,13] While the versatility of traditional chemical catalyst exceeds that of biocatalyst, purely chemical processes often lack the high selectivity and intrinsic sustainability of bioprocesses.[14]

To complement existing efforts in the biocatalysis/biocatalytic cascade space, we are pursuing the development of hybrid chemocatalyst/biocatalytic cascades. Combining biocatalysts with non-enzymatic catalysts in cascade reactions can furnish products inaccessible through biocatalysis alone, while maintaining the associated green chemistry benifits.[15,16–18] A number of reports have successfully incorporated isolated enzymes with organocatalysts in multi-catalytic cascades.[19–21] Our recent work provided the first demonstration of a one-pot cascade reaction using whole-cell biocatalysts with a biocompatible organocatalyst. We showed that *Gluconobactor oxidans* (*G. oxidans)* effectively oxidized n-butanol to n-butyraldehyde. A subsequent aldol condensation catalyzed by a biocompatible lysine catalyst delivered 2-ethyl-2-hexenal (2-EH) in 84% yield and 1.6 g L^{-1} titers in aqueous buffer at 28°C.[22] We not only delivered a commercially valuable precursor in one-pot under mild conditions from a renewable substrate, but also demonstrated that organocatalysts can be leveraged to redirect metabolic flux within the system. At extended processing times *G. oxidans* overoxidize the aldehyde to the corresponding acid. However, the inclusion of an organocatalyst with the whole cell biocatalysts intercepts the aldehyde and drives flux to the industrially relevant α,β-unsaturated aldehyde.



Can other enzyme and/or whole cell biocatalysts be merged with this system?

Figure 1. Our previous work showed that a whole cell biocatalyst (*G. oxidans*) with lysine organocatalysis yields α,β-unsaturated aldehydes.[22] The present work explores the broad applicability of this methodology to isolated enzyme and eukaryotic whole cell biocatalysts.

In the present work, we sought to determine if amino acid-based organocatalysis could be merged with isolated enzymes and other microbial whole cell biocatalysts beyond *G. oxidans* while also exploring the comparative efficiency of systems with isolated enzymes and whole cell biocatalysts (**Figure 1**). Using whole cell biocatalysts requires less intensive preparation than using isolated enzymes: once grown, live cultures can be transferred directly to the reaction medium or substrates can be added directly to the culture media.[23–25] In contrast, enzymes are separated from growth media and cell lysates and then purified before use [26,27], though, in some cases, the crude cell lysate can be used in place of the whole cells or purified enzyme.[28] Whole cells introduce a multitude of other enzymes and pathways to the system, increasing the chance of feedback inhibition or unwanted side products[29] but provide a built-in cofactor recycling system.[30] Isolated enzymes enable more precise control of the enzyme composition of the reaction but often require an auxiliary system for cofactor recycling.[31] The cellular environment can provide some protection from external operating conditions[32]; however, this can come at the cost of impaired substrate/product transport in and out of the cell.[33] Isolated enzymes do not suffer from impaired mass transport but are more susceptible to thermal or chemical denaturation.[34,35]

To explore the comparative advantages and disadvantages of using isolated enzymes and whole cell biocatalysts, we chose alcohol oxidase (AO) because of its wide commercial availability and *Komagataella pastoris (K. pastoris)*, previously known as *Pischa pastoris*[36], the organism from which commercial alcohol oxidase is isolated. Alcohol oxidase (E 1.1.3.13) is a member of the GMC (glucose methanol choline) oxidoreductase superfamily of

enzymes.[37] The short-chain alcohol oxidase oxidizes C_1 to C_6 primary alcohols[38–40], as well as several unsaturated alcohols[40,41] and diols[42] and is used in multi-step syntheses[41,43] and bio-based sensing systems.[44] Alcohol oxidase contains a covalently bound flavin adenine dinucleotide (FAD) prosthetic group, which uses oxygen as an electron acceptor, obviating the need for additional in-situ cofactor recycling. The alcohol substrate is oxidized through a hydride transfer from substrate to FAD, followed by reoxidation of FAD by molecular oxygen, forming hydrogen peroxide.[45–47] Hydrogen peroxide will inactivate the enzyme; however, this process can be reversed with mercaptoethanol to restore activity.[38] To prevent H_2O_2-mediated deactivation, catalase is often added to decompose hydrogen peroxide to water and oxygen and prevent enzyme deactivation.

Komagataella pastoris (ATCC® 28485™), is a strain of methylotrophic yeast that expresses high levels of alcohol oxidase when grown with methanol as the main carbon source.[48] Alcohol oxidase acts as the first step in methanol metabolism and is contained within peroxisomes. In the peroxisome, methanol is oxidized to formaldehyde via alcohol oxidase producing hydrogen peroxide, which is then decomposed by catalase. The compartmentalization of alcohol oxidase with catalase tightly links the production and decomposition of hydrogen peroxide, preventing diffusion into the cytoplasm.[49] Whole cell *K. pastoris* have been used as biocatalysts to oxidize C_1-C_5 primary alcohols[50] and benzyl alcohol.[51] Micro-aqueous systems (<4% water by volume) expand the substrate scope to include higher weight alcohols (C_6-C_{10}).[52] Because *K. pastoris* displays good activity toward aliphatic alcohols and isolated alcohol oxidase does not require an auxiliary co-factor recycling system, the pair permits a straightforward comparison between multi-step reaction efficiencies with enzymes and whole cells.

