Organic Process Research & Development

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Influence of Cofactor Regeneration Strategies on Preparative-Scale, Asymmetric Carbonyl Reductions by Engineered *Escherichia coli*

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Supporting Information

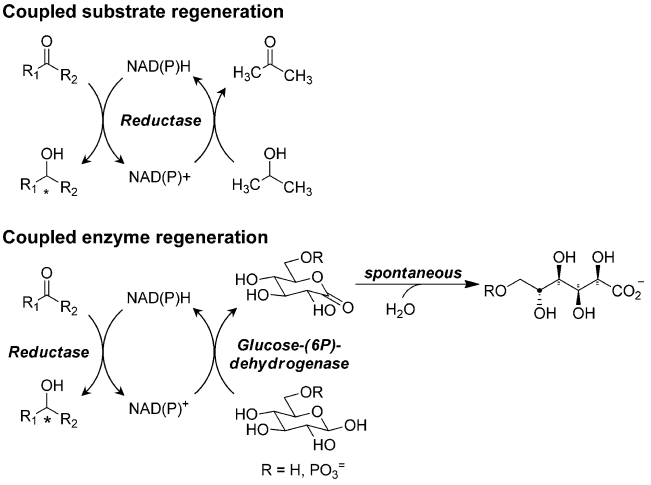
ABSTRACT: This study was designed to determine whether whole cells or crude enzyme extracts are more effective for preparative-scale ketone reductions by dehydrogenases as well as learning which cofactor regeneration scheme is most effective. Based on results from three representative ketone substrates (an α-fluoro-β-keto ester, a *bis*-trifluoromethylated acetophenone, and a symmetrical β-diketone), our results demonstrate that several nicotinamide cofactor regeneration strategies can be applied to preparative-scale dehydrogenase-catalyzed reactions successfully.

1.0. INTRODUCTION

Optically pure alcohols can be readily derivatized and further transformed, making them pivotal intermediates in asymmetric synthesis.[1] Historically, catalytic hydrogenation has proven exceptionally useful in chiral alcohol synthesis,[2,3] although biocatalytic methods have become increasingly popular, with the number of these examples increasing dramatically in recent years.[4,5] The ever-growing number of commercially available dehydrogenases has been a key driving force in making enzyme-catalyzed ketone reduction a first-line choice in chiral synthesis. Recombinant strains (usually engineered *Escherichia coli*) are the typical sources of synthetically useful dehydrogenases. This allows the enzymes to be employed either as catalysts within whole cells or as isolated proteins (purified or semipurified). Intact whole cells simplify carbonyl reductions since glucose can be used to regenerate the nicotinamide cofactor (NADH or NADPH) using the primary metabolic pathways of *E. coli*.[6] Cofactors are supplied by cells, further reducing costs. The main limitation is that the concentrations of organic reactants must be kept sufficiently low to avoid damaging the cell membrane since oxidative phosphorylation (the major source of NADPH in *E. coli* cells under aerobic conditions) depends on an intact cell membrane. It is also possible to permeabilize the membrane somewhat by employing a bisolvent system or by freezing the cells.[7−9] By contrast, using isolated dehydrogenases avoids mass transport and substrate concentration limitations imposed by the cell membrane. The approach does, however, require provision for nicotinamide cofactor regeneration since these are far too costly to be added stoichiometrically.

In most cofactor regeneration schemes for NADPH, the desired dehydrogenase-mediated carbonyl reduction is coupled with another chemical, photochemical, electrochemical, or enzymatic reaction.[10] The last is most likely to be compatible with reaction conditions suitable for the dehydrogenase. NADPH regeneration can be based on a coupled substrate or a coupled enzyme approach (Scheme 1) (for recent examples, see11−15 and references therein). The former is simpler, requiring only a single dehydrogenase that mediates both the desired carbonyl reduction and oxidation of a cosubstrate such as isopropanol (*i*-PrOH). The presence of organic cosolvents (*i*-PrOH and acetone) also aids in substrate solubilization. One drawback, however, is that carbonyl reductions are under thermodynamic control and usually require a large excess of *i*-PrOH to achieve high conversions. The use of alternative ketone acceptors is one strategy that has been used to overcome this problem.[16] In unfavorable cases, the organic cosolvents can also inactivate the dehydrogenase. The coupled enzyme regeneration strategy eliminates this possibility by substituting an innocuous cosubstrate such as glucose or glucose-6-phosphate along with a second dehydrogenase to catalyze its oxidation. The combination of glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-PDH) was the first of these to achieve wide popularity;[17] while

