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Development of a Practical Biocatalytic Process for (*R*)-2-Methylpentanol

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Abstract:

(*R*)-2-Methylpentanol is an important chiral intermediate for the synthesis of certain medicinally important compounds, natural products, and liquid crystals. Here we describe the development of a practical kinetic resolution utilizing an enantiospecific biocatalytic reduction of racemic 2-methylvaleraldehyde. The process utilizes an evolved ketoreductase enzyme to selectively reduce the (*R*)-enantiomer of racemic 2-methylvaleraldehyde to the desired product with high volumetric productivity. A scaleable method for separating the desired product from the off-enantiomer of the starting material is also described. The process is cost-effective, green, and amenable to manufacturing scale.

Introduction

(*R*)-2-Methylpentanol (*R*-2-MP) is an important intermediate for organic synthesis that has been utilized for the production of certain pharmaceuticals[1] and liquid crystals.[2] Several different synthetic approaches to this material based on resolution or asymmetric syntheses have been previously described (Scheme 1). The first report utilized a resolution of racemic 2-MP through crystallization of the tosylate salt of L-valine-2-methylpentyl ester (Scheme 1a).[3] Although somewhat effective based on optical rotation, this procedure was low-yielding, and the enantiomeric excess was not determined. In 1985 Oppolzer described the first asymmetric synthesis via a diastereoselective ester−enolate alkylation of a chiral sultam (Scheme 1b).[4] This procedure was high-yielding but provided material with only modest enantiopurity. A year later Danishefsky utilized *R*-2-MP in the synthesis of the antibiotic Zincophorin.[5] In this work an Evans diastereoselective alkylation of a chiral oxazolidinone-derived imide enolate was employed (Scheme 1c). This alkylation went with 8:1 diastereoselectivity, and enantiopure *R*-2-MP was obtained following separation of the diastereomers and reductive cleavage of the auxiliary. More recently, a lipase-catalyzed resolution of racemic 2-MP was described (Scheme 1d).[6] In this case high ee product could only be isolated in low yield due to relatively poor enantiospecificity of the enzyme. Application of microbial oxidation in a resolution reaction afforded the desired enantiomer with only 40% ee (Scheme 1e).[7] Although effective at small scale, the procedures outlined in Scheme 1 are all unsuitable for industry because each suffers from one or more of the following: high cost of starting materials, large number of processing steps, poor yields, poor selectivity, or difficult purification procedures.

The first industrially viable synthesis of *R*-2-MP was developed at BASF in 2006 (Scheme 2).[8] In this hybrid biocatalytic−chemocatalytic process, the product was prepared by selective hydrogenation of the unsaturated aldehyde to the allylic alcohol followed by asymmetric hydrogenation of the double bond at 200 bar. Due to the relatively low enantioselectivity of the chemocatalyst (~75% ee), an additional step was required to upgrade the product's chiral purity. To that end, a lipase resolution was employed to increase the enantiopurity of the product to 98% ee. Despite the drawbacks of high pressure and the requirement for an extra step to upgrade the ee of the product, this process was effective as demonstrated by successful scale-up in a 3 m^3 reactor.[9]

Enzymes that catalyze the reduction of ketones (or aldehydes) to corresponding alcohols are known as ketoreductases (KRED).[10] The use of KREDs for organic synthesis has been growing rapidly because these biocatalysts can be highly enantioselective in the formation of chiral alcohols[11] and they are becoming more widely available.[12] With the advance of

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(4) Oppolzer, W.; Dudfield, P.; Stevenson, T.; Godel, T. *Helv. Chim. Acta* **1985**, *68*, 212.
(5) Zelle, R. E.; DeNinno, M. P.; Selnick, H. G.; Danishefsky, S. J. *J. Org. Chem.* **1986**, *51*, 5032.
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(7) Clark, D. S.; Geresh, S.; DiCosimo, R. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1383–1388.
(8) Jäkel, C.; Heydrich, G.; Sturmer, R.; Paciello, R. World Patent Application WO 2006/034812, 2006.
(9) Jäkel, C.; Paciello, R. *Chem. Rev.* **2006**, *106*, 2912–2942.
(10) This enzyme type is sometimes referred to as alcohol dehydrogenase (ADH).
(11) (a) Kosjek, B.; Nti-Gyabaah, J.; Telari, K.; Dunne, L.; Moore, J. C. *Org. Process Res. Dev.* **2008**, *12*, 584–588. (b) Lavandera, I.; Kern, A.; Ferreira-Silva, B.; Glieder, A.; de Wildeman, S.; Kroutil, W. *J. Org. Chem.* **2008**, *73*, 6003–6005. (c) Moore, J. C.; Pollard, D. J.; Kosjek, B.; Devine, P. N. *Acc. Chem. Res.* **2007**, *40*, 1421–1419.
(12) A wide selection of KRED biocatalysts are commercially available from Sigma-Aldrich (Milwaukee, WI), IEP GmbH (Wiesbaden, Germany), Enzysource (Hangzhou, China), and Codexis (Redwood City, CA).