Results and Discussion

We focused on the synthesis of 2-ethyl-2-hexenal (2-EH), a key intermediate in the synthesis of 2-ethyl-2-hexanol, which is currently produced on the multi-million ton scale annually using Rh-catalysis. We began by first screening biocompatible amino acid catalysts for in situ aldol condensations.[53] Lysine has previously been reported to be a good catalyst for self-aldol condensations of a number of aliphatic aldehydes (C_3-C_9) in an aqueous or solvent-less system, and we have shown that it is well-tolerated by the gram-negative bacteria *G. oxidans* for single-pot biocompatible reactions.[22,54] When twenty proteinogenic amino acids were screened as homo aldol condensation catalysts in ethanol a number of promising candidates, including lysine, emerged.[55] Thus, we first screened all 20 amino acids under

conditions relevant to our system (50 mM PBS, pH 7.4, RT, 24 h with 200 mM n-butyraldehyde) (**SI Fig 1**). Isooctane (16.7% v/v) was included as a biocompatible extractant to remove the resulting 2-ethyl-2-hexenal (2-EH). Reactions were stirred for 24 h to simulate biocatalysis conditions, at which point, an aliquot of the isooctane layer was analyzed by gas chromatography-mass spectrometry (GC-MS). Expanding the study to include all 20 amino acids revealed that, consistent with previous results, lysine and glycine remained the top performers with a maximum conversion of 50%. Arginine and histidine, which had not been previously explored under these conditions, also performed well, but with overall lower yields of 36 and 37%, respectively.

Next, we tested the top four amino acid catalysts (lysine, glycine, histidine, and arginine) at three concentrations (50, 100, and 250 mM) in a one-pot system with either whole cell *K. pastoris* or isolated alcohol oxidase as a biocatalyst (**Figure 2**). The biocatalyst oxidizes n-butanol to n-butyraldehyde, and an in situ lysine organocatalyst dimerizes n-butyraldehyde to 2-EH. In one-pot reactions with alcohol oxidase (30 U) and an organocatalyst, 2-EH yields were maximized at the highest catalyst loading (250 mM). Lysine (21%) and arginine (18%) gave the highest yields of 2-EH through the two-step process. A similar trend persisted in one-pot reactions with whole cells: histidine (14%) and glycine (19%) delivered lower yields of 2-EH than lysine (36%) and arginine (24%). Taken together, lysine (250 mM) produced the highest yields with both whole cells or isolated enzymes (36% and 21%, respectively). In this initial two-step process, lysine outperformed the other catalysts, and whole cells reactions produced a higher maximum yield than the isolated enzymes. Based on these data, all subsequent reactions were conducted with 250 mM lysine as the organocatalyst.

We next turned toward optimizing the substrate and enzyme loadings (**Figure 3**). When varying alcohol loading in our previous system with *G. oxidans*, we saw a clear trend of increased yields at lower alcohol concentrations across all catalysts tested.[22] Substate concentration is closely tied to enzymatic performance; the rate slows as the active site becomes saturated. In this instance, decreasing substrate loading while maintaining the same