Scheme 1



Special Issue: Biocatalysis 14

Received: October 31, 2013
Published: February 17, 2014

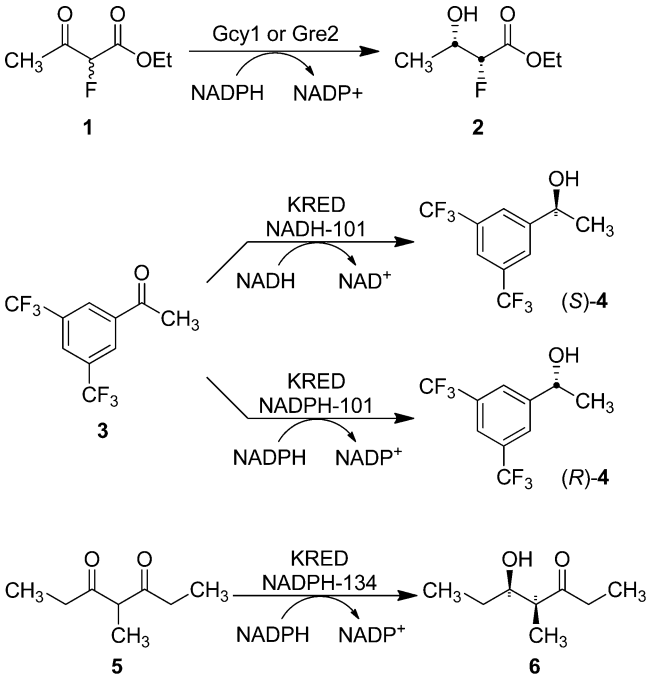
   dx.doi.org/10.1021/op400312n | *Org. Process Res. Dev.* 2014, 18, 793−800

effective, the high cost of G-6-P made this method unattractive for large-scale use. This drawback was overcome by substituting glucose and glucose dehydrogenase (GDH) (for example, see refs 18−21 and references therein). A key advantage of glucose-based NADPH regeneration is the effectively irreversible nature of the reactions since spontaneous lactone hydrolysis under the reaction conditions rapidly removes the products.

This study sought to answer two key questions in dehydrogenase-mediated process development. First, are whole cells or crude enzyme extracts more effective for preparative-scale ketone reductions by dehydrogenases? As noted above, both approaches have advantages and disadvantages. Second, which cofactor regeneration scheme works best? In particular, are whole cell-mediated reductions improved by coexpressing a regeneration enzyme such as glucose or glucose-6-phosphate dehydrogenase?[22,23] As part of this work, we also created an *E. coli* host strain that lacks a major β-keto ester reductase (DkgA, formerly known as YqhE) to avoid competition with overexpressed dehydrogenases.

To enable general conclusions to be drawn from this work, we chose three substrates along with their corresponding dehydrogenases (Scheme 2). Optically active α-fluoro-β-

Scheme 2



hydroxy esters such as **2** have unique chemical and pharmaceutical properties that make them valuable building blocks for complex, fluorinated targets.[24,25] Dehydrogenases such as *Saccharomyces cerevisiae* enzymes Gcy1 and Gre2 mediate dynamic kinetic resolutions of **1**, thereby providing (2*R*,3*S*)-**2** in a single step.[26,27] We tested both G-6-PDH and GDH as NADPH regeneration enzymes for this reduction; on the basis of these results, we applied the optimized conditions to reductions of fluorinated acetophenone **3**. Pollard et al. showed that two commercially available enzymes efficiently reduced acetophenone **3** to the corresponding (*S*)- or (*R*)-alcohols (KRED-NADH 101 and KRED-NADPH 101, respectively) (Scheme 2).[28] The (*R*)-antipode is used for the orally active EMEND for chemotherapy-induced emesis and antidepressant drugs, while (*S*)-**4** is a building block for other Merck NK-1 antagonists.[28] Finally, (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone **6** is a rice weevil pheromone used in traps for early detection of crop infestations; this is critical to avoid massive grain losses.[29] Hydroxy-ketone **6** can be obtained by reducing diketone **5** with commercially available KRED-NADPH 134.

2.0. RESULTS AND DISCUSSION

2.1. dkgA Gene Knockout. Aldo-keto reductase DkgA,[30] the product of the *E. coli dkgA* gene,[31] reduces β-keto esters such as **1**.[32] We created a Δ*dkgA* deletion strain to avoid its interfering with exogenous, overexpressed dehydrogenases. Initial attempts using short homologous regions (~50 bp) flanking an FRT-kan-FRT cassette[33] were unsuccessful; however, by employing the method of Derbise et al., the desired strain was created. The results of several PCR amplifications confirmed that the entire *dkgA* coding region had been deleted precisely and replaced by a kanamycin resistance gene, as designed. This resulting strain was designated BL21(DE3)ΔdkgA::kan. The kanamycin resistance gene was removed by recombination to leave a single FRT site at the original *dkgA* locus (designated *E. coli* BL21(DE3) ΔdkgA).

