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Preparative Asymmetric Synthesis of 4,4-Dimethoxytetrahydro-2H-pyran-3-ol with a Ketone Reductase and in Situ Cofactor Recycling using Glucose Dehydrogenase

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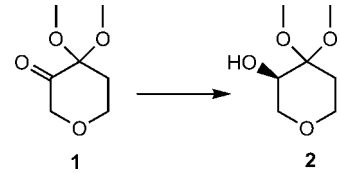
Abstract:

The asymmetric enzymatic ketone reduction of 4,4-dimethoxytetrahydro-2H-pyran-3-one provided the (*R*)-α-hydroxyketal, an important chiral precursor for a pharmaceutical intermediate, with high enantioselectivity (>99% ee). An economical process including in situ NADPH-cofactor regeneration using glucose dehydrogenase has been developed to produce the desired material in high yield (96–98%). The two-enzyme process was employed at pilot-plant scale to produce 80 kg of (*R*)-4,4-dimethoxytetrahydro-2H-pyran-3-ol. Critical factors for scale-up were found to be pH control and agitation speed.

1. Introduction

We recently required a scaleable asymmetric synthesis to produce (*R*)-4,4-dimethoxytetrahydro-2H-pyran-3-ol **2**, a key chiral intermediate in the preparation of a chemokine receptor inhibitor.[1–12] Previous approaches to selectively synthesize **2** involved the use of potassium osmate dehydrate under modified Sharpless dehydroxylation conditions.[13] However, these conditions gave the desired (*R*)-α-hydroxyketal **2** in only 92% ee, which proved difficult to upgrade downstream, produced toxic osmium byproduct requiring special waste treatment, and were expensive. Consequently, we sought a nontoxic, highly efficient asymmetric reduction of 4,4-dimethoxytetrahydro-2H-pyran-3-one, **1**,[14] to provide the corresponding (*R*)-α-hydroxyketal **2** in high enantiopurity and yield (Scheme 1).

Scheme 1. **Asymmetric synthesis route to (*R*)-4,4-dimethoxytetrahydro-2H-pyran-3-ol 2 by asymmetric reduction of 1**



Enzymatic ketone reductions typically proceed highly enantioselectively under mild aqueous reaction conditions without generating harmful waste streams. Biocatalytic processes for the stereoselective reduction of ketones using either whole-cell systems[15–19] or isolated ketoreductases and alcohol dehydrogenases[20–26] have been widely reported. In process development and scale-up at Merck, the use of isolated enzymes is preferred because it allows rapid process development and consistently leads to highly productive processes. Furthermore, recent enzyme discovery efforts have significantly increased the

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Table 1. **Screening results identifying enzyme catalysts selective for either enantiomer of 4,4-dimethoxytetrahydro-2H-pyran-3-ol, 2[a]**

entry	enzyme[b]	area % conversion	% ee
1	KRED101	73	99 (*R*)
2	KRED102	20	68 (*R*)
3	KRED103	0	
4	KRED104	0	
5	KRED105	16	70 (*R*)
6	KRED106	0	
7	KRED107	13	61 (*R*)
8	KRED108	19	86 (*S*)
9	KRED109	0	
10	TB ADH	5	99 (*S*)
11	HLADH	0	
12	BY ADH	0	

[a] 2 mg **1** per reaction in 1 mL 0.2 M KH_2PO_4 pH 7.0, 10 vol% DMSO, 4 mg lyophilized enzyme, 10 mg NAD(P)H at 30 °C, 18 h. [b] KRED = ketoreductase, ADH = alcohol dehydrogenase, TB = *Thermoanaerobium brockii*, HL = horse liver, BY = baker's yeast.

number of commercially available redox enzymes in adequate supplies and with consistent quality.[23,24,27,28] The industrial application of redox enzymes has been limited by the need for expensive nicotinamide cofactors in stoichiometric amounts, but cost-effective methods for nicotinamide cofactor recycling have been reported.[25,29,30]