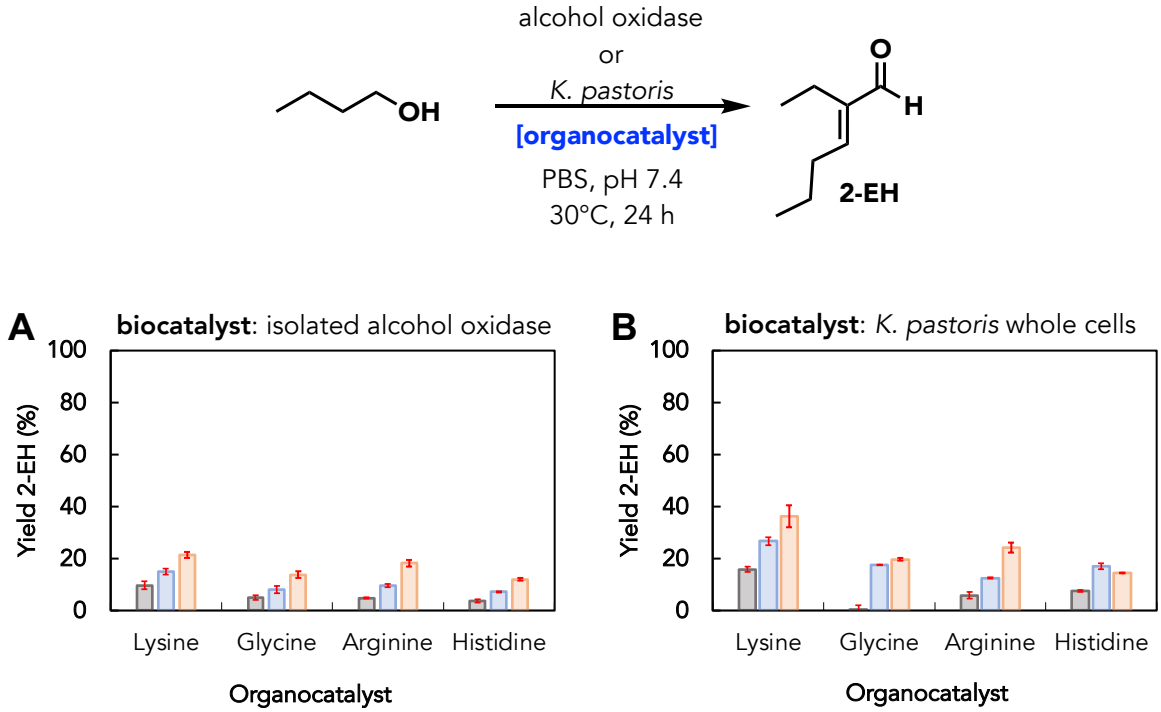


Figure 2. Yield of 2-EH with four different amino acid organocatalysts with isolated alcohol oxidase (A) or whole cell *K. pastoris* (B). Reactions were run with alcohol oxidase (30 U) or *K.* pastoris whole cells (1×) containing 200 mM n-butanol in PBS, pH 7.4 with isooctane (16.7% v/v) at 30°C for 24 h. Organocatalysts were tested at 50 mM (gray), 100 mM (blue) or 250 mM (orange). Each data point represents the average ± SD of three separate reactions.

enzyme concentration could increase the rate of the reaction. Alternatively, increasing the enzyme concentration while maintaining the substrate concentration may achieve the same results. In contrast, high substrate, intermediate, or product concentration could be detrimental to the biocatalyst, leading to inhibition, enzyme inactivation, or cell death. In this instance, decreasing substrate concentration while maintaining biocatalyst loading could potentially increase performance. However, it is more likely that overall performance can be attributed to a combination of competing factors. Due to this complex dynamic, we chose to simultaneously vary both initial substrate concentration and biocatalyst loading.

We tested alcohol oxidase loadings of 15, 30, or 60 units with 100, 200, or 400 mM n-butanol (**Figure 3A**). As with our previous system, higher substrate concentrations lead to decreased yields. For instance, with a constant enzyme loading of 60 units, the yield of 2-EH decreases from 51% at 100 mM butanol to 26% at 400 mM butanol. This trend held across all enzyme loadings studied. We also observed higher yields at higher enzyme loadings. With a constant n-butanol concentration of 100 mM and increasing enzyme loadings, yields increased from 21% (15 U AO) to 51% (60 U AO). Next, we evaluated how these conditions

