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ARTICLE

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Biotechnological Development of a Practical Synthesis of Ethyl (*S*)-2-Ethoxy-3-(*p*-methoxyphenyl)propanoate (EEHP): Over 100-Fold Productivity Increase from Yeast Whole Cells to Recombinant Isolated Enzymes

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S Supporting Information

ABSTRACT: The coupling of the enantioselective reduction catalyzed by Old Yellow Enzymes (OYEs), together with the in situ substrate feeding product removal (SFPR) concept, significantly improved the productivity of the g-scale preparation of ethyl (*S*)-2-ethoxy-3-(*p*-methoxyphenyl)propanoate (EEHP), an important precursor of several PPAR-α/γ agonists, such as Tesaglitazar. The OYEs and the glucose dehydrogenase for cofactor regeneration were cloned, overexpressed in *Escherichia coli*, and purified. The synthetic sequence was completed by a $NaClO_2$ oxidation employing cheap and environmentally friendly conditions. The product was obtained in 94% yield and with an ee of 98% over the two steps.

INTRODUCTION

Nowadays, the chemical industry, besides the optimization of the typical parameters characterizing a process, has to satisfy the increasing societal requirement of more environmentally compatible processes. Biocatalysis is doubtlessly the most promising area in which to search for an answer to such a challenging *demand*.

However, often the discovery of a valuable biotransformation is quite far from a direct practical application. Many development and optimization stages are in between, and the integration of widely different competences (from the subject areas of chemistry, biotechnology, engineering, and so on) becomes mandatory. Among all bioconversions, the clearest example of such a gap is provided by enantioselective baker’s yeast (B.Y.) mediated reduction of prochiral activated C═C double bonds. Indeed, even if thousands of reactions have been described so far,[1] only a few of them have been implemented at industrial scale.[2]

Despite the simplicity and cheapness of the setup, there are several drawbacks from the downstream processing point of view: (i) very low substrate concentrations tolerated by the microorganism that lead to an intrinsically too low productivity; (ii) difficult workup, due to the troublesome separation of product from a huge amount of biomass; (iii) typically incomplete conversion and occurrence of side reactions, which imply the use of industrially unappealing chromatographic steps; (iv) the presence of enzymes with the same specific biocatalytic activity which might have different enantioselectivity.

In this paper, we report the results of the development and optimization of the bioconversion step that lies at the heart of a practical process for the enantiospecific synthesis of ethyl (*S*)-2-ethoxy-3-(*p*-methoxyphenyl)propanoate (EEHP),[3,4] **1**, an important pharmaceutical intermediate for the preparation of antidiabetic drugs of the PPAR-α/γ agonists family,[5] active against type 2 diabetes. The switch from a B.Y. whole cell fermentative synthesis to a more industrially appealing recombinant enzyme catalyzed process, combined with the in situ substrate feeding product removal (SFPR) strategy,[6] led to an outstanding enhancement of productivity and addressed all the above-mentioned issues.[7]

BAKER’S YEAST MEDIATED PROCESS

Recently, we reported a new synthesis of EEHP based on B.Y. mediated reduction of aldehyde **2** to give the corresponding saturated alcohol **3** in a good yield of 78% and an excellent ee of 99% (Scheme 1), using the in situ SFPR technique.[4]

The predominant bioconversions occurring within the resting cell consist of the enantioselective *anti* addition of hydrogen to the C═C double bond[8] catalyzed by enoate reductase (ER) enzymes and/or the reduction of the carbonyl group resulting from one or more alcohol dehydrogenase (ADH) enzymes, as illustrated in Scheme 2. Accordingly, the product distribution typically observed at the end of the bioconversion (by ^{1}H NMR) is composed of a few percent of **2**, over 80% of saturated alcohol **3**, about 10% of allylic alcohol **4**, and less than 10% of other side products (mainly anisaldehyde coming from degradation of **2**).

Even though this step compares well in terms of yield and enantioselectivity with other reported metal-based reductions,[3d,e] it suffers from (i) an extremely low productivity (0.39 g L^{-1} d^{-1}), (ii) a nonquantitative conversion, (iii) a complex purification