In this contribution, we describe the enzymatic asymmetric reduction of 4,4-dimethoxytetrahydro-2H-pyran-3-one, **1**, including an in situ nicotinamide adenine dinucleotide phosphate (NADPH) cofactor regeneration system that employs glucose dehydrogenase (GDH) to reduce the cost of the expensive cofactor.[31] GDH is often active on substrates that are structurally similar to glucose (as is compound **1**), causing significant loss of product enantiopurity. [32] Fortunately, glucose dehydrogenase was not active on **1** and could be used for cofactor recycling. Additionally, **1** has high water solubility, which eliminated the need of a cosolvent. This benefited process development by eliminating the need to balance the mass transfer improvements produced by cosolvents with the negative effects of cosolvent on enzyme performance, as is often seen with less soluble ketones.[32] Process optimization on the bioreduction of **1** therefore focused on scaleability, robustness, and economical feasibility.

2. Results and Discussion

2.1. Process Development. A screen of commercially available ketoreductases and alcohol dehydrogenases in presence of excess NAD(P)H identified KRED101 (ketoreductase101, Table 1, entry #1) giving excellent selectivity for the desired (*R*)-alcohol **2** (>99% EE) as well as high activity under screening conditions. KRED101 was therefore selected for process development.

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In order to achieve a viable process, our experiments focused on a total conversion of **1** at 100 g/L concentration in less than 12 h using a catalytic amount of enzyme. The high solubility of **1** in water allowed us to develop a homogeneous aqueous system without cosolvents or additives. To increase the cost-efficiency, we added a glucose/glucose dehydrogenase (GDH101) recycling system for NADPH to the initial process and then focused on the reaction conditions of the two interlinked enzymatic processes in order to optimize product yield and enzyme/cofactor usage.

We investigated the activity and stability of both enzymes spectrophotometrically by consumption of NADPH at 340 nm to determine preferred temperature and pH conditions for the reaction. KRED101 was known to display high performance at pH 6.0, and at this pH we observed a temperature optimum of 35 °C.[33] Under these conditions, KRED101 retained 50% of its initial activity for up to 50 h.

However, when we examined GDH101 across typical process conditions for enzymatic ketone reductions, we found that pH and temperature significantly impacted GDH101 performance. At 25 °C, the GDH activity was highest at pH 8.0 (which was of concern, given the KRED101 pH optimum), but at 40 °C, the reaction rate was highest at pH 6.5 (Figure 1a). A 3-fold increase in reaction rate was observed as a result

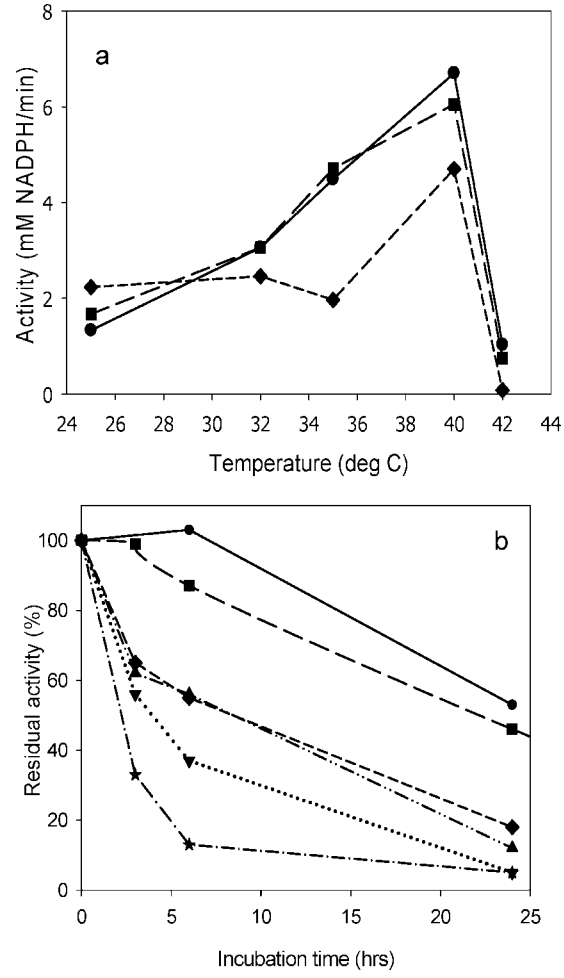


Figure 1. **(a) Temperature optimum of GDH101 at pH 6.5 (●), pH 7.0 (■), and pH 8.0 (◆). (b) Temperature stability of GDH101 at 20 °C (●), 25 °C (■), 28 °C (◆), 30 °C (▲), 32 °C (▼), 37 °C (★).**