The growth rate of BL21(DE3) ΔdkgA was identical to that of the parent BL21(DE3) in rich medium under aerobic conditions (data not shown). To assess the impact of DkgA deletion on carbonyl reductions, both the knockout and parent strains were used to reduce three known DkgA substrates (ethyl 2-methylacetoacetate, ethyl 2-allylacetoacetate, and **1**) at final concentrations of 5 mM. Both ethyl 2-methylacetoacetate and ethyl 2-allylacetoacetate were completely reduced by the parent BL21(DE3) cells in 24 and 40 h, respectively. By contrast, only starting material was observed when the *dkgA* deletion strain was incubated with these two substrates for 48 h. The results for fluorinated β-keto ester **1** were more complex. Deletion of the *dkgA* gene reduced the overall rate of product formation by ~50% and also altered the product distribution. While the parent BL21(DE3) strain reduced **1** mainly to the *threo* diastereomer (~70% de), the *dkgA* knockout strain afforded only 10% de. The lower rate of product formation and diastereoselectivity in the knockout strain was due to significantly diminished production of the *threo* alcohol; the rate of *erythro* alcohol formation remained the same as that of the parent strain. Since deletion of the *dkgA* gene removed a significant level of host reductase activity toward **1**, we did not attempt to carry out additional gene knockout studies to suppress background activity even further.

2.2. Dehydrogenase Strain Construction and Characterization. Plasmids encoding a yeast dehydrogenase (Gcy1 or Gre2) were introduced into *E. coli* BL21(DE3) ΔdkgA cells by electroporation. The resulting strains were cotransformed with compatible plasmids containing genes for glucose dehydrogenase (GDH) or glucose-6-phosphate dehydrogenase (G-6-PDH). All recombinant strains were analyzed for protein overproduction by SDS-PAGE (data not shown) and the appropriate catalytic activities in crude extracts (Table 1). Gcy1 catalytic activity was acceptably high, whether the protein was overexpressed alone or with GDH. Coexpression of G-6-PDH reduced Gcy1 activity by a factor of ~3, however. By contrast, Gre2 specific activity was relatively poor, although it was improved somewhat by coexpression of GDH or G-6-PDH. GDH specific activity was maximal when the enzyme was

dx.doi.org/10.1021/op400312n | Org. Process Res. Dev. 2014, 18, 793−800

Table 1. Specific activities of strains expressing dehydrogenases and/or NADPH regeneration enzymes[a]

		specific activity (U/mg)	
dehydrogenase	coexpressed cofactor regeneration enzyme	dehydrogenase	cofactor regeneration enzyme
Gcy1	none	6–8	–
	GDH	5.1	1.1
	G-6-PDH	2.1	3.9
Gre2	none	0.5	–
	GDH	1.2	0.3
	G-6-PDH	1.6	0.5
none	GDH	–	5–6
	G-6-PDH	–	11

[a]All kinetic measurements used clarified crude extracts, and values are based on 1 unit = 1 μmole NADPH produced or consumed per minute in the presence of the appropriate substrate.

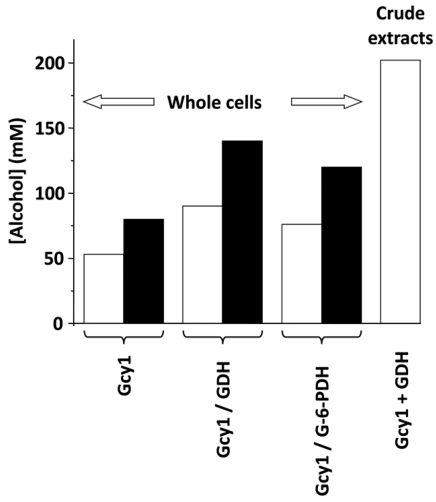


Figure 1. Comparison of whole cells and crude extracts in reducing β-keto ester **1**. The alcohol product was quantitated by GC using an internal standard and a calibration curve prepared with authentic product. Product concentrations were measured at 5.5 h (white bars) and after reaching their final levels at 24 h (black bars).

expressed separately; a 5-fold decrease was observed when a yeast dehydrogenase was coproduced. Finally, G-6-PDH activity was good when coexpressed with Gcy1, but poor in the presence of Gre2. These data demonstrate the difficulty of optimizing and balancing dehydrogenase and regeneration enzyme specific activities in single strains. The alternative strategy of mixing two different strains, each overexpressing a single exogenous enzyme, at the bioconversion stage allows much finer control over activity ratios as well as higher specific activities for each individual enzyme.

Plasmid maintenance by antibiotic resistance is undesirable in large-scale cultures for both cost and environmental reasons. We therefore successfully devised an alternative strategy in which a plasmid-borne *serA* gene complemented a chromosomal deletion in the host strain to restore serine prototrophy.[34] Details are reported in the Supporting Information.

2.3. α-Fluoro-β-keto Ester Reductions. Asymmetric reductions of β-keto esters have been—and remain—very common applications of dehydrogenases in preparative-scale synthesis. To assess the impact of coexpressing cofactor regeneration enzymes on the efficiencies of β-keto ester reductions, we chose Gcy1 and β-keto ester **1** as a representative pair.[35] We first studied reductions in purely aqueous solutions as well as in two-phase mixtures. We then explored strategies to extend the bioconversion period, thereby increasing total product yield.