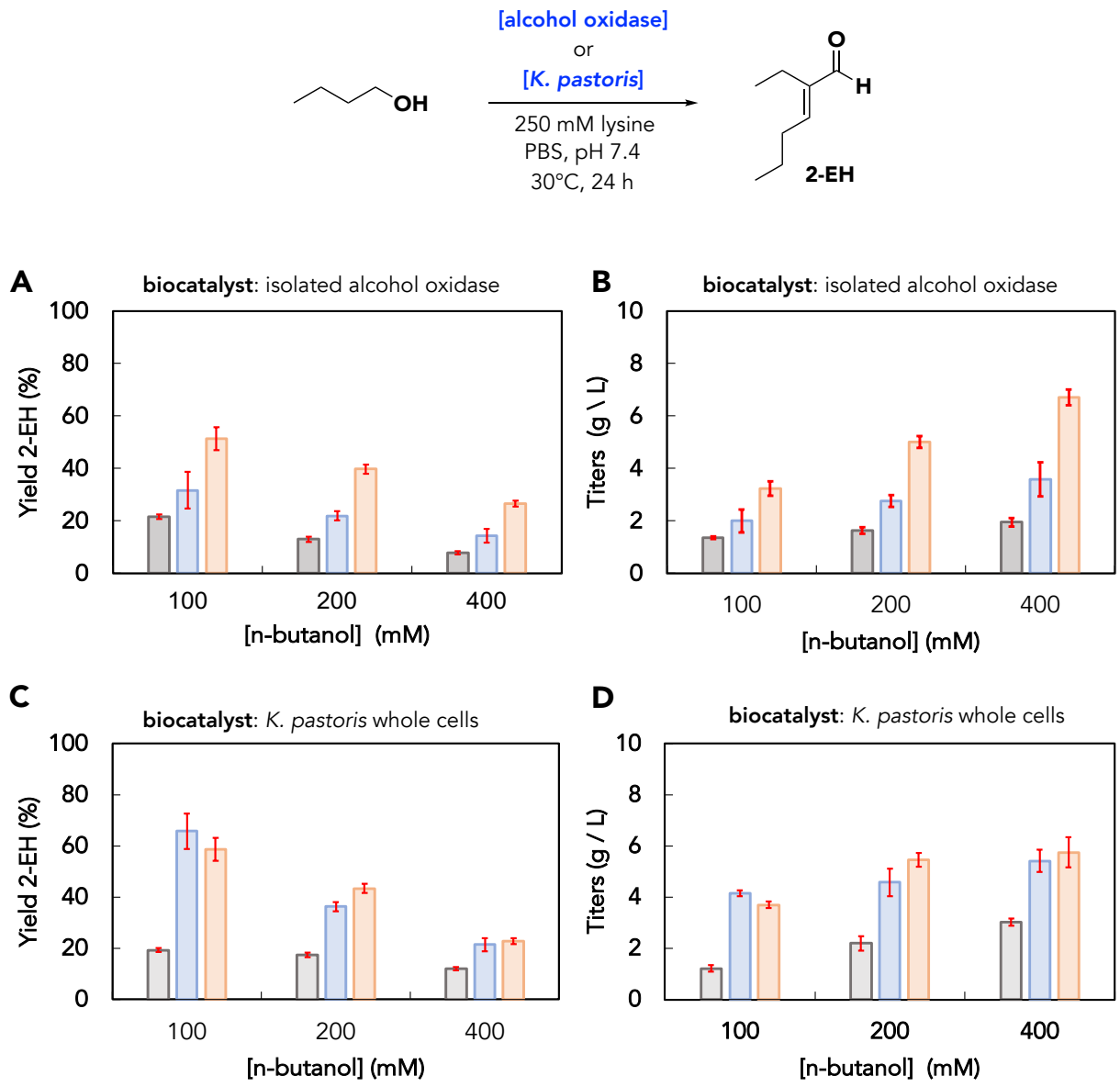


Figure 3 | Effect of biocatalyst and initial alcohol loadings on overall system efficiency. Reactions were run with either isolated alcohol oxidase or *K. pastoris* whole cells and n-butanol at the indicated loadings in PBS, pH 7.4 buffer containing lysine (250 mM) and an isooctane overlay (16.7% v/v) for 24 h at 30°C. Shown are yields (A) and titers (B) with isolated alcohol oxidase at 15 U (grey), 30 U (blue), and 60 U (orange) with the indicated n-butanol loading. Yields (C) and titers (D) for whole cell *K. pastoris* system are shown at 0.5× (grey), 1× (blue), and 2× (orange) cell density with the indicated n-butanol loading. Each data point represents the average ± SD of three separate reactions.

impact the titers of product (**Figure 3B**). Increasing substrate concentration while holding biocatalyst loading constant increases titers across all conditions. The highest alcohol concentration (400 mM) with the highest enzyme loading (60 U) produced the highest titers (6.7 g L^{-1}) with a corresponding yield of 21%, whereas the highest conversion (100 mM n-butanol, 15 U) yielded 3.2 g L^{-1} with a corresponding yield of 51%.

We continued our exploration to determine how whole cell biocatalyst loading impacted yield and titers of the one-pot systems. Yeast cultures grown for 72 h reach an OD_{600} = 2.2, which we define as 1×. Diluting to half the cell density (0.5×) or concentrating to twice the cell density (2×) allowed us to determine how cell density impacts the overall yield. We varied the cell density with the same initial n-butanol concentrations (100, 200, or 400mM) (**Figure 3C**). Decreasing cell loading by half (0.5×) drastically decreased yields across all substrate concentrations. Doubling the cell loading delivers similar yields at all concentrations to the 1× conditions. Consistent with the isolated enzyme experiments, the maximum titers of 5.7 g L^{-1} were achieved with the highest alcohol concentration (400 mM) and biocatalyst loading (2×); however, 1× conditions give very similar yields (**Figure 3D)**. The whole cell biocatalyst maximal titers are 1 g L^{-1} lower than when the enzyme is utilized.

In previous experiments, we terminated all reactions after 24 h to enable straightforward comparison across conditions. Thus, we next examined longer reaction times would deliver higher yields. We tracked the time-course production of 2-EH over the course of 72 h. We explored the lowest n-butanol concentration (100 mM) at all three enzyme loadings (15U, 30 U, and 60 U), as well as the highest enzyme loading (60 U) at three n-butanol concentrations (100 mM, 200 mM and 400 mM) (**Figure 4A**). With fixed enzyme loading at 60 U, the 2-EH yield plateaued after 48 h; however, each reaction reached a different maximum. Low substrate loading (100 mM) delivered the highest yield (60%), while a moderate substrate loading (200 mM) gave 44% 2-EH, and the highest substrate loading (400 mM) gave 23% of 2-EH. A fixed substrate concentration (100 mM) and evaluation of different enzyme loading (15 U and 30 U) showed a continual slow increased in 2-EH production, even up to 72 h, providing 27% and 60% yield, respectively. In contrast, 60 U reached its maximal yield at 48 h (60%) and plateaued. Similar trends were seen with the yeast cells acting as biocatalyst (**Figure 4B**). The yields for all conditions plateaued after 48 hours. The reactions with double the yeast loading (2×) and the lowest alcohol concentration (100 mM) maintained the highest yields for the first 24 hrs. After 24 hours both 1× and 2× cell densities showed comparable yields. Both whole cell and enzymes provided maximal yields of ~60%.