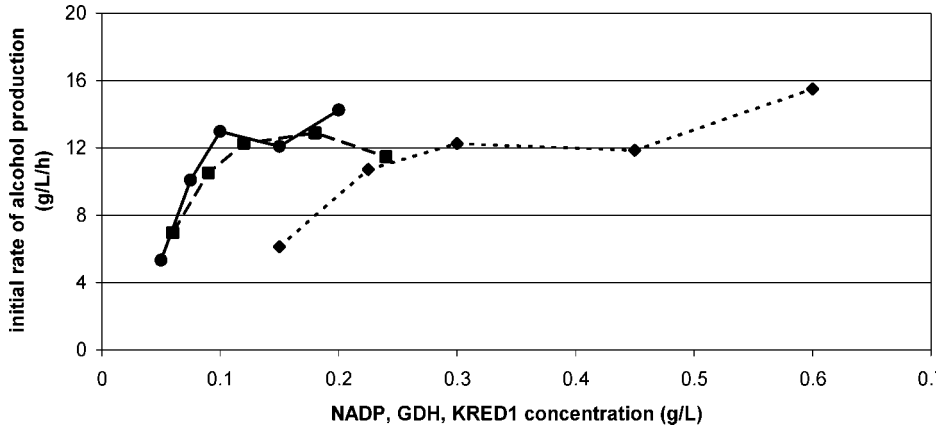


Figure 2. **Initial reaction rate of 2 formation depending on KRED101 concentration (●), NADP concentration (■), and GDH101 concentration (◆).**

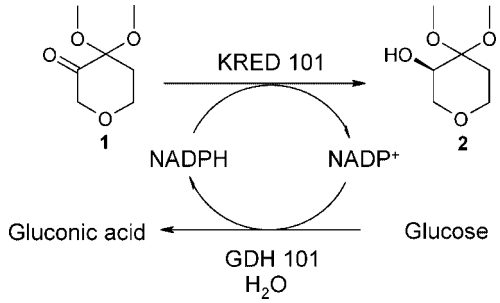
of increasing the temperature from 25 to 40 °C. The downside of this higher GDH activity at elevated temperature was a 5-fold decreased stability, with about 80% of the initial activity lost within less than 5 h at the highest temperature (Figure 1b).

The pH optimum of KRED101 and GDH101 coincided at pH 6.5, and our initial expectation was that glucose dehydrogenase would be more active on glucose as its native substrate than the ketoreductase was on **1**, therefore compensating for its rapid activity loss at 37 °C. This turned out not to be the case (Figure 2).

With GDH101 being highly active but not as stable as KRED101, the process required GDH101 in greater concentration than KRED101 to ensure the ketone reduction is not limited by the cofactor recycling rate. Under established conditions of pH 6.5 and 37 °C, we measured conversion over 2.5 h of a base reaction system of 100 g/L **1**, 0.1 g/L KRED101, 0.3 g/L GDH101, 0.12 g/L NADP and 130 g/L glucose (1.1 mol equiv compared to **1**) and varied the concentrations of KRED101, glucose dehydrogenase and NADP in three separate experiments (Figure 2). The catalyst and cofactor concentrations of this base process proved to be essentially colimiting for all components and 3 times as much GDH101 compared to KRED101 weight was required.

Given a similar cost of both enzymes, we focused on GDH as the largest cost component of the reaction and settled on 35 °C based on retaining ~20% activity in 10 h (Figure 1b). The optimized process is shown in Scheme 2.