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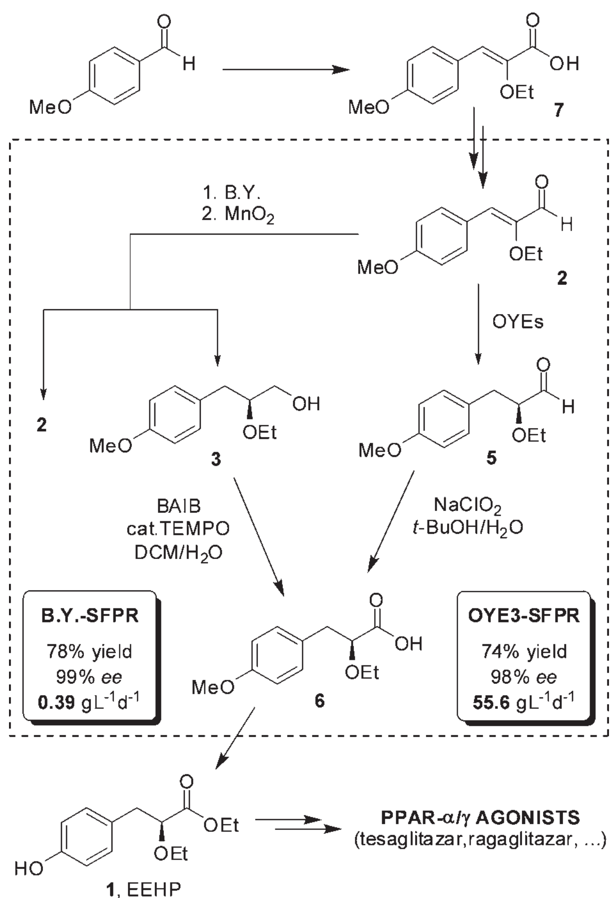
Scheme 1. Comparison between the Two Biocatalytic Approaches to the Synthesis of EEHP



process based on the chemoselective oxidation of the undesired allylic alcohol **4** to give the starting material (by MnO_2), which only then can be isolated from the product by chromatography, and (iv) a useless and counterproductive reduction of the carbonyl group (by ADH) since the final target is an ester, i.e., **1**.

IDENTIFICATION OF ENZYMES WITH SUBSTRATE-SPECIFIC ENOATE REDUCTASE (ER) ACTIVITY

Given that α-enolether aldehydes like **2** were reduced in unusually high yields[4] and are unconventional substrates, we thought that it was worth investigating whether an unknown ER activity could be identified.

For the enzyme identification procedure, instead of the commercially available yeast strain that we employed for the reduction, we chose a model *S. cerevisiae* strain (BY4741) since its genome has been completely sequenced,[9] facilitating later assignment of genes to enzymes. Biotransformations of **2** run in parallel with whole cells of each strain showed an identical product distribution (by GC-MS analysis), validating the use of the model yeast.

To identify the catalytic activities, the cell-free extract (CFE) was prepared from a BY4741 culture and subjected to ion exchange (IEX) chromatography on a quaternary ammonium stationary phase for a preliminary separation of proteins based on their different isoelectric points. A typical chromatogram is shown in Figure 1.

The ER activity test on the fractions collected according to the chromatogram was not carried out spectrophotometrically on typical model substrates like 2-cyclohexenone[10] but directly in biotransformation conditions with both NAD(P)H cofactors on our substrate **2** to give **5**, to be sure to find the specific activity.

Only the fractions corresponding to the first sharp peak of the chromatogram showed an evident ER activity and a negligible ADH activity. However, a still too large number of proteins were present in the fraction (as proven by SDS−PAGE analysis), making the identification of the ER impossible at this stage.

Therefore, fractions containing the ER activity were pooled, concentrated by centrifugal filtering units, and further separated by means of a native PAGE, which was partially stained to reveal the protein distribution and partially cut to enable (i) an activity assay and (ii) MS/MS analysis of the present proteins (Figure 2).

The proteins in one slice of the gel which afforded 51% conversion of **2**, the highest value of all measured slices, were digested and analyzed by MS/MS spectrometry. The bioinformatics analysis (with ProteinLynx and Mascot search programs, see Supporting Information) returned agreement with predicted fragments from four proteins. Only one of them belongs to the class of the oxidoreductases, the well-known Old Yellow Enzyme 2 (OYE2),[11] widely studied for the bioreduction of activated C=C double bonds.[12] This result unambiguously rules out the presence of novel ER activities. Moreover, it is known that another homologue ER enzyme, named OYE3, is present in B.Y.,[10b] but its expression level is so low[13] that its activity could not be detected in our enzyme identification procedure.