Strains overexpressing Gcy1, either alone or in combination with GDH or G-6-PDH, were grown in rich medium and induced. To determine the impact of an intact cell membrane on reaction rate, half the cells were lysed to yield crude extracts, while the remaining biomass was used for whole cell-mediated reductions. For strains that overproduced only a single enzyme, crude extracts prepared from equal masses of cells were combined. Reactions with whole cells were carried out in 1 L volumes under conditions used successfully for other β-keto ester reductions[6] in the presence of excess ketone and glucose. Both whole cell and cell free reductions were carried out under the same conditions, except that 50 μM $NADP^+$ was added to the latter.[36]

The data in Figure 1 show that coexpressed GDH or G-6-PDH modestly increased the reduction rate of β-keto ester **1**. As in our previous studies,[6] a strong correlation between initial rate and the final achievable product titer was observed. These data also show that membrane transit was at least partially rate-limiting in whole cell-mediated reductions and underscore the significant advantages of using crude extracts for preparative-scale reactions. Here, cell-free conditions allowed at least 25-fold higher rates compared to whole cell-mediated reactions using the same quantity of biomass.

To avoid the need for a separate cell lysis step, we explored the possibility of creating crude extracts *in situ* by carrying out the reductions of **1** using whole cells in the presence of an immiscible cosolvent (*n*-BuOAc or MTBE). Reaction conditions similar to those described above were employed, and excess β-keto ester **1** and glucose were present at all times (Figure 2). In the absence of an organic solvent, whole cells overexpressing Gcy1 alone afforded 40 mM alcohol **2**, both in the absence and presence of added $NADP^+$. Under these conditions, the cell membranes remained intact, and the nicotinamide cofactor was unable to reach the intracellular compartment where carbonyl reduction occurred. On the other hand, when *n*-BuOAc was added, no alcohol product was observed, even though additional $NADP^+$ had been added. It was clear that *n*-BuOAc had lysed the cells; unfortunately, NADPH was no longer supplied by the enzymes and/or cofactors of host cell metabolism. To overcome this problem, we repeated the experiments with mixtures of cells that overexpressed either Gcy1 or GDH. Under these conditions, it was clear that MTBE was the better solvent for *in situ* cell lysis and facilitating the desired reduction of β-keto ester **1**.

One drawback to the above-mentioned reductions is no further reduction occurred after 6 h, even when additional β-keto ester **1** and glucose were still present (Figure 3). This could be due to loss of reductase activity, loss of the cofactor regeneration enzyme activity, or a combination of both. We therefore carried out reductions of **1** for 6 h with 25 units of both Gcy1 and GDH and 100 μM $NADP^+$. Substrates (β-keto ester **1** and glucose) were added periodically to maintain saturating conditions. After 6 h, an additional 25 units of Gcy1, GDH, or both were added. No further additions were made to the control reaction. While there is some scatter in the data (Figure 3), it is clear that adding Gcy1 has the most significant impact, suggesting that this enzyme is the main determinant of reaction longevity.

dx.doi.org/10.1021/op400312n | Org. Process Res. Dev. 2014, 18, 793–800

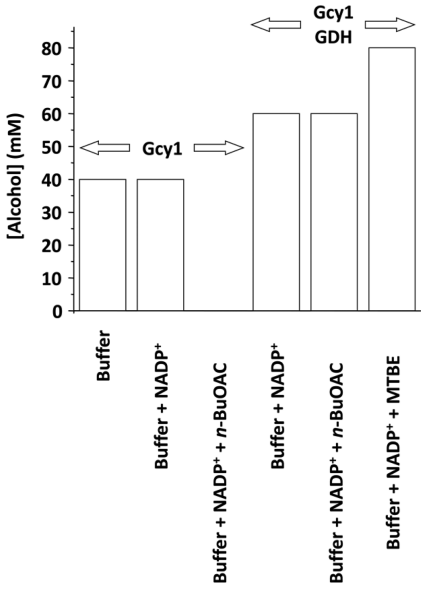


Figure 2. Reductions of β-keto ester **1** under two-phase conditions. Reductions were carried out with approximately 1 g of cells overexpressing Gcy1, supplemented with 1 g of cells overexpressing GDH where indicated. For reactions under two-phase conditions, an equal volume of the organic solvent was included, and mixtures were stirred rapidly. Conversions were carried out in the presence of excess β-keto ester **1** and glucose to afford the maximum product yield.

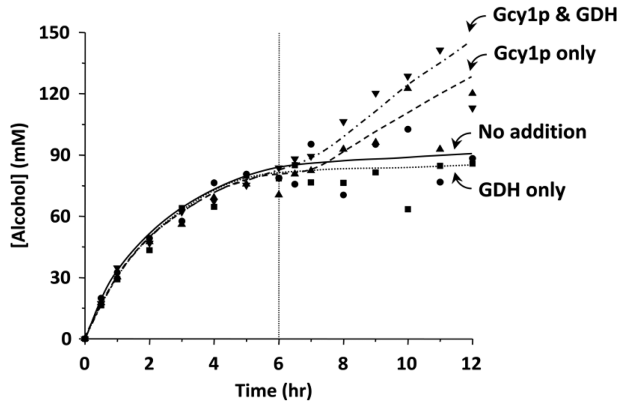


Figure 3. Assessing the stabilities of Gcy1 and GDH under reaction conditions. The reduction of β-keto ester **1** was carried out with crude extracts under standard conditions. Additional crude extract from Gcy1 and/or GDH overexpression strains were added after 6 h, and product formation was monitored for an additional 6 h.