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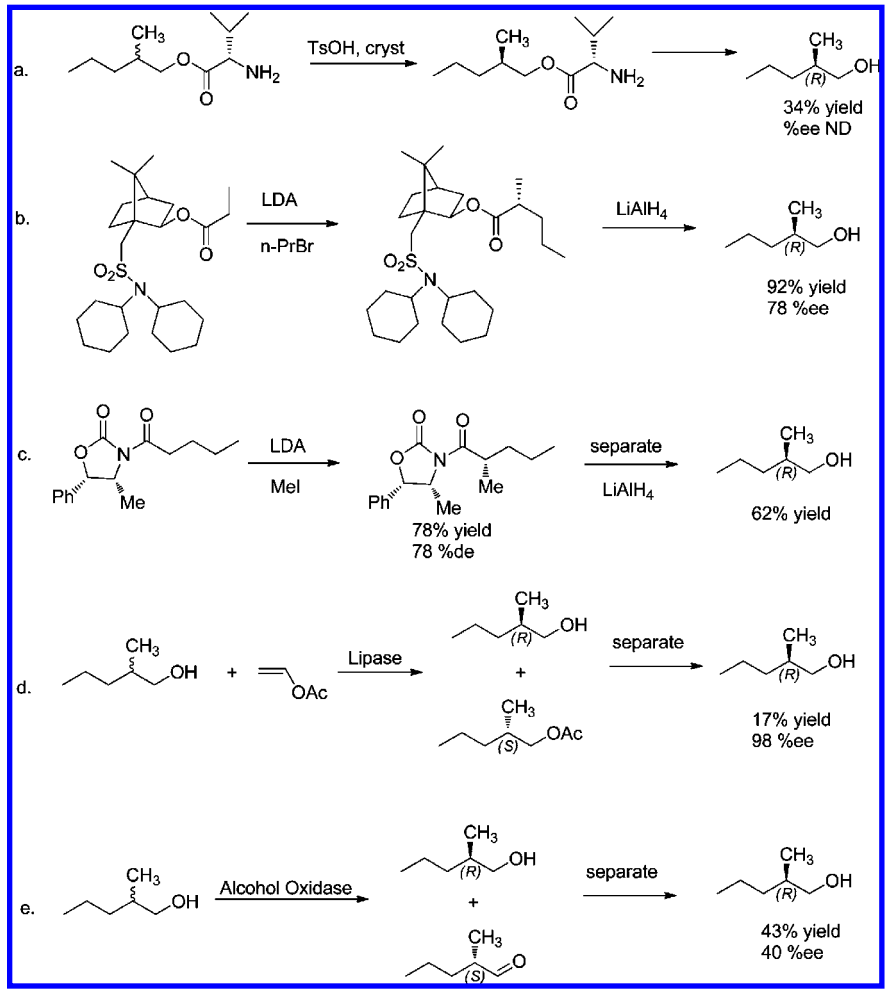
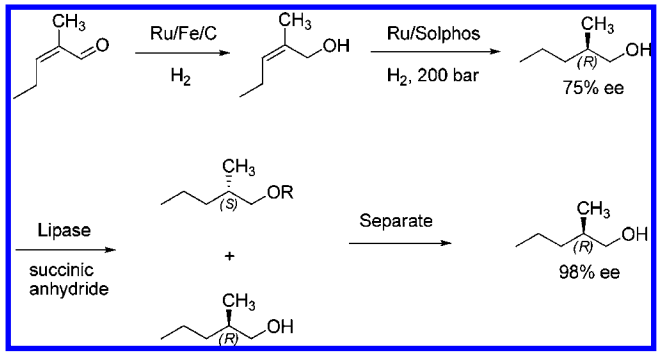
Scheme 1. **Known small-scale routes to (*R*)-2-methylpentanol**



Scheme 2. **BASF process for the production of(*R*)-2-methylpentanol**



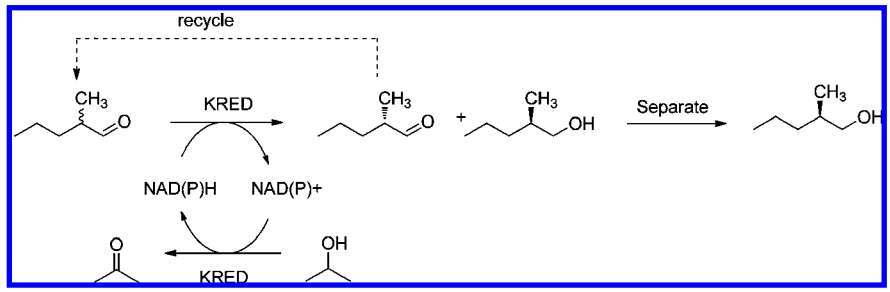
modern protein engineering techniques enzymes can be tailored to accept specific substrates and function within commercially relevant process constraints. In comparison with typical chemical catalysts for asymmetric reduction, enzymes have an added benefit of being relatively low cost, easy to manufacture, renewable, and enabling environmentally friendly manufacturing processes.[13]

Here we report on the development of an efficient process for preparing *R*-2-MP from racemic 2-methylvaleraldehyde based on enantiospecific enzymatic reduction (Scheme 3). In this process, a highly active KRED biocatalyst was engineered to convert only (*R*)-2-methylvaleraldehyde to the desired product leaving the (*S*)-enantiomer unreacted. The KRED enzyme is dependent on the cofactor β-nicotinamide adenine dinucleotide phosphate, in its reduced form (NADPH), for effecting hydride transfer to the carbonyl moiety. This cofactor should be required in only catalytic amounts when its regeneration is coupled with the oxidation of isopropyl alcohol (IPA) to acetone.[14] Moreover, the reduction of 2-methylvaleraldehyde and the oxidation of IPA are both effected by a single enzyme with IPA serving as

(13) For selected reviews of biocatalysis in a process chemistry environment, see: (a) Ran, N.; Zhao, L.; Chen, Z.; Tao, J. *Green Chem.* **2008**, *10*, 361–372. (b) Tao, J.; Zhao, L.; Ran, N. *Org. Process Res. Dev.* **2007**, *11*, 259–267. (c) Sylvestre, J.; Chautard, H.; Cedrone, F.; Delcourt, M. *Org. Process Res. Dev.* **2006**, *10*, 562–571. (d) Leresche, J. E.; Meyer, H.-P. *Org. Process Res. Dev.* **2006**, *10*, 572–580. (e) Wells, A. *Org. Process Res. Dev.* **2006**, *10*, 678–681. (f) Chikusa, Y.; Hirayama, Y.; Ikunaka, M.; Inoue, T.; Kamiyama, M.; Moriwaki, M.; Nishimoto, Y.; Nomoto, F.; Ogawa, K.; Ohno, T.; Otsuka, K.; Sakota, A.; Shirasaka, N.; Uzura, A.; Uzura, K. *Org. Process Res. Dev.* **2003**, *3*, 289–296. (g) Gotor, V. *Org. Process Res. Dev.* **2002**, *6*, 420–426. (h) Lye, G. J.; Dalby, P. A.; Woodley, J. M. *Org. Process Res. Dev.* **2002**, *6*, 434–440. (i) Wandrey, C.; Liese, A.; Kihumbu, D. *Org. Process Res. Dev.* **2000**, *6*, 286–290. (j) Bommarius, A. S.; Riebel, B. R. *Biocatalysis*; Wiley-VCH: New York, 2003. (k) Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations*, 2nd ed.; Wiley-VCH: New York, 2006. (l) Drauz, K.; Waldmann, H. *Enzyme Catalysis in Organic Synthesis*; Wiley-VCH: New York, 2002; (m) Patel, R. *Stereoselective Biocatalysis*; Marcel Dekker: New York, 2000.