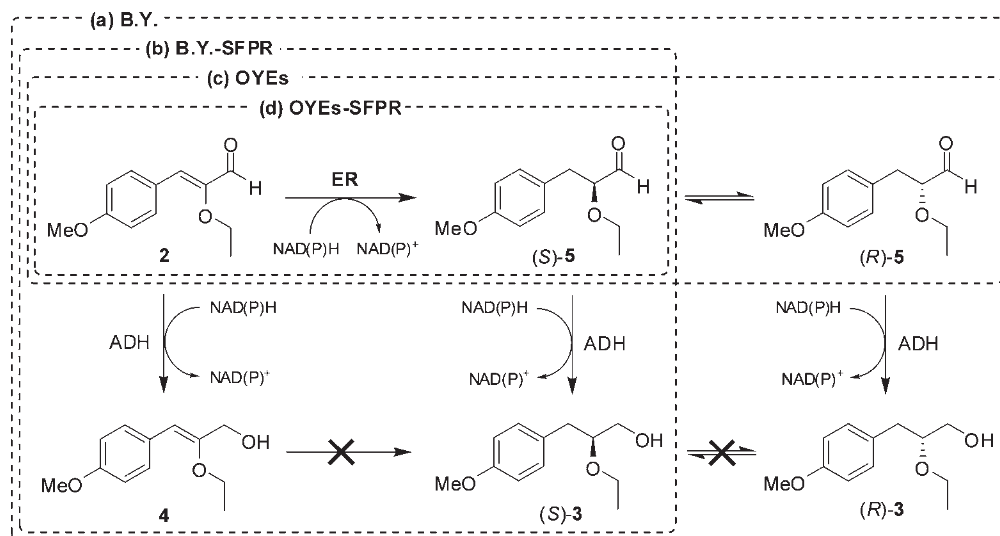
CLONING, OVEREXPRESSION, AND PURIFICATION OF OYE2/OYE3

Although the IEX fraction already showed a remarkable decrease in side activities, the isolation of the OYEs from yeast cells is unconvenient because of their low expression levels.[13] Therefore, the most practical approach consisted of cloning and overexpressing each OYE, e.g., in the bacterial model host *Escherichia coli*.

From the process development point of view, the direct use of whole cells of *E. coli* overexpressing OYEs or its CFE as biocatalysts without the need of any purification could be convenient and desirable. Thus, we compared the activity of the CFE obtained from a typical *E. coli* cloning strain (DH5α) with that of yeast (Table 1). Unfortunately, very similar results were obtained, indicating that also in *E. coli* interfering side activities are present. In the attempt to find a host lacking undesirable activities towards our substrate, we tested the multiple-deletion strain MDS41, which is deprived of 14% of its original genome,[14] but without success.

To ensure an easy and highly efficient purification, we selected affinity purification via the well-established His-tag system. The *oye2* and *oye3* genes were amplified by polymerase chain reaction (PCR) from chromosomal DNA of BY4741; in the process the stop codon was removed, and appropriate restriction sites were introduced. Then each gene was inserted into the commercial plasmid pET-30a bearing a C-terminal His-tag sequence. The resulting plasmids were used to transform *E. coli* BL21 cells which served as an expression host. Cultures of the two strains were induced with IPTG, and the cells were harvested and disrupted affording the corresponding CFEs, from which the recombinant OYEs were isolated by immobilized metal affinity chromatography (IMAC).

Scheme 2. Main Biocatalytic Reactions Occurring during the Biotransformation of 2 by (a) B.Y., (b) B.Y.-SFPR, (c) OYEs, and (d) OYEs-SFPR



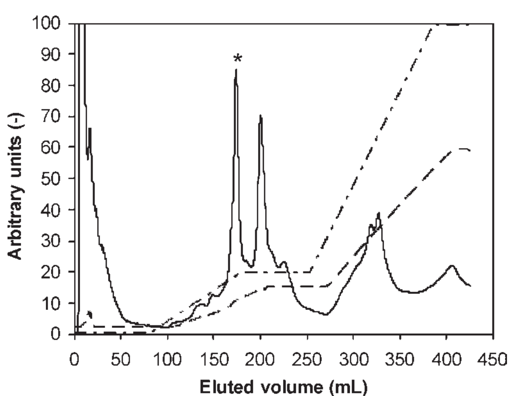


Figure 1. IEX chromatogram of yeast CFE. The peak which exhibited ER activity is marked with an asterisk (*). Absorbance 280 nm (—), conductivity (— —), NaCl gradient 0−500 mM (— · —).

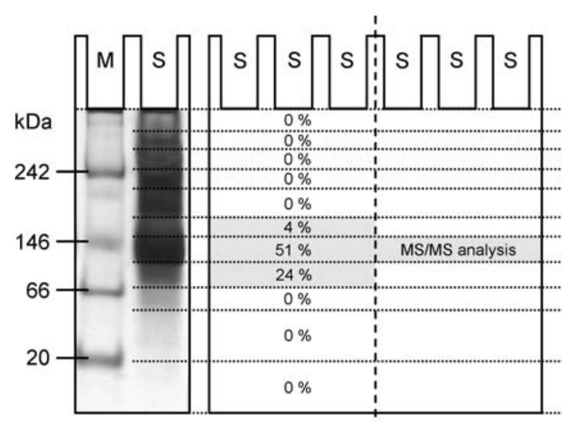


Figure 2. Native PAGE separation of the active IEX fraction. Conversions of **2** into **5** obtained from the activity tests are shown on the bands. The slice portion submitted to the MS/MS analysis is highlighted. M: protein marker lane. S: sample lane.

COFACTOR REGENERATING SYSTEM

The progress curves registered for the conversion of **2** into **5** by OYEs in a batch reaction with overstoichiometric NAD(P)H cofactors (Figure 3) show that OYE2 catalyzes the bioreduction faster than OYE3 and that both enzymes exhibit a clear preference for NADPH, making the utilization of the latter more a need than a choice, especially if a high productivity is sought.