2.4. Large-Scale Applications. Previous studies on the reductions of **3** used purified enzyme preparations.[28] Our goal was to see whether these reductions could be carried out more economically by employing whole cells that overexpressed the appropriate dehydrogenases or *in situ*-prepared cell lysates.

The specific activity of purified KRED NADH-101 for ketone **3** was 8 U/mg. Since this was nearly the same as that of the Gcy1/β-keto ester **1** pair investigated previously, we hoped that the same methods might also be applicable. Unfortunately, all attempts to reduce **3** in two-phase systems with *n*-BuOAc or MTBE were unsuccessful, even when whole cells expressing GDH were included. Much better results were obtained when crude extracts from KRED NADH-101 and GDH cells were employed under aqueous conditions and the ketone substrate was solubilized by 10% EtOH. This allowed 50 mM **3** to be completely reduced after 3.3 h. Whole cells could also be substituted for the corresponding crude extracts. KRED NADH-101 had the same specific activity for *i*-PrOH oxidation as for reducing **3**, which allowed the same dehydrogenase to be used for both for ketone reduction and cofactor regeneration.

Small-scale reductions of acetophenone **3** were carried out with magnetic stirring. This substrate is poorly soluble in water, and Pollard et al. showed that mixing efficiency significantly impacted its rate of reduction when purified dehydrogenases were employed.[28] We therefore carried out preparative-scale reductions in a 2 L fermenter equipped with Rushton impellers. All reductions contained 1 g/L NAD^+, and **3** was added to a final concentration of 390 mM (100 g/L) in a reaction volume of 700 mL.

We surveyed four different conditions for large-scale reductions of **3** to the corresponding (*S*)-alcohol **4**. Within experimental error, all four methodologies performed equally well, affording crystalline (*S*)-**4** with an average yield of 80% and >98% ee (Table 2). The first trial involved mixing crude extracts from strains individually overexpressing KRED NADH-101 or GDH. Ethanol (10%) was used to help dissolve the substrate, and a glucose stock solution was added continuously. Essentially all of the acetophenone substrate was consumed after 24 h. To avoid the need for cells overexpressing GDH, we substituted *i*-PrOH oxidation to regenerate NADPH. The initial *i*-PrOH concentration (10% ≡ 1.3 M), represented a 3.3-fold molar excess with respect to ketone **3**. Because the reaction had not reached completion after 24 h, the initial quantity of KRED NADH-101 (3000 U) was supplemented with an additional 500 U of enzyme and 5% *i*-PrOH, which provided a final 5-fold molar excess of *i*-PrOH versus ketone **3**. The reaction reached 95% completion after 79 h, and the desired product was isolated in 79% yield. Very similar results were obtained when whole cells overexpressing KRED NADH-101 were substituted for the crude extract. In an attempt to decrease the reaction time, a more aggressive *i*-PrOH feed schedule was adopted so that a 9.8-fold molar excess of *i*-PrOH versus ketone **3** was achieved within 13 h. Under these conditions, the reaction reached 95% completion after 25 h (Figure 4), nearly

Table 2. Large-scale reductions of acetophenone 3

catalyst form	KRED NADH-101 quantity	NADH regeneration method	cosolvent	reaction time (h)	purified yield of (*S*)-**4**
crude extracts	3000 U	3000 U GDH, excess glucose	10% EtOH	24	61 g (86% yield)
crude extract	3000 U; additional 500 U after 24 h	*i*-PrOH oxidation	10% *i*-PrOH; additional 5% *i*-PrOH after 24 h	79	57 g (79% yield)
whole cells	10 g (~3000 U); additional 2 g (~600 U) after 24 h	*i*-PrOH oxidation	10% *i*-PrOH; additional 2.5% *i*-PrOH after 24 h	78	57 g (79% yield)
whole cells	10 g (~3000 U)	*i*-PrOH oxidation	10% *i*-PrOH; additional 10% *i*-PrOH after 6 h; additional 10% *i*-PrOH after 13 h	25	53 g (75% yield)

dx.doi.org/10.1021/op400312n | *Org. Process Res. Dev.* 2014, 18, 793−800

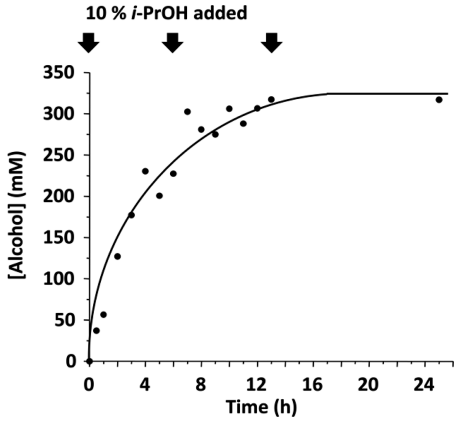


Figure 4. Time course for reduction of acetophenone **3** by whole cells overexpressing KRED NADH-101. Isopropanol (10% v/v) was added at times indicated by vertical arrows. The concentration of (*S*)-**4** was determined by GC along with a standard curve.

the same as when GDH was used for NADH regeneration. Since it requires only a single enzyme from cell paste, this strategy is extremely straightforward and economical to employ.