(14) (a) Bradshaw, C. W.; Hummel, W.; Wong, C. H. *J. Org. Chem.* **1992**, *57*, 1532–1536. (b) Bastos, F. M.; Dos Santos, A. G.; Jones, J., Jr.; Oestreicher, E. G.; Pinto, G. F.; Paiva, L. M. C. *Biotechnol. Tech.* **1999**, *3*, 661–664. (c) Bastos, F. M.; Franca, T. K.; Machado, G. D. C.; Pinto, G. F.; Oestreicher, E. G.; Paiva, L. M. C. *J. Mol. Catal. B: Enzym.* **2002**, *19−20*, 459–465.

Scheme 3. **Enantiospecific reduction of 2-methylvaleraldehyde**



the stoichiometric reductant in the catalytic cycle. This makes process economics quite favorable, since a second enzyme is not required to regenerate the cofactor. We also report a purification and isolation method for separating the product from the unreacted substrate. The process was developed at the 2-L scale and has been successfully scaled to produce 100 kg of material suitable for use in the production of an API.

Results and Discussion

Kinetic vs Dynamic Kinetic Resolution. The reaction as outlined in Scheme 3 is a kinetic resolution where (without recycle) the possible maximum yield is 50%. If this type of reaction can be run under conditions where the aldehyde is undergoing racemization at a rate comparable to or faster than reduction, then a dynamic kinetic resolution is possible. This is of course highly desirable because the maximum theoretical yield increases to 100%. For this to be successful, the racemization reagents and conditions must be compatible with the enzyme. It is well documented that racemization of chiral 2-alkyl carbonyl compounds (including aldehydes) is rapid in polar solvents with either acid or base catalysis.[15] In our hands racemization of (*R*)-2-methylvaleraldehyde was incomplete at pH = 11, and rapid at pH = 12. Unfortunately, our enzymes were not stable above pH 9 so high pH conditions supportive of racemization were precluded. Other reagents known to catalyze aldehyde racemization at lower pH, such as 4-dimethylaminopyridine and L-proline were tested without success. On the basis of these findings we did not pursue the dynamic kinetic resolution approach further.[16]

Initial Survey of Potential Biocatalysts. The first step in the development of a biocatalytic process for 2-methylvaleraldehyde resolution was the identification of a KRED enzyme with some level of activity for carbonyl reduction. Screening of our internal collection of wild-type enzymes revealed activity for a KRED isolated from *Lactobacillus kefir* (GenBank accession #AAP94029.1) that gave *R*-2-MP of 85% ee at 40% conversion under initial screening conditions.[17] The screening reaction conditions utilized 0.225 M (23 g L^{-1}) substrate and 0.5 g L^{-1} $NADP^+$ cofactor to give a calculated selectivity factor of $E = 20$.[18] This data suggested that both enzyme enantiospecificity and volumetric productivity needed to be improved to achieve an economically viable chemical process. On the basis of this analysis, an enzyme evolution project was initiated utilizing the *L. kefir* KRED as a starting point.

Table 1. **Process targets for evolved biocatalyst performance**

parameter	targets
substrate] (g L^{-1})	≥220
[enzyme] (g L^{-1})	≤2
reaction time (h)	≤24
NADP cost contribution ($/kg)	≤10
conversion (%)	≥45
ee (%)	≥98

Enzyme Evolution. For successful evolution of an enzyme it is critical to screen libraries of enzyme variants under conditions that resemble the target process conditions.[19] Table 1 lists the performance targets required to achieve a commercially desirable process that were defined at inception of this project. Volumetric productivity (g L^{-1} day^{-1}) is a critical metric that is directly related to substrate loading (g L^{-1}) and reaction time (day^{-1}). Our analysis indicated that a substrate loading of >220 g L^{-1} and a reaction time of <24 h giving a product volumetric productivity of >100 g L^{-1} day^{-1} would meet the requirement. Other critical targets included enzyme charge (≤2 g L^{-1}), cofactor cost contribution (≤$10 per kg), conversion (≥45%), and selectivity (≥98% ee).[20] We estimated that our commercialization goals could be realized through achievement of these targets.

Full details of our directed-evolution approach are beyond the scope of this work, and thus, only a brief summary is

(15) (a) Badin, E. J.; Pascu, E. *J. Am. Chem. Soc*. **1945**, *67*, 1353–1356. (b) Braddock, D. C.; Brown, J. M. *Tetrahedron: Asymmetry* **2000**, *11*, 3591–3607. (c) Cram, D. J.; Rickborn, B.; Kingsbury, C. A.; Haberfield, P. *J. Am. Chem. Soc*. **1961**, *83*, 3678–3687. (d) Ebbers, E. J.; Ariaans, G. J. A.; Houbiers, J. P.; Bruggink, A.; Zwanenburg, B. *Tetrahedron* **1997**, *53*, 9417–9476.

(16) The off-line recycling of the unreacted substrate is viable but was deemed impractical for our scale in light of its relatively low cost of ~5 U.S.$/kg at metric ton level.

(17) The initial screening conditions utilized glucose/glucose dehydrogenase (GDH) cofactor recycling. See: Faber, K. *Biotransformations in Organic Chemistry*, 5th ed.; Springer-Verlag: Berlin, Heidelberg, NY; 2004; p 179.

(18) The selectivity factor (*E*) takes both conversion and selectivity into account, see: Kagan, H. B.; Fiaud, J. C. In *Topics in Stereochemistry*; Eliel, E. L., Wilen, S. H., Eds.; Interscience: New York, 1988; Vol. 18, pp 249−330.