To keep the incidence of cofactor costs very low, the setup of a cofactor regeneration system is mandatory since it allows the use of a catalytic amount of the latter, preferentially in the cheaper oxidized form ($NAD(P)^+$).

Among all types of NADPH regeneration systems that have been reported so far, the most common and efficient is based on the oxidation of glucose to give δ-gluconolactone, which in turn hydrolyzes irreversibly to gluconic acid, driving the reaction to completion.[15] The oxidation of the sacrificial substrate is catalyzed by a glucose dehydrogenase (GDH), as illustrated in Scheme 3. The conversions of **2** at different reaction times achieved using two efficient commercial GDHs (from *Pseudomonas* sp. and from *Thermoplasma acidophilum*) resulted satisfactory (Table 2). However, for reasons of convenience and economy, we undertook the overexpression and the purification of the regeneration enzyme as well. Therefore, a *gdh* gene was amplified by PCR from the *Bacillus megaterium* DSM509 strain, with primers designed on the

Table 1. Background Activity Tests on 2, Performed on CFEs of Different Strains

strain	product distribution (%)[a]				
	2	3	4	5	subprod.
S. cerevisiae BY4741	2	82	9	0	7
E. coli DH5α	2	26	41	5	26
E. coli MDS41	3	0	48	11	39

[a] By GC-MS.

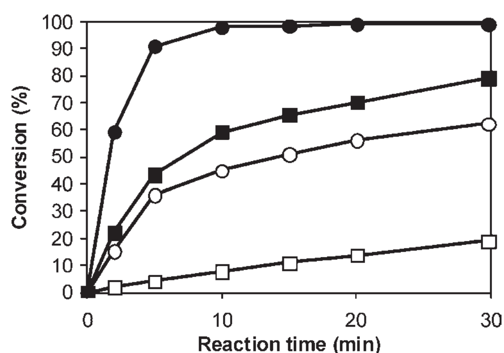
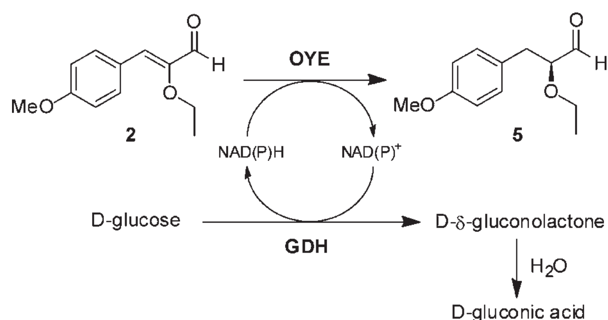


Figure 3. Progress curves for the conversion of **2** into **5** by OYEs with overstoichiometric cofactors (OYE2/NADPH (●), OYE3/NADPH (■), OYE2/NADH (○), OYE3/NADH (□)). Expt. cond.: 20 mM HEPES buffer pH 7.0, 1 mM substrate, 1.1 mM NAD(P)H, 25 μg/mL of OYE, 30 °C, 160 rpm. Conversions were determined by GC-MS.

Scheme 3. Glucose/GDH Cofactor Regeneration System



known *gdh* sequence of the DSM319 strain, incorporating the His-tag sequence in the forward primer (leading to an N-terminal His-tag). The purified PCR product was cloned into a pKTS vector,[16] and then the resulting pKTS-GDH plasmid was introduced in *E. coli* BL21 (DE3) cells for overproduction. A culture of the latter was induced with IPTG/anhydrotetracycline and subjected to the same procedure described above for the OYEs, yielding the purified recombinant GDH.[17]

In terms of performance, this new GDH from *B. megaterium* (GDH-BM) compares well with its commercial counterparts (Table 2), validating its use as a cofactor regenerating system, with the additional advantage that it can be produced without being dependent on a supplier.

OPTIMIZATION OF EXPERIMENTAL PARAMETERS FOR THE BIOCONVERSION

Since quantitative conversions with both the OYEs after 12 h were obtained, the necessity to achieve high conversions in the shortest reaction time led us to set the latter to 12 h as a reasonable trade-off. Although apparently at these conditions the OYEs seem to give identical performances (Table 2), actually OYE3 is slightly more stereoselective as proven by the optical purity of **5** (85% ee with OYE2 vs 90% ee with OYE3, by chiral GC). Disappointingly, both ee's results were lower than that achieved with yeast whole cells (99% ee),[4] most likely due to an epimerization of **5** (Scheme 2). Consequently, we decided to work at neutral pH to minimize such a potential racemization,