Preliminary experiments revealed that KRED NADPH-101 reduced acetophenone **3** to the corresponding (*R*)-alcohol with very high optical purity. Unfortunately, the specific activity of this enzyme toward **3** was only 2 U/mg, significantly lower than that of (*S*)-selective KRED NADH-101. In addition, KRED NADPH-101 did not accept *i*-PrOH as a substrate, so GDH was used to regenerate NADPH. Several reaction conditions were screened on a small scale (20 mL). The best results were obtained by mixing whole cells that individually overexpressed KRED NADPH-101 or GDH with no cosolvents. These conditions were scaled up using the same fermenter with 10 g of each cell type. The initial substrate concentration was 78 mM (20 g/L), and $NADP^+$ was present at 1 g/L. Glucose was maintained at ~100 mM. After 24 h, only a small amount of **3** had been consumed, so additional portions of both cell types (5 g) were added. The reaction was halted after 48 h, when its progress had stopped at approximately 50% conversion. The crude product was recovered by solvent extraction, and (*R*)-**4** was purified by column chromatography, affording 2.6 g of (*R*)-**2** in >98% purity and 89% ee along with 2.8 g of recovered **3**. Given these disappointing results, this conversion was not pursued further.

The final reaction subjected to scale-up study involved the highly selective monoreduction of symmetrical diketone **5** by KRED NADPH-134 to yield the corresponding (4*S*,5*R*)-keto alcohol **6** (Scheme 2).[29] This enzyme oxidized *i*-PrOH with good specific activity (17 U/mg), nearly equal to that toward **6** (15 U/mg). All studies were carried out with a partially purified preparation of KRED NADPH-134 in the presence of $NADP^+$. While *i*-PrOH could be used to regenerate NADPH successfully, reactions were limited to substrate loading of ~200 mM, and long times (50 h) were required to achieve completion. Far superior results were obtained when GDH was used for cofactor regeneration. For example, 700 mM **6** (50 g) was reduced with a 95% yield by KRED NADPH-134 (100 U) and GDH (100 U) in an open beaker (500 mL) with manual glucose addition and pH control.

3.0. CONCLUSIONS

Taken together, our results demonstrate that both crude extracts and whole cells can be used to carry out asymmetric ketone reductions simply and economically. This is particularly useful when large-scale applications are contemplated. The ability to create crude extracts *in situ* is especially convenient since the biocatalyst can be stored as frozen cell paste, which can be added directly to the reaction mixture. When dehydrogenases accept *i*-PrOH, a single enzyme can be used for cofactor regeneration and substrate reduction.[12−14,37,38] The main limitation of this strategy is that high *i*-PrOH levels can be required to provide sufficient thermodynamic driving force unless more complex cosubstrates are employed (for example, see ref 16). For those dehydrogenases that cannot utilize *i*-PrOH, *E. coli* cells that overexpress GDH offer a very convenient alternative for cofactor regeneration.

4.0. EXPERIMENTAL SECTION

4.1. General Procedures. ^1H NMR spectra were measured in $CDCl_3$ at 300 MHz, and chemical shifts were referenced to residual protonated solvent. Optical rotation values were determined at room temperature in the indicated solvent. Ethyl 2-fluoroacetoacetate was purchased from Sigma (St. Louis, MO), 3,5-*bis*-trifluoromethyl acetophenone was obtained from SynQuest Laboratories (Alachua, FL), and nicotinamide cofactors and 4-methyl-3,5-heptanedione were provided by BioCatalytics and Codexis. Other reagents were obtained from commercial suppliers and used as received. Thin-layer chromatography (TLC) was performed using precoated silica gel plates (EMD Chemicals). Products were purified by flash chromatography on Purasil silica gel 230−400 mesh (Whatman). Gas chromatographic analyses utilized either DB-17 (0.25 mm × 30 m, 5 μm film thickness; J&W) or Chirasil-Dex CB (0.25 mm × 25 m, *X* μm film thickness; Varian) columns with detection by either FID or EI-MS (70 eV). Trinder reagent was purchased from Fisher.