Scheme 2. **Biocatalytic synthesis of 4,4-dimethoxytetrahydro-2H-pyran-3-ol 2 (100 g/L, 0.63 M) with ketoreductase KRED101 (0.1 g/L) and in situ NADPH (0.12 g/L NADP) recycling using glucose dehydrogenase GDH101 (0.3 g/L) and glucose (130 g/L, 0.72 M) as hydrogen source; reaction was run in 0.5 M KH_2PO_4 pH 6.5, 35 °C**



With the base process in place, a substrate loading study was run to confirm final substrate concentrations. Although the 120 g/L reaction achieved completion, the extended time required suggested the reaction was near the limit of productivity (Figure 3). To ensure successful completion of the ketone reduction, the substrate concentration was maintained at 100 g/L rather than increasing the GDH101 concentration. The lower protein content in the reaction mixture also simplified the product isolation development and was key for easy scale-up.

2.2. pH Control. The cofactor recycling system produced gluconic acid which decreased the pH during the course of the

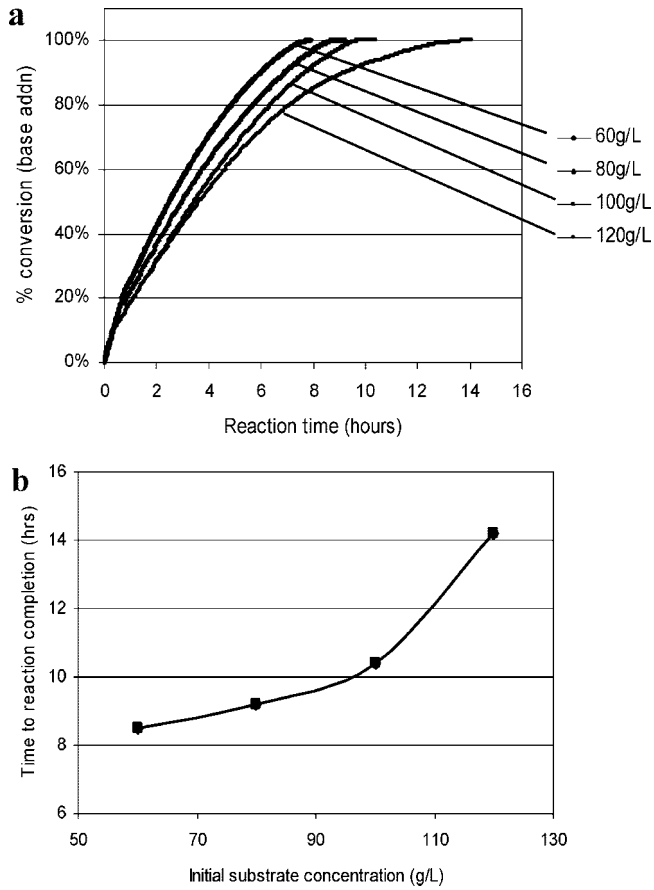


Figure 3. **Reaction time depending on substrate loading. (a) Conversion by base addition versus time at different starting concentrations of 1. (b) Time to reaction completion versus initial substrate charge.**

reaction. In order to maintain consistent optimal reaction conditions, pH adjustment was required. The use of a weak base ($KHCO_3$) avoided the stability issues of substrate **1** in the presence of high local concentrations of NaOH.

At laboratory scale (up to 20 g), we used an automated system providing continuous pH control and monitoring of all reaction parameters. The reduction proceeded to completion at a consistent rate, demonstrating the well-balanced conditions for the two-enzyme system (Figure 4).

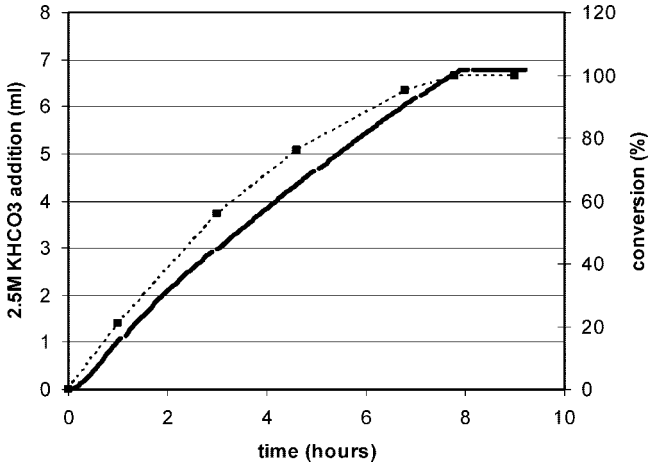


Figure 4. **Continuous pH control at small scale. The conversion as measured by gas chromatography (■) mirrors the addition of 2.5 M $KHCO_3$ (solid line).**