Having optimized concentrations and time-course production profiles, we next turned to optimizing the temperature (**Figure 5A-B**) and pH conditions (**Figure 5C-D**) for isolated enzyme and whole cell reactions. The optimal pH and temperature for alcohol oxidase has

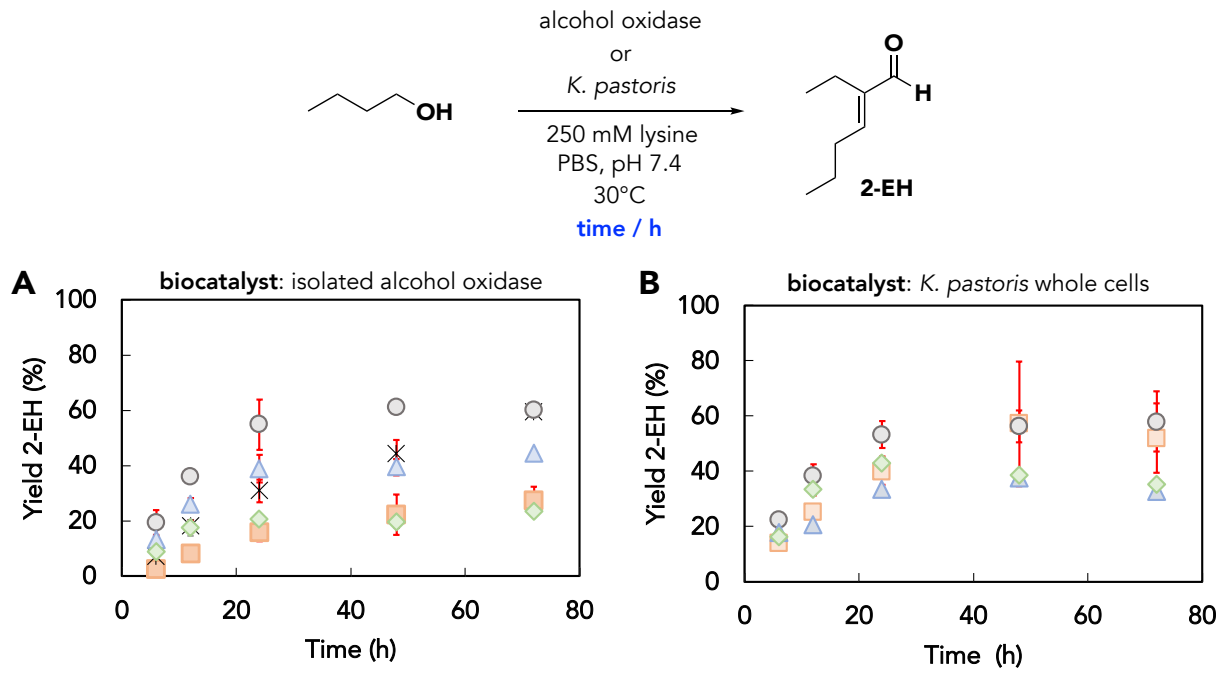


Figure 4 | Time course production of 2-EH with alcohol oxidase or *K. pastoris* whole cells at the indicated biocatalyst and n-butanol (BuOH) loading in PBS, pH 7.4 containing 250 mM lysine and an isooctane overlay (16.7% v/v) at 30°C. Aliquots were removed at the indicated times, and the yield determined by GC-MS. (A) Isolated alcohol oxidase (■) 15 U, 100 mM BuOH (×) 30 U, 100 mM BuOH (●) 60 U, 100 mM BuOH (▲) 60 U, 200 mM BuOH (♦) 60 U, 400 mM BuOH. (B) Whole cell *K. pastoris* (■) 1× cell density, 100 mM BuOH (●) 2× cell density, 100 mM BuOH (▲) 1× cell density, 200 mM BuOH (♦) 2×, 200 mM BuOH. Each data point represents the average 2-EH yield at the indicated timepoint ± SD of three separate reactions.

been reported as either pH 7.4 at 30°C[46] or pH 7.5 at 37°C[38]. Yields for both whole cells (63%) and isolated enzymes (74%) peaked at 30°C and pH 8. Overall, the enzymes were more tolerant of the highest temperature tested (45°C) while the whole cells were more tolerant of the highest pH tested (pH 9.2). Yields were poor (<20%) for both whole cells and enzymes at the lowest pH tested (pH 6). Because *K. pastoris* is able to grow with on methanol at pH 6, the low yields are likely a result of slower lysine catalysis, which is impeded at acidic conditions.[50]

We next probed why the reaction yield plateaued for both the isolated enzymes and whole cell biocatalysts. We reasoned that this observation could be the result of either incomplete biocatalyzed oxidation of the alcohol to aldehyde, incomplete organocatalyzed aldol formation, or creation of a side-product. Other flavin-dependent oxidases have been reported to over-oxidize past the aldehyde to the carboxylic acid.[56,57] Analysis of both the aqueous and organic layers by NMR shows the majority of the remaining mass is unreacted