Oligonucleotides were purchased from IDT (Coralville, IA), and long primers were purified by ion-exchange HPLC. Standard methods for molecular biology procedures were employed, and plasmids were purified by CsCl buoyant density ultracentrifugation.[39] Electroporation was used to introduce nucleic acids into *E. coli* cells. LB medium used for bacterial cultivation contained 1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract and 1% NaCl. Superbroth (SB) contained 3.2% Bacto-Tryptone, 2.0% Bacto-Yeast Extract, 0.5% NaCl and 5 mL of 1 M NaOH (per liter of medium). SOB medium contained 2.0% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 0.05% NaCl; 2.5 mL of 1 M KCl and 2 mL of 1 M $MgCl_2$ was added after sterilization. Agar (15 g/L) was included for solid medium. Plasmids pKD13, pKD46, and pCP20 were obtained from the *E. coli* Genetic Stock Center. PCR amplifications were carried out for 25−30 cycles of 94 °C (1 min), 54 °C (2 min), and 72 °C (3 min) followed by 10 min at 72 °C in buffers recommended by the suppliers. Enzymes were obtained as frozen whole cells of *E. coli* overexpression strains or as lyophilized powders of purified enzymes (GDH-102, both forms; KRED-NADH-101, frozen cells; KRED-NADPH-101, both forms; KRED-NADPH-134, purified enzyme).

Biotransformation reactions were monitored by GC. Samples were prepared by vortex mixing a portion of the aqueous reaction mixture (50−100 μL) with twice the volume of EtOAc. The organic phase was separated and analyzed by GC.

dx.doi.org/10.1021/op400312n | *Org. Process Res. Dev.* 2014, 18, 793−800

When needed, methyl benzoate was used as an internal standard for quantitation, and standard curves were prepared by extracting aqueous samples with varying concentrations of authentic products.

4.2. β-Keto Ester Reductions by *E. coli* BL21(DE3) ΔdkgA::kan. Overnight precultures of BL21(DE3) and BL21(DE3) ΔdkgA::kan were diluted 1:100 into 100 mL of SB in 500 mL Erlenmeyer flasks. The BL21(DE3) ΔdkgA::kan culture was supplemented with 25 μg/mL kanamycin. Cultures were shaken at 37 °C. Upon reaching $O.D._{600} \approx 0.4$, neat β-keto ester was added to a final concentration of 5.0 mM, and shaking was continued at 37 °C. Reductions were monitored by GC.

4.3. Recombinant Strain Creation and Characterization. All dehydrogenases were overexpressed in *E. coli* from IPTG-inducible T7 promoters. Compatible origins of replication and different antibiotic resistance markers were used to construct coexpression strains. Gcy1: pBC964, p15A origin, chloramphenicol; pBC063, *colE1* origin, ampicillin. Gre2: pBC965, p15A origin, chloramphenicol; pBC688, *colE1* origin, kanamycin. GDH: pBC951, p15A origin, chloramphenicol; pBC303, *colE1* origin, ampicillin. G-6-PDH: pBC971, p15A origin, chloramphenicol; pBC972, colE1 origin, kanamycin. All eight plasmids were used individually to transform the *E. coli* BL21(DE3) ΔdkgA::kan strain. In addition, four coexpression strains were also created in the same host: Gcy1 + GDH (pBC603, pBC951), Gcy1 + G-6-PDH (pBC603, pBC971), Gre2 + GDH (pBC688, pBC951) and Gre2 + G-6-PDH (pBC688, pBC971).

Recombinant cells were cultured at 37 °C in a New Brunswick Scientific M19 fermenter in 4 L of LB medium supplemented with the appropriate antibiotic(s) at 700 rpm and an air flow rate of 4 L/min. When the culture reached an $O.D._{600\ nm}$ of 0.5, protein overexpression was induced by adding IPTG to a final concentration of 100 μM, then continuing the culturing at 30 °C for an additional 6 h. Cells were harvested by centrifugation at 8500 × *g* for 20 min at 4 °C. Cells were stored at 4 °C (short-term) or at −20 °C (long-term). To prepare crude extracts, cells were washed with water, resuspended in 100 mM KP_i (pH 7.0) containing 0.1 mM phenylmethylsulfonylfluoride (PMSF) and passed twice through a French pressure cell at 16,000 psi. Insoluble materials were removed by centrifuging at 70,000 × *g* for 20 min at 4 °C. The pellet was discarded, and the supernatant was used as the cell-free extract.

Enzyme activities were determined spectrophotometrically at 25 °C by monitoring A_{340} (ε = 6220 L/mol·cm) in 100 mM KP_i (pH 7.0). Assay mixtures contained 0.2 mM NADH or NADPH (KRED-NADH-101 and KRED-NADPH-101) or $NAD(P)^+$ (GDH or *i*-PrOH oxidation measurements), 2.5 mM substrate and the appropriate amount of the enzyme cell-free extract in a final volume of 1.0 mL. Stock solutions (1 M in EtOH) were prepared for lipophilic substrates. One unit of enzyme activity catalyzed the conversion of 1.0 μmol of cofactor per minute. Protein concentrations were estimated by the method of Bradford,[40] using bovine serum albumin (BSA) as the standard.

4.4. Reductions of Ethyl 2-Fluoroacetoacetate 1. Small-scale trial reactions were carried out in an open beaker with magnetic stirring at room temperature using manual cosubstrate addition and pH control (3.0 M KOH titrant). Standard reaction mixtures contained either whole cells (final concentration of 0.04 g/mL in 100 mM KP_i (pH 7.0)) or crude extracts (final concentration of 0.70 U/mL in M9 medium lacking NH_4Cl) in to volumes of 20−50 mL. Reactions in two-phase systems were carried out under the same conditions by adding an equal volume of organic solvent to the buffer mixture.