Table 2. Comparison of the Performances of Three GDHs with Respect to the Cofactors Employed and the Biotransformation Time[a]

			conversion[c] (%)		
GDH[b]	cofactor	OYE	5 h	12 h	24 h
PS	NAD^+	OYE2	10.0	39.7	43.0
		OYE3	1.5	6.9	5.8
	$NADP^+$	OYE2	94.1	**100**	100
		OYE3	85.6	**100**	100
TH	NAD^+	OYE2	–	–	–
		OYE3	–	–	–
	$NADP^+$	OYE2	95.1	**100**	100
		OYE3	86.1	**100**	100
BM	NAD^+	OYE2	1.9	3.3	3.4
		OYE3	0.6	1.6	1.8
	$NADP^+$	OYE2	67.9	**100**	100
		OYE3	74.7	**100**	100

[a] Expt. cond.: 50 mM phosphate buffer, pH 7.0, 5 mM substrate, 0.1 mM $NAD(P)^+$, 20 mM glucose, 4 U GDH (ref 17), 1% DMF, 30 °C, 160 rpm (ref 18a and 19). [b] BM: recombinant from *B. megaterium*. PS: commercial from *Pseudomonas* sp. TH: commercial from *Thermoplasma acidophilum*. [c] By GC-MS.

even if it is known that some OYEs exhibit their maximum activity around pH 8.0.[18]

In summary, it was decided to proceed with the optimization of other parameters using OYE3, GDH-BM, $NADP^+$, and pH 7.0.

Anyway, the productivity (1.9 g L^{-1} d^{-1}, Table 3 entry 1), obtained using such conditions with a typical substrate loading of 1.0 g L^{-1},[19] remained unsatisfactory, although it improved considerably with respect to that of yeast (0.39 g L^{-1} d^{-1}). In the attempt to increase it further, we tested a higher substrate loading (15.0 g L^{-1} vs 1.0 g L^{-1}), but the conversions dropped down, even employing higher cosolvent concentrations and/or liquid–liquid biphasic mixtures[19d] (entries 2–7).

The criterion adopted for our optimization process requires the highest possible productivity with quantitative yields, to avoid any purification procedure. Hence, although in the best case (entry 5) we achieved a very high productivity (24.3 g L^{-1} d^{-1}), it is impaired by a noncomplete conversion and therefore unacceptable. In contrast, by introducing the in situ SFPR strategy we obtained rewarding results.[7]

The winning concept of SFPR developed by Vicenzi et al. at Eli Lilly is based on maintaining both substrate and product concentrations at a level not toxic for the microorganism and not inhibiting the catalytic activity of enzyme deputed to the biotransformation.[6] To this purpose, the substrate, which is typically lipophilic, is adsorbed on a hydrophobic resin, ensuring its release into the aqueous medium at extremely low concentrations; the same is true for the product, mostly adsorbed on the resin as soon as it is formed. Such an absorption/desorption equilibrium is almost instantaneous, and the concentration in the aqueous phase can be easily adjusted by changing the ratio between resin and substrate ($X_{r/s}$). Another interesting aspect of this technique is that by lowering the concentrations using higher $X_{r/s}$ the enantioselectivity usually increases.[20,4]

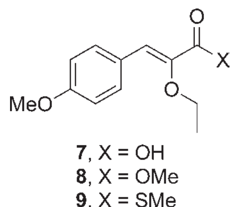
The results show clearly that by using a much lower $X_{r/s}$ with respect to those usually employed with the yeast ($X_{r/s}$ 10–20)[4,21]

Table 3. Optimization of the Substrate Loading, $X_{r/s}$, OYE3 Concentration, and Kind of Substrate for the Preparative Scale Application

entry	substrate	loading (g L^{-1})	cosolvent/biphasic system	$X_{r/s}$	OYE3 conc .(mg L^{-1})	Conv.[a] (%)	ee[b] (%)	productivity[c] (g L^{-1} d^{-1})
1	**2**	1	DMF 1%	–	25	100	90	1.9
2	**2**	15	DMF 1%	–	25	20	–	–
3	**2**	15	DMF 1%	–	125	66	90	18.8
4	**2**	15	DMF 5%	–	125	18	–	–
5	**2**	15	DMSO 5%	–	125	86	88	24.3
6	**2**	15	MTBE 20%	–	125	4	–	–
7	**2**	15	*i*-PrOAc 20%	–	125	2	–	–
8	**2**	15	resin	20	125	49	99	14.7
9	**2**	15	resin	3	125	100	99	29.9
10	**2**	30	resin	3	125	32	99	19.1
11	**2**	30	resin	3	175	74	99	44.2
12	**2**	30	resin	2	175	86	98	51.1
13	**2**	30	resin	1	175	**100**	**98**	**59.4**
14	**2**	50	resin	1	175	63	98	62.4
15	**2**	50	resin	1	250	16	–	–
16	**7**	15	resin	3	125	–	–	–
17	**8**	15	resin	3	125	–	–	–
18	**9**	15	resin	3	125	–	–	–

[a] By GC-MS. [b] By chiral GC. [c] Defined as the product of substrate loading, conversion, % eutomer (calculated from the ee) divided by the biotransformation time.