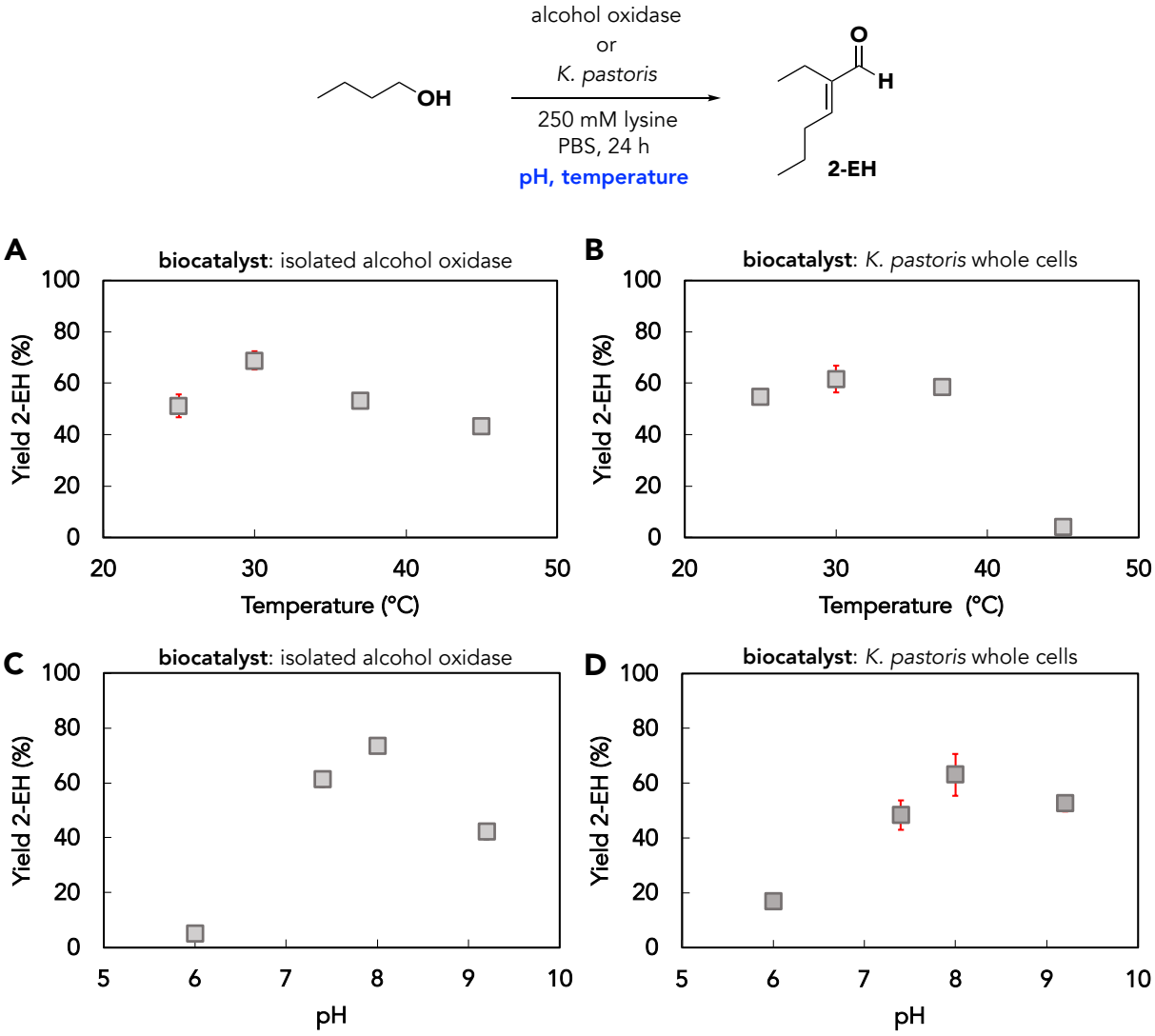


Figure 5. Effect of temperature on two-step, one-pot conversion of n-butanol to 2-EH using isolated alcohol oxidase (A) or whole cell *K. pastoris* (B) and effect of pH on the two-step, one-pot conversion of n-butanol to 2-EH with isolated alcohol oxidase (C) or *K. pastoris* (D). Variable temperature studies were run with alcohol oxidase (60 U), 100 mM n-butanol in PBS, pH 7.4 containing lysine (250 mM) and an isooctane overlay (16.7%) at the indicated temperature for 24 h. Variable pH studies were run with *K. pastoris* (2×), 100 mM n-butanol in PBS at 30°C with lysine (250 mM) and an isooctane overlay (16.7% v/v) at the indicated pH for 24 h. Each data point represents the average yield of 2-EH ± SD of three separate reactions.

butanol; however, a small amount of both butyraldehyde and butanoic acid exist in the aqueous phase. To probe the state of the biocatalyst, a second aliquot of alcohol was added to the reaction after 24 hrs. Both K. *pastoris* and alcohol oxidase showed reduced activity with the second addition of alcohol (**SI Figure 2**), indicating that some deactivation of the biocatalyst occurs during the course of the reaction. Repeating the experiment in media instead of PBS did not significantly impact the biocatalyst activity.