(19) This is a most critical aspect of the Codexis enzyme evolution technique. In a classical process development program available catalysts are screened to discover the best performers. Then the process is adjusted to fit the catalyst. In our method the catalyst is adjusted/optimized to fit the desired chemical process, rather than the other way around. This represents a new paradigm in catalytic process development.

(20) The selectivity factor (*E*) takes both conversion and selectivity into account. For a kinetic resolution with 45% conversion and 98% ee an $E \approx 250$ is required, see: Kagan, H. B.; Fiaud, J. C. In *Topics in Stereochemistry*; Eliel, E. L., Wilen, S. H., Eds.; Interscience: New York, 1988; Vol. 18, pp 249−330. Extremely high *E* values are required when the desired product is formed from the enantiomer undergoing reaction rather than the other way around. Thus, the present type of kinetic resolution is the most difficult case.

Table 2. **Evolution of *L. kefir* ketoreductase: the variants listed were generated by various directed-evolution methods**

variant	*E*	active mutations (compared to wild-type)	number of amino acid mutations
wild-type	<50	none	0
042	51−100	L153Q; K236R	2
202	101−200	G82S; E145S; L153Q; I223 V	4
374	201−300	G82S; S96A; E145S; L153Q; I223 V	5

Table 3. **Summary of activity and selectivity data for variants 202 and 302 at 25 °C and 10 °C**

variant	temperature (°C)	reaction time (h)	conversion (%)	specificity (% ee)	*E*
202	25	24	40	97.2	138
302	25	24	52	92.9	−
202	10	19	30	98.9	275
302	10	19	38	98.1	193
202	10	45	42	98.2	235
302	10	45	50	96.2	206

provided.[21] Libraries of mutant enzymes were generated through standard molecular biology techniques and screened for activity and selectivity under the prescribed reaction conditions in the high throughput format of 96-well plates. The reactions were assayed in a tiered format: the first tier screen was an active/inactive spectrophotometric assay for cofactor turnover; the second tier screen was a GC analysis which was applied to the active hits to determine activity (conversion) and enantiospecificity. These measurements were made relative to the control enzyme so that improved variants could be culled out. The hits were then validated under more representative process conditions in small-scale chemical reactors where product isolation was also studied. The genes encoding validated protein hits were sequenced, and their translated protein sequences were analyzed via statistical modeling [protein structure−activity relationships (ProSAR)] methods. On the basis of this treatment, follow-on libraries were designed, prepared, and screened. This process was repeated in an iterative fashion until activity and selectivity targets were reached.

The *L. kefir* KRED exhibited reasonable activity towards 2MP, but the product was formed in only 85% ee; thus enantiospecificity became the key target for enzyme evolution. The first improved variant, known internally as 042, contained two mutations compared to the natural enzyme from *L. kefir* (Table 2). This enzyme displayed better selectivity and was also amenable to the preferred IPA cofactor recycling conditions. This enzyme was capable of reaching 45% conversion giving product of 95% ee in 23 h. This performance was achieved at 110 g L^{-1} substrate, 2 g L^{-1} KRED, and 0.15 g L^{-1} $NADP^+$ in 35% IPA/buffer. Although substantially improved over that of the wild-type, this performance fell short of our process targets so enzyme evolution was continued. The next substantially improved enzyme variant was 202. This variant contained four mutations relative to the natural enzyme. The performance was improved in terms of both selectivity and activity, affording 38% conversion and 97.8% ee in 40 h at the target substrate loading of 220 g L^{-1}; however, reaction time and selectivity were still below the performance goals. The next round of evolution yielded 374, the first variant that met all the process targets. This enzyme contained five mutations relative to the natural enzyme. The performance was improved both in terms of selectivity and activity affording product of 98.2% ee in a 23-h reaction at which point conversion was 46% at the target substrate loading of 220 g L^{-1}. Thus, a total of three rounds of evolution were required to generate a biocatalyst capable of meeting all of the targeted process conditions.

Process Development

Effect of Temperature. In parallel with the identification of improved enzyme variants during enzyme optimization, gram quantities of the best variants in each round of evolution were produced for process development studies. Enzyme optimization and process development studies were performed concomitantly to ensure that improved variants identified in high throughput screening indeed exhibited improvements under process conditions. In addition, these hits were used to fine-tune the process conditions utilizing laboratory reactors. For example, the effect of temperature on selectivity was evaluated utilizing two biocatalysts at two different temperatures, 25 and 10 °C (Table 3). Biocatalyst 202 was the top performer from the second round of evolution (40% conversion in 24 h), while 302 was identified as more active than 202 in round 3 screening (48% conversion in 24 h) albeit with lower selectivity at 92.9% ee. In an effort to improve selectivity, both variants were further tested at 10 °C. The reactions were repeated in duplicate at 10 °C for two different reaction times. Similar to what was observed at 25 °C, 302 showed higher reactivity but less selectivity than 202. Both variants provided *R*-2-MP with higher enantiopurity at lower temperature; however, this increased selectivity came at the expense of reaction rate.

Stopping the Reaction. As described above, reactions run with 302 showed that *S*-2-MP was detected as the reaction approached 50% conversion. This was of concern because in a kinetic resolution where the reaction product is desired, it is imperative to stop the reaction before conversion exceeds 50%. Since the *L. kefir*-based KRED variants require Mg^{2+} to maintain activity,[22] it seemed plausible to use a chelating agent, such as EDTA, to inactivate the enzyme so that the reaction could be stopped at a desired conversion. A series of experiments were conducted in which various amounts of EDTA (25−85 equiv based on 2 mM Mg^{2+} added during reaction set up) were added to a 202-catalyzed reaction. The enzyme was completely inactivated within minutes when at least 50 equiv of EDTA was added at a point where the reaction had proceeded to 25% conversion. This procedure was effective for stopping the reaction at desired conversion and as such was incorporated into the final process.

Process Optimization Utilizing the Final Variant. Once variant 374 had been shown to meet the project targets of 45% conversion and >98% ee further process development was undertaken prior to validation and scale-up. Statistical design

(21) (a) Fox, R. J.; Huisman, G. W. *Trends Biotechnol.* **2007**, *3*, 132–138. (b) Fox, R. J.; Davis, S. C.; Mundorff, E. C.; Newman, L. M.; Gavrilovic, V.; Ma, S. K.; Chung, L. M.; Ching, C.; Tam, S.; Muley, S.; Grate, J.; Gruber, J.; Whitman, J. C.; Sheldon, R. A.; Huisman, G. W. *Nat. Biotechnol.* **2007**, *25*, 338–344.