To fully understand the pH control limitations, we stress tested a stepwise pH control between pH 6.0 and pH 6.5 at 0.62 kg of **1** scale and the reduction completed as expected (Figure 5).

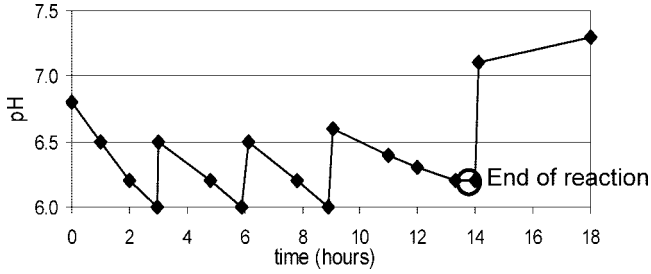


Figure 5. **Stepwise pH control at 6.5 L. Addition of 2.5 M $KHCO_3$ is correlated to the formation of 2 and displays the reaction rate.**

Due to the addition of larger volumes of $KHCO_3$ for neutralization at a time, a significant amount of CO_2 was formed at each addition causing excessive foaming. The foaming did not appear to degrade the enzymes as the reaction rate was unaffected, but it did require additional precautions such as sufficient head space and ventilation of the vessel at any scale.

2.3. Tip Speed Optimization. Another important parameter for the scale-up was the sensitivity of the enzymes to shear forces.[34–36] An agitation stress test showed that higher tip speed had a negative effect on reaction completion time, presumably by reducing the stability of one or both enzymes (Figure 6). An upper limit of 1.5 m/s was set for agitation in the pilot plant to maintain the 10–12 h end of reaction target. This limitation did not pose any concerns with obtaining adequate mixing at scale to ensure appropriate homogeneity of the added base.

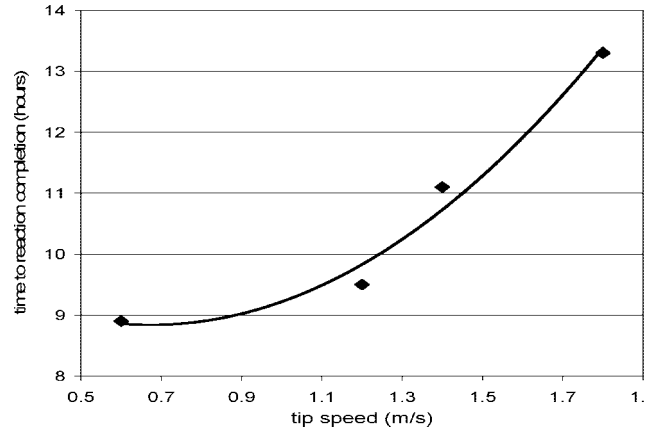


Figure 6. **Time to reaction completion depending on tip speed correlated to shear sensitivity of the enzymatic system.**

2.4. Isolation. For isolation, the high water solubility of the product **2** as well as the protein content provided a challenge that required some preparation of the reaction mixture before extraction. Upon completion of the asymmetric reduction, the pH was adjusted to pH 7.0 using $KHCO_3$, and the aqueous batch was saturated with KCl (~150 g/L). We then added 1.5 volumes of acetonitrile which precipitated the enzyme and formed an organic phase promoted by saturation with KCl. The α-hydroxyketal **2** preferentially migrated into the acetonitrile layer, leaving cell debris, salts, and other impurities in the aqueous phase. To dry the organic phase and to reduce the level of salt carryover, we added 0.5 volume of toluene before the aqueous layer was cut to waste. After a final concentration step and solvent switch into toluene to simplify the follow-up chemistry, the process yielded enantiopure (*R*)-**2** (>99% ee) in reproducible 96–98% isolated yield.