Conclusions

To close, we have shown that lysine organocatalyzed aldol condensations are compatible with both isolated enzymes and whole cell biocatalysts. This is the first methodology that combines a eukaryotic whole cell biocatalyst with an organocatalyst in the same flask for multi-step transformations. Yields for systems with isolated alcohol oxidase peaked at 73% (4.7 g/L) at 100 mM n-butanol, pH 8.0 at 30°C. Whole cell *K. pastoris* delivers maximal yields of 61% (3.9 g/L titers) at 100 mM n-butanol, pH 8.0 at 30°C. Higher titers are available at higher n-butanol concentrations (400 mM n-butanol; 5.7 – 6.7 g/L). However, these titers come at the sake of decreased yield, as significant amounts of unreacted n-butanol are observed. Compared to our initially reported system with the gram-negative bacteria *G. oxidans* as a whole-cell biocatalysts, which delivered 2-EH in 84% yield and 1.8 g/L titers, both the isolated alcohol oxidase and whole cell *K. pastoris* significantly improve upon the titers. At the shaker flask level, using commercially available enzymes is the easier option. The purified enzymes are stored in the freezer, giving them the convenience of a traditional chemical reagent. *K. pastoris* grow slowly on methanol, requiring approximately three days from start up to reach an appropriate cell density. However, should this be run in recurring batches or at a larger scale, commercially sourced enzymes quickly become expensive and use of the whole cells becomes more prudent. A drawback of both systems, however, is the high concentration of organocatalyst needed to drive an aqueous condensation dimerization at low aldehyde concentrations. Our current work is focused on engineering the catalyst microenvironment for aldehyde activation chemistry.

References

1 R. A. Sheldon and J. M. Woodley, *Chem. Rev.*, 2018, 118, 801–838.
2 R. A. Sheldon and D. Brady, *ACS Sustainable Chem. Eng.*, 2021, 9, 8032–8052.
3 C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman and G. J. Hughes, *Science*, 2010, 329, 305–309.
4 K. S. Dalal, M. A. Chaudhari, D. S. Dalal and B. L. Chaudhari, *Catalysis Communications*, 2021, 152, 106289.
5 M. Gavrilescu and Y. Chisti, *Biotechnology Advances*, 2005, 23, 471–499.
6 S. Wenda, S. Illner, A. Mell and U. Kragl, *Green Chemistry*, 2011, 13, 3007–3047.
7 E. P. Greenberg, J. R. Chandler and M. R. Seyedsayamdost, *J. Nat. Prod.*, 2020, 83, 738–743.
8 P. Vojáčková, L. Michalska, M. Nečas, D. Shcherbakov, E. C. Böttger, J. Šponer, J. E. Šponer and J. Švenda, *J. Am. Chem. Soc.*, 2020, 142, 7306–7311.
9 D. Röthlisberger, O. Khersonsky, A. M. Wollacott, L. Jiang, J. DeChancie, J. Betker, J. L. Gallaher, E. A. Althoff, A. Zanghellini, O. Dym, S. Albeck, K. N. Houk, D. S. Tawfik and D. Baker, *Nature*, 2008, 453, 190–195.
10 J. B. Siegel, A. Zanghellini, H. M. Lovick, G. Kiss, A. R. Lambert, J. L. St.Clair, J. L. Gallaher, D. Hilvert, M. H. Gelb, B. L. Stoddard, K. N. Houk, F. E. Michael and D. Baker, *Science*, 2010, 329, 309–313.
11 Q. Tang, C. W. Grathwol, A. S. Aslan-Üzel, S. Wu, A. Link, I. V. Pavlidis, C. P. S. Badenhorst and U. T. Bornscheuer, *Angewandte Chemie International Edition*, 2021, 60, 1524–1527.
12 R. A. Sheldon and D. Brady, *Chem. Commun.*, 2018, 54, 6088–6104.
13 B. Hauer, *ACS Catal.*, 2020, 10, 8418–8427.
14 S. Wallace and E. P. Balskus, *Curr Opin Biotechnol*, 2014, 30, 1–8.
15 K. N. Stewart and D. W. Domaille, *ChemBioChem*, , DOI:10.1002/cbic.202000458.
16 D. Kracher and R. Kourist, *Current Opinion in Green and Sustainable Chemistry*, 2021, 32, 100538.
17 N. Losada-Garcia, Z. Cabrera, P. Urrutia, C. Garcia-Sanz, A. Andreu and J. M. Palomo, *Catalysts*, 2020, 10, 1258.
18 Y. Liu, P. Liu, S. Gao, Z. Wang, P. Luan, J. González-Sabín and Y. Jiang, *Chemical Engineering Journal*, 2021, 420, 127659.
19 G. Rulli, N. Duangdee, K. Baer, W. Hummel, A. Berkessel and H. Gröger, *Angewandte Chemie International Edition*, 2011, 50, 7944–7947.
20 K. Baer, M. Kraußer, E. Burda, W. Hummel, A. Berkessel and H. Gröger, *Angewandte Chemie International Edition*, 2009, 48, 9355–9358.
21 E. Liardo, N. Ríos-Lombardía, F. Morís, F. Rebolledo and J. González-Sabín, *ACS Catal.*, 2017, 7, 4768–4774.
22 K. N. Stewart, E. G. Hicks and D. W. Domaille, *ACS Sustainable Chem. Eng.*, 2020, 8, 4114–4119.
23 A.-D. Cheng, S.-S. Shi, Y. Li, M.-H. Zong and N. Li, *ACS Sustainable Chem. Eng.*, 2020, 8, 1437–1444.
24 P. Kottenhahn, K. Schuchmann and V. Müller, *Biotechnol Biofuels*, 2018, 11, 93.
25 R. Villa, A. Romano, R. Gandolfi, J. V. Sinisterra Gago and F. Molinari, *Tetrahedron Letters*, 2002, 43, 6059–6061.
26 Z. Liang, G. Li, J. Xiong, B. Mai and T. An, *Chemosphere*, 2019, 237, 124461.
27 S. A. Kelly, J. Megaw, J. Caswell, C. J. Scott, C. C. R. Allen, T. S. Moody and B. F. Gilmore, *ChemistrySelect*, 2017, 2, 9783–9791.
28 H. Land, P. Ceccaldi, L. S. Mészáros, M. Lorenzi, H. J. Redman, M. Senger, S. T. Stripp and G. Berggren, *Chemical Science*, 2019, 10, 9941–9948.
29 B. Lin and Y. Tao, *Microbial Cell Factories*, 2017, 16, 106.
30 J. Wachtmeister and D. Rother, *Current Opinion in Biotechnology*, 2016, 42, 169–177.
31 W. Liu and P. Wang, *Biotechnology Advances*, 2007, 25, 369–384.