(22) Hummel, W. *Appl. Microbiol. Biotechnol.* **1990**, *34*, 15–19.

Table 4. **Variables, constants, and responses in the DoE design utilizing variant 374**

variables	constants	responses
0.75 g L^{-1} ≤ KRED ≤ 2.5 g L^{-1}	[substrate] = 220 g L^{-1}	conversion (%)
0.04 g L^{-1} ≤ $NADP^+$ ≤ 0.13 g L^{-1}	IPA = 45% (v/v)	selectivity (% ee)
15 °C ≤ temperature ≤ 25 °C	pH = 7.5	

of experiments (DoE) methods were employed to identify the key reaction parameters.[23] The screening design described in Table 4 was carried out to determine which variables were most significant to the reaction. Three key variables—$NADP^+$ concentration, reaction temperature, and biocatalyst concentration—were included in the design. The reason was that concentrations of KRED and cofactor were known to affect activity, as indicated by historical data, while temperature had been shown to affect both activity and enantiospecificity as suggested by Table 3. The substrate concentration, pH, %IPA, and mixing rate were kept constant.

A total of 19 runs were conducted in a single experiment utilizing a manual parallel synthesis device[24] and the response data points were collected at 4 h, 8 h, and 23 h. Analysis of variance and regression analysis of the 8-h sample data for conversion provided the calculated response surface graphs shown in Chart 1. In Chart 1a, it can be seen that the most significant variable affecting the conversion was the KRED concentration, while the cofactor concentration had no effect. A minor variable affecting the conversion was temperature (Chart 1d). It was noteworthy that the $NADP^+$ concentration did not affect conversion within this design space, which suggested that it might be lowered without substantially affecting the process performance and thereby providing cost savings. Analysis of the 4- and 23-h data led to similar conclusions.

Analyses of the selectivity response data for 8-h samples indicated that the KRED concentration and the temperature were both significant variables as shown in b and c of Chart 1. Consistent with previous observations, lowering the reaction temperature resulted in the product with increased ee at a given % conversion. Lowering the KRED concentration gave an apparent increase in specificity, which could be completely explained by the lower conversion.[25] Again the $NADP^+$ concentration had no significant effect on the specificity, which suggested that the amount could be lowered.

A summary of the model for this design space is shown in Chart 1d. From this response surface model the % conversion at 23 h for various combinations of KRED concentration and temperature may be predicted directly.[26] Accordingly, it was predicted that a 23-h reaction run at 20 °C with a KRED concentration of 1.3 g L^{-1} would achieve 45% conversion, giving product of 98.3% ee. This model prediction was tested in duplicate reactions using the lowest amount of cofactor (0.05 g L^{-1} $NADP^+$). It was found that the reaction was slower than expected, reaching only 41% conversion in 22 h (Table 5, entries 2 and 3). Reactions using the original (pre-DoE) conditions reached 45% conversion in 7 h (Table 5, entry 1). However, under the new conditions, the enantiopurity of the product was excellent at 99.0% ee.

Product Isolation. The final reaction mixture of this kinetic resolution contained at least 50% 2-methylvaleraldehyde, in addition to isopropyl alcohol, water, acetone, and the product. The separation of the product *R*-2-MP from the starting material was challenging as both are low molecular weight liquids with similar boiling points. Calculations indicated that ~9 theoretical plates would be required for their separation by fractional distillation. In theory this is straightforward; however, in practice it was quite difficult. Thus, several alternative procedures were explored, including: (1) base treatment to convert unreacted aldehyde to higher molecular weight products; and (2) bisulfite adduct formation to capture the unreacted aldehyde as a water-soluble compound.

Base Treatment Approach. Aliphatic aldehydes readily undergo aldol condensations upon treatment with strong base. A simple aldol condensation of 2-methylvaleraldehyde should consume the aldehyde to afford a product with about twice the molecular weight and a higher boiling point, thereby facilitating the recovery of *R*-2-MP via a simple distillation. A drawback to this approach is the destructive nature of the workup, precluding the possibility to recycle the (*S*)-aldehyde. A concentrated reaction mixture was treated with excess 50% aqueous NaOH at 75 °C for 2 h. This led to complete consumption of aldehyde without noticeable decomposition of the alcohol. Subsequent fractional distillation afforded *R*-2-MP in 30% isolated yield. Surprisingly however, the enantiopurity of *R*-2-MP had decreased from 97.3 to 87.8% ee. Because of the degradation in enantiopurity this approach was abandoned.

Co-distillation with IPA. During the course of isolation experiments it was discovered that 2-methylvaleraldehyde codistilled with IPA at atmospheric pressure, yielding a distillate of the aldehyde/IPA mixture with a composition of 17:83 w/w. This property was exploited to reduce the levels of 2-methylvaleraldehyde prior to the bisulfite adduct formation described below.

Bisulfite Addition Product Approach. Unhindered aldehydes readily react with sodium bisulfite to form addition products which are highly insoluble in organics.[27] The reaction is reversible on treatment with either acid or base and is useful for separation of aldehydes from other organic-soluble compounds. The addition of one equivalent of sodium bisulfite to a concentrated reaction mixture led to the formation of a water-soluble adduct. This allowed the separation of *R*-2-MP from the aldehyde−bisulfite adduct through extraction with MTBE.

This approach was successfully implemented into the production process. The final workup and isolation strategy involved: (1) low temperature distillation to remove part of the

(23) For a review of parallel synthesis and design of experiment (DoE) methods in pharmaceutical chemistry research see: Gooding, O. W. *Curr. Opin. Chem. Biol.* **2004**, *8*, 297–304.

(24) Two 12-place Radley's Carousel instruments were utilized (Radleys Discovery Technologies, U.K.).

(25) It is typical for an inverse relationship to exist between conversion and % ee in resolution processes where the reacting enantiomer is the desired product.