2.6. Scale-Up. This two-enzyme process in a linked cofactor recycle system was demonstrated successfully at 80 kg scale in the pilot plant using standard glass-lined process equipment in a simple configuration (Figure 7). The pH was monitored in

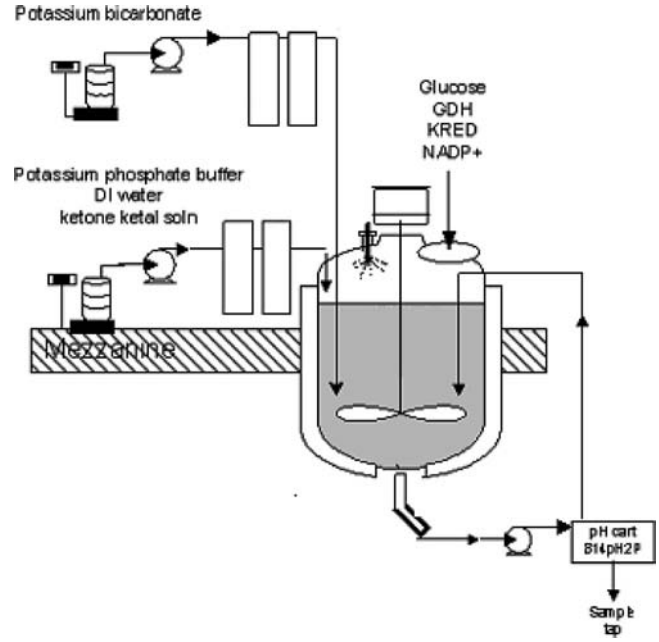


Figure 7. **Schematic illustration of pilot-plant assembly for biocatalytic reduction of 1 at 80 kg scale.**

a recirculation loop and controlled stepwise between pH 6.25 and pH 6.5. Narrowing the pH window compared to the pH window in the 0.62 kg stress test allowed pH adjustment by an hourly base addition due to the consistent reaction rate and better control over the foaming caused by CO_2 formation.

2.7. Conclusion. The asymmetric enzymatic reduction of 100 g/L **1** provides the enantiopure (*R*)-α-hydroxyketal **2**, an important chiral precursor for a pharmaceutical intermediate, in high yield. A robust and efficient homogeneous process has been developed including an in situ NADPH-cofactor regeneration system using glucose dehydrogenase. Temperature and pH conditions were established, thus balancing activity optimum and stability of both enzymes and achieving a consistent reaction rate for optimum product yield and enzyme/cofactor usage. Process optimization towards scale-up focused on adapting parameters for pH control, tip speed, and product isolation to large scale. The optimized two-enzyme process was demonstrated successfully at 80 kg pilot-plant scale.

3. Experimental Section

The in-house library of isolated redox enzymes consisted of a ketoreductase screening kit including KRED101-109 (Biocatalytics, Inc.), and alcohol dehydrogenases from *Thermoanaerobium brockii*, horse liver, and yeast (all Sigma).

Glucose dehydrogenase, GDH101, and nicotinamide adenine dinucleotide phosphate cofactors NA(D)P and NAD(P)H were purchased from Biocatalytics, Inc.

Dextrose (glucose) monohydrate, KH_2PO_4, and $KHCO_3$ were obtained in high purity from Sigma.

3.1. Analysis. The concentrations of **1** and **2**, were determined by gas chromatography (GC 6850, Agilent Technologies) using a Restek Rtx 1701 column (30 m, 0.32 mm ID, 0.1 um). The temperature program held 50 °C for 1 min before ramping to 250 at 60 °C/min and holding 250 °C for 1.2 min.

The enantiomeric excess of **2** was determined by GC using a chiral column Restek Rt-bDEXsa (30 m, 0.32 mm ID, 0.25 um) with a gradient from 120 to 180 °C over 15 min.