Chart 1. Non-Aldehyde-Based Substrates



a quantitative conversion was achieved (entry 8 vs 9), thus allowing us to further increase the loading from 15.0 to 30.0 g L^{-1} provided that OYE3 concentration is increased (entry 10 vs 11). The same trend was confirmed by the decrease of $X_{r/s}$ from 3 down to 1 (entries 11–13), achieving in the best case a productivity of 59.4 g L^{-1} d^{-1}.

However, such an approach cannot be reiterated once more at a higher protein concentration (entry 14 vs 15) because too high substrate loadings (e.g., 50.0 g L^{-1}) require high amounts of cosubstrate (glucose). In these conditions, significant protein precipitation was observed, with a detrimental effect on the conversion (entry 15).

Another beneficial effect that we recently observed in coupling the SFPR methodology with isolated OYEs in reducing α-substituted unsaturated aldehydes is that the ee improves with respect to the corresponding homogeneous biotransformations. Indeed, also in this case the ee increases from 90% to an excellent 99% (entry 1 vs 8) reaching the same value obtained with yeast. Thus, we concluded that racemization of **2** occurs during the biotransformation; however, in the presence of resin the product is readily removed, and such a phenomenon is likely kinetically suppressed.[7]

In support of this hypothesis, the bioreduction of **2** with fermenting B.Y. but without the SFPR methodology gave after 5 days the alcohol **3** with a modest ee of 50%,[7] indicating indeed that such a racemization occurs, and it is slightly faster than the carbonyl reduction catalyzed by ADH. Moreover, a sample of (*S*)-**5**, incubated at the same experimental conditions used in the biotransformation, but without any enzyme, showed an appreciable loss of optical purity, indicating that **5** epimerizes spontaneously.

Another significant improvement of this process could have resulted by cutting down the number of synthetic steps. Since in the literature several examples in which OYEs catalyzed the reduction of α,*β*-unsaturated methyl esters or acids have been reported,[19a−c,22] we tested the acid **7** (precursor of **2**, Scheme 1) and its methyl ester **8** but without success (entries 16 and 17). Even the activation of C═C double bonds provided by a stronger electron-withdrawing group such as thiomethylester, i.e., **9**, resulted equally ineffective (entry 18).

■ DEVELOPMENT OF THE OXIDATIVE STEP AND G-SCALE IMPLEMENTATION OF THE TWO-STEP PROCESS

The formation of aldehyde **5** instead of alcohol **3** is clearly more profitable because it enables the application of a cheaper and more environmentally compatible oxidative step with respect to that used in the original synthesis (TEMPO/BAIB system).[4] Thus, the saturated aldehyde **5** was quantitatively oxidized to the corresponding acid **6** by treatment with a solution of $NaClO_2$, phosphate buffer, and a chlorine scavenger (2-methyl-2-butene) in *t*-BuOH/water (1:1), without any loss of optical purity.[23]

Furthermore, we found it extremely convenient to oxidize directly the aldehyde still adsorbed on the resin after previous filtration from the enzyme solution and water washing of residual cofactor and proteins. Indeed, after the usual acid−base workup we isolated the acid in a 94% yield over the steps, with 98% ee.

Finally, we tested the bioconversion of **2** using the best setup (OYE3, GDH-BM, $NADP^+$, $X_{r/s}$ = 1, substrate loading 30 g L^{-1}, pH 7.0, 30 °C) coupled with the oxidative step on a preparative scale (1.0 g) affording **6** in almost quantitative yield (94%) and a similar ee (98%) with an astonishing productivity of 55.6 g L^{-1} d^{-1} over the two steps.

■ CONCLUSIONS

To the knowledge of the authors, this is the first example of OYE3 catalyzed bioreduction performed on g-scale and used for the synthesis of APIs such as Tesaglitazar. The use of overexpressed OYE3 combined with the in situ SFPR has allowed the following improvements with respect to the original synthesis: (i) the productivity increased 2 orders of magnitude from 0.39 to 59.4 g L^{-1} d^{-1}; (ii) the conversion is quantitative, simplifying the purification procedure; (iii) the ready recovery of products by absorption on resin minimizes the epimerization process preserving the high optical purity of **5**; and (iv) the oxidative step is carried out in cheaper and more environmentally compatible conditions, making it much more feasible from the perspective of an industrial scale-up.

Finally, someone might infer that even if the isolated enzymes based process proved to be superior in terms of productivity and efficiency with respect to its B.Y. mediated counterpart the latter might be cheaper, because it does not require any effort for the production of the noncommercially available enzymes. However, the comparison between the quantities of biocatalyst (1:10^5, enzymes vs B.Y.) and water (1:30) employed to process an equal amount of substrate shows clearly that the whole cells based route is highly unpractical on an industrial scale and therefore economically unconvenient, even if B.Y. is extremely inexpensive. Indeed, all the efforts (technological and economical) required for the production of the isolated enzymes are largely counterbalanced by the higher level of practicality and simplicity introduced in the process.