(26) The prediction is made by projecting values for the *x*-axis (amount KRED) and *y*-axis (temperature) onto the *z*-axis response surface (% conversion).

(27) (a) Soffer, M. D.; Bellis, M. P.; Gellerson, H. E.; Stewart, R. A. *Organuic Syntheses*; Wiley and Sons: New York, 1963; Collect.Vol. 4, pp 903−906.

Chart 1. **Predicted response surface diagrams from the model generated through statistical analysis of the DoE data**

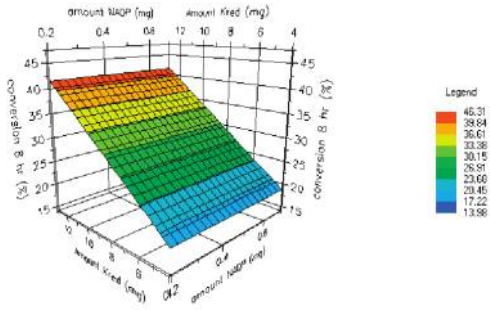


Chart 1a. Response surface showing the effect of changing the amount of KRED and the amount of NADP on % conversion at 8 h and 20 °C. Amount of KRED shows substantial effect while amount of NADP shows no effect.

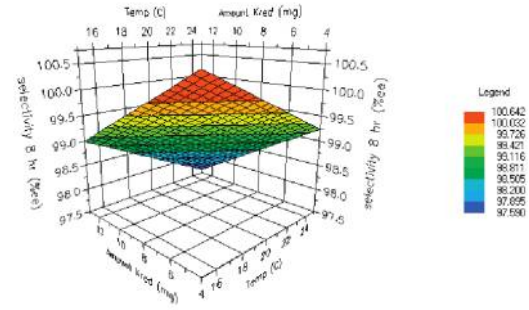


Chart 1b. Response surface showing the effect of changing the amount of KRED and the temperature on %ee of the product at 8 h and 0.085 g L^{-1} NADP. Both factors show an effect.

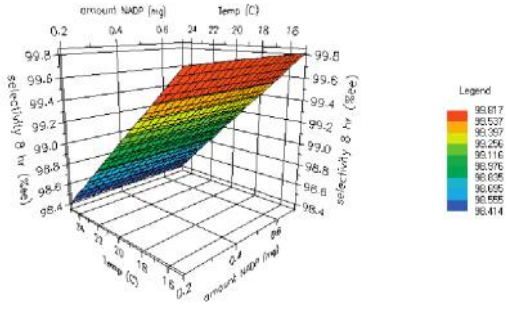


Chart 1c. Response surface showing the effect of changing the temperature and the amount of NADP on %ee of the product at 8 h and 1.6 g L^{-1} KRED. Temperature shows substantial effect while amount of NADP shows no effect.

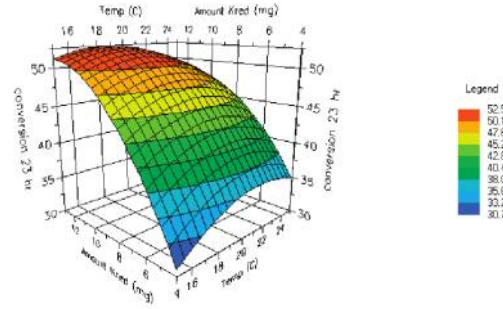


Chart 1d. Response surface showing the effect of changing the amount of KRED and the temperature on %conversion at 23 h and 0.085 g L^{-1} NADP. 45% conversion is predicted when amount KRED = 6.6 mg (1.3 g L^{-1}) and temperature = 20 °C.

Table 5. **Testing of model predictions: conversion and selectivity in MPH374-catalyzed reactions at reduced biocatalyst loadings and $NADP^+$ concentrations**

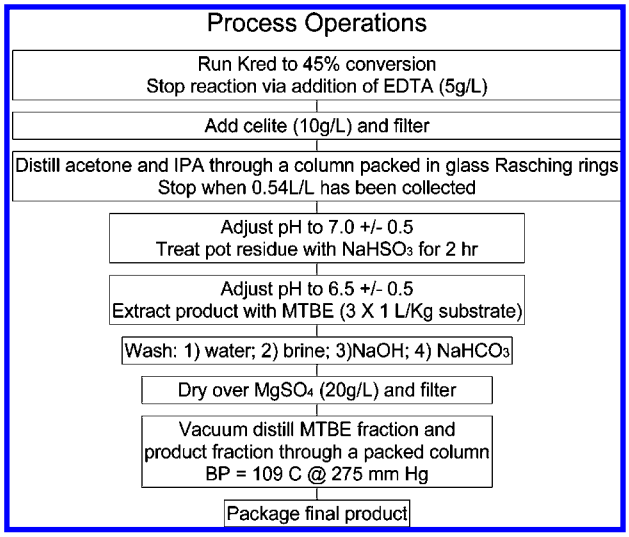
entry	amount of KRED (g L^{-1})	amount of $NADP^+$ (g L^{-1})	reaction time (h)	temp (°C)	conversion (%)	selectivity (% ee)
1	2	0.1	7	25	45	98.0
2	1.3	0.05	22	20	41	98.9
3	1.3	0.05	22	20	41	99.1

unreacted aldehyde as a codistillate with IPA, (2) treatment with sodium bisulfite to render the remaining residual aldehyde water-soluble, (3) product extraction with MTBE, (4) concentration and final simple distillation to isolate the product. A flow diagram describing the final process is shown in Chart 2. The process was validated at 2-L scale, affording product of excellent quality in 67% theory as described in the Experimental Section. Following this a pilot run was conducted at 400 kg substrate charge. The process scaled nearly directly, requiring 28 h to reach 45% conversion (8 h longer). Isolation of the product through distillation was accomplished in two batches due to equipment size limitations. The distillation proceeded as expected, affording a total of 96.4 kg of product, 53.6% theory (assuming 45% conversion). The production batch showed chemical purity of 99.93% (GC/FID area %) and enantiomeric purity of 98.22% ee. Based on this the initial process targets shown in Table 1 were all met or exceeded, and the final product quality was within specification for API production.