3.2. Catalyst Screening. Two milligrams (12.5 μmol) of **1** in 0.1 mL of DMSO was added to 4 mg of lyophilized enzyme catalyst and 10 mg of NAD(P)H in 0.9 mL of 0.2 M KH_2PO_4 buffer pH 7.0. The reactions were incubated at 30 °C and 220 rpm. After 18 h, the reactions were extracted with ethyl acetate and analyzed by GC.

3.3. Enzyme Activity Assay. All enzyme activities were measured using a 3-min kinetic assay on a Bio-Tek Power Wave HT spectrophotometer.

Temperature performance of KRED101 was determined by following the oxidation of 0.12 g/L (0.14 mM) NADPH at 340 nm in the presence of 2.1 mg/L KRED101 and 0.1 g/L (0.63 mM) **1** in 0.2 mL of 0.5 M KH_2PO_4 buffer pH 6.0. The temperature ranged from 20 to 45 °C.

The activity of glucose dehydrogenase GDH101 was determined by following the reduction of 0.12 g/L (0.16 mM) NADP at 340 nm in the presence of 7.5 mg/L GDH101 and 65 g/L (0.33 M) glucose monohydrate (Sigma) in 0.2 mL of 0.5 M KH_2PO_4 buffer pH 6.0−8.5. The temperature ranged from 20 to 45 °C.

3.4. General Procedure for the Biocatalytic Synthesis of 4,4-Dimethoxytetrahydro-2h-pyran-3-ol 2 with Ketoreductase KRED101 and in Situ Cofactor Recycling Using Glucose Dehydrogenase GDH101. Typical reactions were run at 100 g/L (0.63 M) **1** and 0.1 g/L KRED101 in 0.5 M KH_2PO_4 buffer pH 6.5. 0.3 g/L GDH101, 0.12 g/L NADP, and 130 g/L (0.72 M) glucose was added for the cofactor recycling system. To a solution of **1** and glucose in 0.5 M KH_2PO_4 buffer pH 6.5 were added adequate volumes of stock solutions of NADP (typically 13 g/L), GDH 101 (typically 32 g/L), and KRED101 (typically 10 g/L) in 0.5 M KH_2PO_4 buffer pH 6.5. The reactions were heated to 35 °C, and the pH was held at pH 6.5 by addition of 2.5 M $KHCO_3$.

After completion of the reaction, the pH was adjusted to pH 7.0 using 2.5 M $KHCO_3$, and the aqueous batch was saturated with KCl (∼150 g/L). The α-hydroxyketal was extracted into 1.5 volumes of acetonitrile, and 0.5 volumes of toluene was added. The organic layer was distilled to yield **2**.

Laboratory-scale reactions were performed at 5–50 mL scale in automated reactors (Multimax, Mettler Toledo) with temperature, pH control, and overhead stirring. Larger-scale reactions (5–10 L) were run in a 50-L Buchi reactor equipped with temperature control, pH control, and overhead mixing.

3.5. Loading Studies. Loading of **1** was investigated at 10-mL scale according to the general procedure but with varying substrate charges at 60, 80, 100, 120 g/L. The reaction rate was determined by base consumption using continuous pH control at pH 6.5. Conversion of the reaction was determined by GC at 1, 3.5, and 18 h.

Experiments to determine KRED101, GDH101, and loading were run at 5-mL scale using the general procedure at 37 °C. The concentration of KRED101 was investigated at 0.05, 0.075, 0.1, and 0.15 g/L, while all other concentrations were according to the general procedure. GDH101 concentration was varied at 0.15, 0.225, 0.45, and 0.6 g/L. The limitation of NADP was investigated at 0.06, 0.09, 0.12, 0.18, and 0.24 g/L. The reaction rate was determined by base consumption using continuous pH control at pH 6.5. Conversion of the reaction was determined by GC at 2.5 h.

3.6. Agitation Stress Test. Shear sensitivity experiments were performed at 40-mL scale according to the general procedure. The tip speed of a mechanical overhead stirrer was varied at 0.6, 1.2, 1.4, and 1.8 m/s. The reaction rate was followed by base consumption using continuous pH control at pH 6.5. Conversion of the reaction was determined by GC at 1 and 18 h.

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(33) pH optimum for KRED101 was provided by Biocatalytics, Inc.

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