■ EXPERIMENTAL SECTION

Materials and Strains. Chemicals, solvents, and commercial enzymes were obtained from suppliers and used without further purification. Compounds **2**, **3**, **4**, **6**, and **7** were prepared as described in ref 4. A reference sample of racemic **5** was prepared by Dess−Martin periodinane oxidation of **4**.[24] Strains were obtained from the following sources: *S. cerevisiae* BY4741 from EUROSCARF collection (Heidelberg, Germany), *B. megaterium* DSM509 from DSMZ (German Collection of Microorganisms and Cell Cultures), *E. coli* MDS42 from Scarab Genomics, and *E. coli* BL21 (DE3) and *E. coli* DH5α from New England Biolabs.

General Methods. Yeast strains were grown in standard YPD medium (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of glucose) at 30 °C and *E. coli* strains in standard LB medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl) at 37 °C, with constant shaking at 220 rpm in both cases.

Analytical Methods. Protein concentration was determined with the Bio-Rad Protein Assay reagent according to Bradford,[25] using bovine serum albumine (BSA) as a standard. ^1H and ^{13}C NMR spectra were recorded on a Bruker ARX 400 spectrometer (400 MHz ^1H, 100.6 MHz ^{13}C) in $CDCl_3$ solution at rt, using TMS as an internal standard for ^1H and $CDCl_3$ for ^{13}C; chemical shifts δ are expressed in ppm relative to TMS; *J* values are given in Hz. GC-MS analyses were performed on an Agilent HP 6890 gas-cromatograph equipped with a 5973 mass detector and an Agilent HP-5 (30 m × 0.25 mm × 0.25 μm) column. Method: 60 °C (1 min)/6 °C/min/150 °C (1 min)/12 °C/min/280 °C (5 min). Chiral GC analyses were performed on a DANI HT 86.10 gas chromatograph equipped with a Varian Chirasil-Dex CB (25 m × 0.25 mm) column. Method: 75 °C (1 min)/3 °C/min/119 °C (17 min)/30 °C/min/180 °C (5 min). Chiral HPLC analyses were performed on a Merck−Hitachi L-4250 chromatograph equipped with a Chiralcel OD column and UV detector (210 nm); mobile phase, *n*-hexane/*i*-PrOH 99:1; flow rate, 0.6 mL/min. Optical rotations were determined on a Dr. Kernchen Propol digital automatic polarimeter. TLC analyses were performed on Merck Kieselgel 60 F_{254} plates.

Preliminary Whole Cell Experiments. Yeast cells (200 mg CWW) were suspended in water (2.0 mL) containing glucose (40 mg) and incubated at 30 °C with mechanical stirring. After 1 h the substrate solution (5.0 mL, 1.6 mM) was added, and the reaction was monitored every few hours by withdrawal of a 500 μL sample, extraction with EtOAc (350 μL), and GC analysis of the organic phase dried over Na_2SO_4.

Overexpression of the Enzymes in *E. coli* BL21 (DE3). A 5 mL culture in LB medium containing the appropriate antibiotic (50 μg/mL of kanamycin for pET-30a, 100 μg/mL of ampicillin for pKTS) was inoculated with a single colony from a fresh plate and grown overnight at 37 °C and 220 rpm. This starter culture was used to inoculate a 200 mL culture, which was in turn grown overnight at the same conditions and used to inoculate a 1.5 L culture. The latter was vigorously aerated at 37 °C and 220 rpm until OD_{600} reached 0.4−0.5, and then enzyme expression was induced by the addition of 0.1 mM IPTG (50 ng/mL of anhydrotetracycline was also added in the case of the pKTS plasmid). After 5−6 h the cells were harvested by centrifugation (5000*g*, 20 min, 4 °C), resuspended in 50 mL of lysis buffer (20 mM phosphate buffer pH 7.0, 300 mM NaCl, 10 mM imidazole), and homogenized (Haskel high-pressure homogenizer). The CFE, after centrifugation (20 000*g*, 20 min, 4 °C), was chromatographed on an IMAC stationary phase (Ni-Sepharose Fast Flow, GE Healthcare) with a mobile phase composed of 20 mM phosphate buffer pH 7.0, 300 mM NaCl, and a 10−300 mM imidazole gradient. Protein elution was monitored at 280 nm, and the fractions were collected according to the chromatogram and dialyzed twice against 1.0 L of 20 mM phosphate buffer pH 7.0 (12 h each, 4 °C) to remove imidazole and salts. Purified protein aliquots were stored frozen at −80 °C.