Conclusions

An effective process for the preparation of *R*-2-MP from racemic 2-methylvaleraldehyde was developed. Directed enzyme evolution was applied to create a process-fit KRED biocatalyst capable of operating at high substrate loading and low enzyme and cofactor loading. The same KRED enzyme was able to utilize IPA to regenerate the reduced form of the cofactor so that the need for a second auxiliary enzyme, such as GDH, could be circumvented. The biocatalytic reaction was characterized and optimized utilizing DoE and parallel synthesis techniques. Two approaches to product isolation were explored, and an effective procedure was developed based on separation of the product via bisulfite adduct formation of the unreacted

Chart 2. **Flow diagram showing the operations employed in the final process**



aldehyde. The developed process was validated at the 2-L scale and was subsequently applied to produce 100 kg of material suitable for use in production of an API. Compared with previous routes, this one-step process is shorter and utilizes a renewable biocatalyst.[28]

Experimental Section

Materials and Methods. β-nicotinamide adenine dinucleotide phosphate in its oxidized form (NaNADP) was obtained from Oriental Yeast Company (Andover, MA). Screening panels of enzyme variants and lyophilized enzyme powders were obtained through standard molecular biology techniques described elsewhere.[29] Screening panels and lyophilized enzyme powders of KRED enzymes similar to those described here are available from Codexis (Redwood City, CA).

All gas chromatographic analysis was conducted utilizing an Agilent 6850 series II gas chromatograph (injection port temperature, 200 °C; detector temperature, 300 °C; split ratio 100:1; He carrier gas, 0.6 mL/min). Specific methods are as follows: Conversion of 2-methylvaleraldehyde to 2-methylpentanol was determined using an Agilent 19091J-433E column (phenyl methyl siloxane, capillary 30.0 m × 250 μm × 0.25 μm nominal); ramp profile: 60 °C for 3 min, ramp from 60 to 200 °C at 20 °C per minute, hold for 1 min, injection volume, 1 μL; sample concentration, 15 mg/mL in IPA or MTBE; retention time: 2-methylvaleraldehyde, 1.84 min; 2-methylpentanol, 2.53 min. Calibration curves for substrate and product were developed utilizing standard serial dilution techniques for precise quantitation of conversion. The enantiomeric purity of (*R*)-2-methylpentanol was determined using a Chiraldex B-DP column (30 m × 0.25 μm, cat no. 78023: Astec Inc., Whippany, NJ), oven temperature 65 °C (isothermal), injection volume 3 μL, sample concentration 15 mg/mL in IPA or MTBE, retention times: (*R*) 2-methylpentanol, 9.6 min, (*S*) 2-methylpentanol, 10.9 min.

(28) The process mass intensity (PMI) for this process is 29.3, 54% of which is recyclable solvent (IPA and MTBE), 30% is water, 12% is substrate, and 5% is reagents.

(29) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Plainview, New York, 1989; Vol. 1−3. (b) Provisional U.S. Patent application submitted; filing date August 27, 2008, 61/092,647, 2008.

First-Tier High Throughput NADPH Fluorescence Prescreen to Identify Enzymes for the Oxidation of 2-Propanol. This first-tier NADPH fluorescence-based prescreen was developed to identify active variants and to assess the thermal stability of KRED variants. Plasmid libraries obtained by directed evolution and containing evolved ketoreductase genes were transformed into *Escherichia coli* and plated on Luria−Bertani (LB) broth containing 1% glucose and 30 μg/mL chloramphenicol (CAM). After incubation for at least 16 h at 30 °C, colonies were picked using a Q-bot robotic colony picker (Genetix U.S.A., Inc., Beaverton, OR) into 96-well shallow-well microtiter plates containing 180 μL of Terrific broth (TB), 1% glucose, and 30 μg/mL CAM. Cells were grown overnight at 30 °C with shaking at 200 rpm. Twenty microliters of this culture was then transferred into 96-deep-well plates containing 350 μL of Terrific broth (TB), 2 mM $MgSO_4$, and 30 μg/mL CAM. After incubation of deep-well plates at 30 °C with shaking at 250 rpm for 2−3 h recombinant gene expression by the cell cultures was induced by addition of isopropyl thiogalactoside (IPTG) to a final concentration of 1 mM. The plates were then incubated at 30 °C with shaking at 250 rpm for 15−17 h.

Cells were pelleted via centrifugation, resuspended in 300 μL of lysis buffer, and lysed by shaking at room temperature for at least 1 h. The lysis buffer contained 100 mM triethanolamine(chloride) buffer (pH 7.0−7.2), 1 mg/mL lysozyme, 200 μg/mL polymixin B sulfate, and 2 mM $MgSO_4$. The plates were then spun at 4000 rpm for 10 min at 4 °C, and the clear supernatant was used in the fluorescent assay. In 96-well black microtiter plates 20 μL of clear supernatant was added to 180 μL of an assay mixture consisting of 90 mM triethanolamine(chloride) buffer pH 7.0, 1 mM $MgSO_4$, 1 g/L NADP, and 2% 2-propanol. Reaction progress was measured by following the increase in fluorescence of NADPH at 445 nm after excitation at 330 nm in a Flexstation (Molecular Devices, U.S.A.). Variants with initial rates higher than the negative control (cells transformed with empty vector) were consolidated and tested in the cofactor/IPA recycling system.

Second-Tier High Throughput Screening to Identify Improved Enzymes for the Stereospecific Reduction of 2-Methylvaleraldehyde Using Isopropyl Alcohol for Cofactor Regeneration. Fifty microliters of cell lysate obtained as described above was transferred to a deep well plate (Costar #3960). Forty microliters of 1 M triethanolamine(chloride) buffer pH 7.0, 50 μL of 0.875 mg/mL Na-NADP (Oriental Yeast), 70 μL of 2-methylpentanal, and 140 μL of isopropyl alcohol were added to each well. After sealing the plates, the reaction samples were shaken for at least 16 h at ambient temperature.