32 J. Yan, X. Zheng and S. Li, *Bioresource Technology*, 2014, 151, 43–48.
33 R. R. Chen, *Appl Microbiol Biotechnol*, 2007, 74, 730–738.
34 A. Merz, M. Yee, H. Szadkowski, G. Pappenberger, A. Crameri, W. P. C. Stemmer, C. Yanofsky and K. Kirschner, *Biochemistry*, 2000, 39, 880–889.
35 P. A. Dalby, J. P. Aucamp, R. George and R. J. Martinez-Torres, 2007.
36 C. P. Kurtzman, *International Journal of Systematic and Evolutionary Microbiology*, 2005, 55, 973–976.
37 D. R. Cavener, *Journal of Molecular Biology*, 1992, 223, 811–814.
38 R. Couderc and J. Baratti, *Agricultural and Biological Chemistry*, 1980, 44, 2279–2289.
39 R. N. Patel, C. T. Hou, A. I. Laskin and P. Derelanko, *Archives of Biochemistry and Biophysics*, 1981, 210, 481–488.
40 G. Dienys, S. Jarmalavičius, S. Budrien≐, D. Čitavičius and J. Sereikait≐, *Journal of Molecular Catalysis B: Enzymatic*, 2003, 21, 47–49.
41 A. Siebum, A. van Wijk, R. Schoevaart and T. Kieboom, *Journal of Molecular Catalysis B: Enzymatic*, 2006, 41, 141–145.
42 M. Kjellander, K. Götz, J. Liljeruhm, M. Boman and G. Johansson, *Biotechnol Lett*, 2013, 35, 585–590.
43 M. Pérez-Sánchez, C. R. Müller and P. Domínguez de María, *ChemCatChem*, 2013, 5, 2512–2516.
44 T. Monteiro, R. Zumpano, C. M. Silveira and M. Gabriela Almeida, in *Enzymes for Solving Humankind's Problems: Natural and Artificial Systems in Health, Agriculture, Environment and Energy*, eds. J. J. G. Moura, I. Moura and L. B. Maia, Springer International Publishing, Cham, 2021, pp. 303–362.
45 J. Vonck, D. N. Parcej and D. J. Mills, *PLoS One*, 2016, 11, e0159476.
46 C. Koch, P. Neumann, O. Valerius, I. Feussner and R. Ficner, *PLOS ONE*, 2016, 11, e0149846.
47 V. Menon, C. T. Hsieh and P. F. Fitzpatrick, *Bioorganic Chemistry*, 1995, 23, 42–53.
48 P. Ozimek, M. Veenhuis and I. J. van der Klei, *FEMS Yeast Research*, 2005, 5, 975–983.
49 H. Yurimoto, M. Oku and Y. Sakai, *International Journal of Microbiology*, 2011, 2011, e101298.
50 S. J. B. Duff and W. D. Murray, *Biotechnology and Bioengineering*, 1988, 31, 44–49.
51 S. J. B. Duff and W. D. Murray, *Biotechnology and Bioengineering*, 1989, 34, 153–159.
52 W. D. Murray and S. J. B. Duff, *Appl Microbiol Biotechnol*, 1990, 33, 202–205.
53 D. W. Domaille, G. R. Hafenstine, M. A. Greer, A. P. Goodwin and J. N. Cha, *ACS Sustain Chem Eng*, 2016, 4, 671–675.
54 Y. Watanabe, K. Sawada and M. Hayashi, *Green Chemistry*, 2010, 12, 384–386.
55 K. A. Ostrowski, D. Lichte, M. Stuck and A. J. Vorholt, *Tetrahedron*, 2016, 72, 592–598.
56 D. Monti, G. Ottolina, G. Carrea and S. Riva, *Chem. Rev.*, 2011, 111, 4111–4140.
57 M. Pickl, M. Fuchs, S. M. Glueck and K. Faber, *Appl Microbiol Biotechnol*, 2015, 99, 6617–6642.