Screening-Scale Biotransformation of 2 into 5. The substrate **2** (according to the desired substrate loading, see Table 3) was dissolved in the required cosolvent or adsorbed on XAD 1180 resin (by adding the resin to a solution of the substrate in Et_2O and removing the solvent under reduced pressure). Either dissolved or adsorbed, the substrate was then added to a solution of glucose (4 equiv with respect to **2**), $NAD(P)^+$ (0.1 mM), GDH (4 U), and OYE (25−250 μg/mL) in phosphate buffer (1.0 mL, 50 mM, pH 7.0). The mixture was stirred for 12 h in an orbital shaker (160 rpm, 30 °C). The resins were filtered on a sieve, and both the resins and the aqueous phase were extracted with EtOAc, centrifuging after every extraction (15 000*g*, 1.5 min). The combined organic solutions were dried on Na_2SO_4 and concentrated under reduced pressure, yielding **5** (or a mixture of **5** and **2**) as a yellowish oil.

Data of (*S*)-2-Ethoxy-3-(4-methoxyphenyl)propanal (*S*)-5. Prepared according to Table 3, entry 13. Colorless oil: 99% purity by GC (t_R 19.31 min); 98% ee by chiral GC

(t_R 30.6 min (*S*), 31.1 min (*R*)); $[\alpha]_D^{20}$ = −68.7 (*c* 1.1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 9.68 (d, *J* = 2.3, 1H), 7.16 (d, *J* = 8.7, 2H), 6.84 (d, *J* = 8.7, 2H), 3.81−3.83 (m, 1H), 3.80 (s, 3H), 3.61 (m_{AB}, 1H), 3.46 (m_{AB}, 1H), 2.95 (m_{AB}, 1H), 2.86 (m_{AB}, 1H), 1.20 (t, *J* = 7.0, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 203.5, 158.5, 130.4, 128.6, 113.9, 85.1, 66.5, 55.2, 35.8, 15.2. MS: *m*/*z* (%) 208 $[M]^+$ (8), 179 (6), 151 (11), 121 (100), 91 (16).

Oxidation of Aldehyde 5 to Acid 6. The saturated aldehyde **5** (100 mg, 0.48 mmol) and 2-methyl-2-butene (280 μL, 2.64 mmol) were dissolved in *t*-BuOH (3 mL) and cooled to 0 °C in an ice bath under magnetic stirring. Then, a solution of $NaClO_2$ (217 mg, 2.40 mmol) and $NaH_2PO_4 \cdot 2H_2O$ (112 mg, 0.72 mmol) in water (3 mL) was added dropwise. After completion of the oxidation (checked by TLC), the reaction mixture was quenched with oxalic acid dihydrate (189 mg, 1.5 mmol), poured into brine (10 mL), and extracted with EtOAc (3 × 10 mL). The combined organic phases were dried over Na_2SO_4 and concentrated under reduced pressure to give **6** as a yellowish solid: 104 mg, 97% yield, 95% purity by GC (of the methyl ester derivative obtained by treatment with CH_2N_2 in Et_2O, t_R 20.72 min). Physical data are consistent with those described in ref 4.

Preparative-Scale Biotransformation and Oxidative Step (Aldehyde 2 to Acid 6). To a solution of substrate **2** (1.0 g, 4.85 mmol) in Et_2O (15 mL) was added XAD 1180 resin (1.0 g, $X_{r/s}$ = 1), and the solvent was removed under reduced pressure. The adsorbed substrate was added to a solution of glucose (3.5 g, 19.4 mmol), $NADP^+$ (2.6 mg, 3.3 μmol), GDH (10 U), and OYE3 (overall 175 μg/mL) in phosphate buffer (33 mL, 50 mM, pH 7.0). The mixture was stirred for 12 h in an orbital shaker (160 rpm, 30 °C). The resins were filtered on a sieve, washed with phosphate buffer (2 × 25 mL, 50 mM, pH 7.0), and added to a solution of 2-methyl-2-butene (2.8 mL, 26.4 mmol) and $NaH_2PO_4 \cdot 2H_2O$ (1.12 g, 7.2 mmol) in *t*-BuOH/H_2O (50 mL, 1:1). After 5 h the reaction mixture was quenched as described above, and the resins and the aqueous phase were extracted with EtOAc (4 × 20 mL for the resins, 1 × 20 mL for the aq phase). The combined organic solutions were washed with aq NaOH (2 × 30 mL, 1 M), and the aq phase was acidified with HCl (1 M) and extracted with EtOAc (2 × 30 mL). The organic solution was dried over Na_2SO_4 and concentrated under reduced pressure, yielding the saturated acid **6** as a yellowish solid: 1.02 g, 94% yield, 95% purity by GC (of the methyl ester derivative t_R 20.72 min), 98% ee by chiral HPLC (of the methyl ester derivative t_R 16.2 min (*R*), 17.1 min (*S*)).

ASSOCIATED CONTENT

Ⓢ Supporting Information. Detailed enzyme identification procedure, MS/MS analysis report, preparation of the overexpressing strains, plasmid maps, amino acid sequence of the GDH, SDS−PAGE analysis of the purified proteins, synthesis of substrates **8** and **9**, and NMR spectra of **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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