After centrifugation (4000 rpm, 1 min, ambient temperature) 1 mL of MTBE was added per well. Plates were resealed and shaken for 2 to 3 min, and the organic and aqueous layer were separated by centrifugation (4000 rpm, 5 min, ambient temperature). Five hundred microliters of the organic layer was transferred into a new deep-well plate containing ~100 mg of Na_2SO_4. The plates were sealed and shaken for 2−3 min. The plates were resealed and shaken for 2−3 min. After centrifugation (4000 rpm, 5 min, ambient temperature), 100 μL of the

organic layer from each well was added to 100 μL of ethyl acetate in wells of a fresh microtiter plate. After sealing the plates, samples were subjected to chiral GC analysis described above. The *E* value was calculated from the ee and % conversion. Potential improved variants over the parent variant were regrown in quadruplicates and retested under the described conditions to identify the hit variants. In order to validate the HTP results shake flask powders of improved variants were prepared and tested in small-scale reactions according to the procedures described below.

Production of Ketoreductase Powders; Shake Flask Procedure. A single microbial colony of *E. coli* containing a plasmid with the ketoreductase gene of interest was inoculated into 50 mL of Tryptic broth (12 g/L bacto-tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 65 mM potassium phosphate, pH 7.0) containing 30 μg/mL chloramphenicol and 1% glucose in a 250 mL Erlenmeyer flask. Cells were grown overnight (at least 16 h) in an incubator at 30 °C with shaking at 250 rpm. The culture was diluted into 250 mL of Terrific Broth (1 mM $MgSO_4$, 30 μg/mL chloramphenicol) in 1 L flask to an optical density at 600 nm (OD_{600}) of 0.2 and allowed to grow at 30 °C. Expression of the ketoreductase gene was induced with 1 mM IPTG when the OD_{600} of the culture was 0.6−0.8 and the culture was incubated overnight (at least 16 h). Cells were harvested by centrifugation (5000 rpm, 15 min, 4 °C), and the supernatant was discarded. The cell pellet was resuspended with an equal volume of cold (4 °C) 100 mM triethanolamine (chloride) buffer, pH 7.0, and harvested by centrifugation as above. The washed cells were resuspended in 12 mL of the cold triethanolamine (chloride) buffer and passed through a French Press twice at 12000 psi while the temperature was maintained at 4 °C. Cell debris was removed by centrifugation (9000 rpm, 45 min, 4 °C). The clear lysate supernatant was collected and stored at −20 °C. Lyophilization of the frozen clear lysate provided a dry powder of crude ketoreductase enzyme.

Small-Scale Reduction of 2-Methylvaleraldehyde by Engineered Ketoreductases Derived from *L. kefir*. Improved KRED variants were evaluated on a small scale with magnetic stirring. A 25 mL glass vial was charged as follows: to 11 mL of 100 mM triethanolamine hydrochloride buffer (pH 7) were added 31 mg of lyophilized KRED powder, 32 μL of 1 M $MgSO_4$, 3 mg of Na-NADP, 2.2 g of 2-methylvaleraldehyde, and 7 mL of isopropyl alcohol. Reactions were run at 25 °C for 18−24 h. Reaction samples were analyzed by the GC methods described above.

Preparative-Scale Production of (*R*)-2-Methylpentanol. To a 2 L jacketed reaction vessel fitted with a mechanical stirring paddle, pH probe, nitrogen inlet, and thermocouple were added 684 mL of isopropyl alcohol, 285 mL of 100 mM triethanolamine (pH 9.5), 4 mM $MgSO_4$, and 342 g of 2-methylvaleraldehyde. Stirring was started at ∼100 rpm, and the temperature was set to 20.0 °C. The pH was adjusted to pH = 7.4 + 0.1 by addition of 10 vol % hydrochloric acid. After the desired temperature and pH were reached, the reaction was initiated by adding the following in order: 0.154 g of Na-NADP dissolved in 15 mL of deionized water, 3.05 g of KRED enzyme 374 dissolved in 100 mL of deionized water. The conversion was monitored by GC as described above. Samples were prepared for analysis as follows: 50 μL of reaction mixture was partitioned between 0.5 mL of water and 1.0 mL of MTBE. The MTBE layer was withdrawn and injected onto the GC. When the reaction reached 45% conversion (20 h), the enzyme was inactivated by charging with 7.5 g of EDTA and 15 g of Celite 545. The mixture was stirred for 15 min prior to proceeding. The reaction mixture was filtered through an "M" sintered glass funnel, and the cake was washed with 75 mL of isopropyl alcohol. The biphasic mixture was transferred to a 2 L distillation flask and the pH was adjusted to 7 ± 0.5 using 20% NaOH solution. The distillation flask was fitted with a thermocouple connected to a temperature controller, a heating mantle, and a jacketed distillation column (1.5 cm diameter × 15 cm length) packed with glass Rasching rings (6 mm diameter × 6 mm length, Chemglass p/n CG-1283-01). The distillation was allowed to proceed until a total of 810 mL of distillate was collected. The residue was cooled to ∼25 °C, and 95 g of sodium bisulfite dissolved in 200 mL of deionized water was added over a 6 min period such that the temperature remained ≤35 °C. The reaction mixture was stirred for 2 h at 35 °C during which time it became monophasic. The pH was adjusted to 6.5 using 20% NaOH. GC analysis (sample preparation as described above) determined a product-to-substrate ratio of 96:4. The solution was extracted with 3 × 300 mL of MTBE, followed by washing of the combined organic extract with 75 mL of deionized water, 75 mL of brine, 75 mL of 0.5 N NaOH, 50 mL of saturated $NaHCO_3$, and 25 mL of brine. The organic solution was dried over 30 g of $MgSO_4$ for 3 h and filtered into a 2 L distillation flask fitted with a thermocouple connected to a temperature controller, heating mantle, and a jacketed distillation column. MTBE and residual IPA were removed by atmospheric distillation until the pot temperature reached 130 °C. The product was obtained by vacuum distillation at 275 mmHg pressure. The fraction with a boiling range of 109−110 °C was collected, affording 101 g of colorless liquid (67% of theory). The chemical purity was 97.7%, and the enantiomeric purity was 98.5% ee. All physical and spectroscopic properties were identical to those of an authentic sample of racemic 2-methylpentanol obtained commercially (Aldrich 214019-1 L).

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