Larger-scale, whole cell-mediated reductions were carried out at 30 °C in 1 L of M9 medium lacking NH_4Cl using 15−22 g (wet weight) of the appropriate cells (overexpressing Gcy1, Gcy1, and GDH or Gcy1 and G-6-PDH). The initial concentrations of **1** and glucose were 20 mM and 4 g/L, respectively. Glucose (10% aqueous solution) was fed at approximately 15 mL/h to maintain its concentration at ∼4 g/L. Feed rates were adjusted based on the results of Trinder assays and the pH was controlled at 7.0 by automated addition of 3.0 M KOH. Neat substrate was added portionwise (in 10 or 20 mM increments) over time, and product formation was measured by GC/MS. The reaction using whole cells overexpressing Gcy1 was carried out for 24 h, then the crude product was recovered by continuous extraction with 2 L of CH_2Cl_2 over 2 days.[41] The organic phase was dried with $MgSO_4$ and concentrated under reduced pressure to yield 9.1 g of the desired alcohol (76% yield, 95% purity by GC) as a yellow oil. GC analysis showed 85% de, with each diastereomer having >98% ee.

The reduction of **1** using crude cell extracts was carried out in 1 L of 100 mM KP_i (pH 7.0) at 30 °C. Cells overexpressing Gcy1 (13 g wet weight) and GDH (16 g wet weight) were used to prepare crude extracts as described above. The reaction mixture initially contained 30 mM β-keto ester **1**, 6 g of glucose, and 50 μM $NADP^+$. Both **1** and glucose were added periodically to maintain approximately steady-state levels, and the pH was controlled at 7.0 by automatic addition of 3.0 M KOH. After 5.5 h, complete conversion of 400 mM β-keto ester **1** had been achieved and the reaction was stopped. The alcohol product was isolated as described above to yield 27.9 g of the desired alcohol (92% yield, 96% purity by GC) as a yellow oil. GC analysis showed 80% de, with each diastereomer having >98% ee.

4.5. Reductions of 3,5-Bistrifluoromethyl Acetophenone 3. Reactions were carried out at 30 °C in a 2 L Biostat B2 vessel using 700 mL of buffer: M9 medium lacking NH_4Cl for whole cell-mediated conversions or 100 mM KP_i (pH 7.0) for reactions involving crude extracts. The pH was maintained at 7.0 by automated addition of 3 M KOH. Glucose and substrates were added by manually controlled pumps. For whole cell-mediated reactions, the dissolved oxygen was maintained at 25% saturation by varying the stirring rate (between 120 and 1200 rpm) while the airflow was kept constant at 0.5 L/min. For reactions involving crude extracts, the stirring rate was set at 600 rpm.

Reductions were carried out similarly to those described above. When GDH was used for NADPH regeneration, 10% EtOH was included in the buffer to enhance substrate solubility. It was omitted when *i*-PrOH was used for cofactor regeneration. Reaction mixtures initially contained 70 g of acetophenone **3** and 700 mg of $NAD(P)^+$. Conversions were terminated when the remaining substrate concentration dropped below 20 mM according to GC/MS. The product was collected by filtration after cooling the reaction mixture overnight at 4 °C. The aqueous filtrate was saturated with NaCl and extracted with CH_2Cl_2, then the combined organic phases were dried with $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by recrystallization from heptanes at 45 °C.[28] ^1H NMR data matched those

dx.doi.org/10.1021/op400312n | *Org. Process Res. Dev.* 2014, 18, 793−800

reported previously.[42] $[\alpha]_D = -22.9$ (c = 0.015 in MeOH); lit. $[\alpha]_D = +22$ (c = 1.04 in MeOH) for (*R*)-**4**.[42]

4.6. Reduction of 4-Methyl-3,5-heptanedione 5. The reaction was carried out in an open beaker containing 500 mL of 100 mM triethanolamine (pH 7.0), 700 mM diketone **5** (50 g), 2 mM $MgSO_4$, 500 mg of $NADP^+$, 15 g of glucose, and 1500 units each of KRED-NADPH-134 and GDH. The conversion was terminated when the remaining substrate dropped below 30 mM according to GC/MS. The product was recovered by continuous extraction with CH_2Cl_2 over 2 days. The organic phase was dried with $MgSO_4$ and concentrated under reduced pressure. The crude product (48.1 g) was 92% pure according to GC (90% de with each diastereomer >98% ee) and was not purified further. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.80 (d, J = 3.2 Hz, 1H), 2.41−2.63 (m, 3H), 1.27−1.63 (m, 2H), 1.12 (s, 3H), 1.00−1.07 (m, 3H), 0.88−0.97 (m, 3H).

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Generous financial support by the NIH (SBIR 76124) and the NSF (CHE-0615776) is gratefully acknowledged. We also thank Dr. Despina Bougioukou for providing the *DkgA* knockout strain.

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