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One-pot, single-step deracemization of 2-hydroxyacids by tandem biocatalytic oxidation and reduction†

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A facile and efficient one-pot, single-step method for deracemizing a broad range of 2-hydroxyacids to (*R*)-2-hydroxyacids was established by combination of resting cells of an (*S*)-hydroxyacid dehydrogenase-producing microorganism and an (*R*)-ketoacid reductase-producing microorganism.

The resolution of racemates is the most current approach to obtain enantiomerically pure compounds.[1,2] Enantiopure 2-hydroxyacids (2-HAs) are particularly important building blocks for the preparation of pharmaceuticals and fine chemicals. Their classical kinetic resolution by enzymatic or non-enzymatic catalysts is abundantly documented.[3] However, the yield of a kinetic resolution is limited to only 50%. As a consequence, alternative methods able to directly convert a racemate mixture to a single enantiomer (deracemization) are highly advantageous.[4]

Tandem oxidation and reduction reactions in a one-pot, single-step procedure operate the two processes concurrently in one pot, circumventing the often time-consuming and yield-reducing isolation and purification of intermediates in the multiple synthesis system. This approach is one of the most attractive deracemization methods which allow complete transformation of a racemate into a single stereoisomeric product.[2] One enantiomer is usually oxidized to the ketone while the other enantiomer remains unchanged. The ketone is then reduced to the opposite enantiomer subsequently. The net result is the conversion of the racemate to a single enantiomer in potentially 100% yield with 100% ee (enantiomeric excess).[5] This promising technology has been successfully applied to the deracemization of secondary alcohols[6] and amines.[7] The combination of an oxidation and a reduction reaction in a cascade allows performing deracemization in an economic and efficient fashion.[8]

Unfortunately, it is still a challenge for chemists to run oxidation and reduction reactions simultaneously in organic synthesis due to the diverging reaction conditions required for each transformation.[9,10] Moreover, it is very difficult to meet the requirements of such double selectivities. To date, no general comparable process has been reported for the deracemization of a broad range of racemic 2-HAs in a one-pot, single-step method. Tsuchiya *et al.*[10] reported the deracemization of mandelic acid by biocatalytic oxidation–reduction. The asymmetric oxidation of (*S*)-mandelic acid by *Alcaligenes bronchisepticus* required aerobic conditions, while *Streptococcus faecalis* employed for the reduction of the intermediate benzoylformate was inactivated under these conditions.

Recently, we established high-throughput screening methods to isolate a stereoselective 2-HA dehydrogenase-producing strain for asymmetric oxidation of 2-HAs.[11] *Pseudomonas aeruginosa* CCTCC M 2011394 harboring FMN-dependent (*S*)-2-HA dehydrogenase was isolated. Asymmetric oxidation of a wide range of racemic 2-HAs by *P. aeruginosa* CCTCC M 2011394 produced the corresponding 2-ketoacids (2-KA) and (*R*)-2-HAs with excellent ee.[12] Therefore, we envisaged that we might be able to couple our asymmetric oxidation of racemic 2-HAs with the opposite stereoselective reduction of 2-KAs for the efficient oxidoreductive deracemization to (*R*)-2-HAs (Scheme S1, ESI†).

In the initial experiments we attempted to screen microbes featuring a broad substrate spectrum with satisfactory 2-KA reductase (2-KAR) activity and (*R*)-stereoselectivity. A total of 124 strains of microbes were isolated, of which 16 strains reduced 2-KAs to the corresponding (*R*)-2-HAs. According to the overall performance including conversion, ee of the product, reaction stability and substrate specificity, the best strain, designated ZJB5074, was chosen for further studies. According to the physiological and biochemical characterization as well as the comparison of the 18S rDNA gene sequence, strain ZJB5074 was identified as *Saccharomyces cerevisiae* (Table S1 and Fig. S1, ESI†). The whole cells of *S. cerevisiae* ZJB5074 exhibit high 2-KAR activity at pH 7.5–8.0 and at 30–35 °C using benzoylformic acid as the substrate. Interestingly, the ee (%) values of the product were all kept above 99% and showed little variation in the temperature range from 25 to 50 °C and pH range from 6.0 to 9.0 (Fig. S2, ESI†), which is in contrast to the previous observation that enantioselectivity decreased at higher reaction temperatures and pH during the bioreduction by *S. ellipsoideus* GIM2.105.[13] The bioreduction of

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† Electronic supplementary information (ESI) available: Scheme S1, Fig. S1–S3, Table S1, and detailed experimental procedure. See DOI: 10.1039/c3cc46240d

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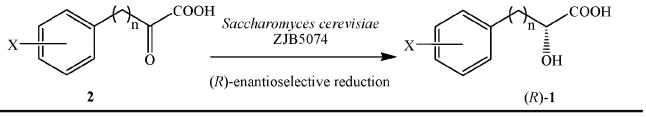
2-ketoacids mediated by resting cells of *S. cerevisiae* ZJB5074 proceeded smoothly because of the existence of a regeneration system within the biocatalyst, which avoids the extra addition of expensive cofactors. However, co-substrates are needed for cofactor recycling.[14] The addition of co-substrates such as glycerol, fructose, lactose and glucose to the reaction media remarkably enhanced the conversion efficiency (Fig. S2, ESI†). Glycerol was the most suitable co-substrate for *S. cerevisiae* ZJB5074. When 10 g L^{-1} of glycerol was added to the reaction system, the yield of (*R*)-**1a** formed from its racemate reached above 95% after 28 h of reduction, which was a 140% increase compared to that obtained without addition of a co-substrate. The positive effect on this resting-cell catalyzed reaction was mainly achieved through the cellular metabolism of glycerol to promote the regeneration of coenzymes.[15] The cofactor requirements in this reduction reaction were also investigated. The activities of the permeabilized *S. cerevisiae* ZJB5074 with NADPH as a cofactor were much higher than those with NADH or without addition of a cofactor, suggesting that an NADPH-dependent reductase catalyzing asymmetric reduction was contained in the whole cells of *S. cerevisiae* ZJB5074 (Fig. S3, ESI†). The operational stability of *S. cerevisiae* ZJB5074 was evaluated by successive batch bioreduction of **2a** 20 times. Compared with the first batch, no obvious decrease in ee and activity was observed, indicating that *S. cerevisiae* ZJB5074 was a reusable biocatalyst with excellent operational stability. To explore the catalytic capacity of *S. cerevisiae* ZJB5074, the bioreduction of various 2-ketoacids was performed. *S. cerevisiae* ZJB5074 exhibited a broad substrate spectrum and efficiently catalyzed the bioreduction of all the 2-ketoacids tested with excellent enantioselectivity (ee > 99%). The nature of the substituent, position of the substituent, number of substituents on the benzene ring, and the length of the carbon chain between the hydroxy group and the benzene ring had some effect on the activity but little effect on the enantioselectivity. Among all the 2-HAs tested, substrates **2b** (Table 1, entry 2) and **2e–2o** (Table 1, entries 5–15) were efficiently reduced by *S. cerevisiae* ZJB5074 with higher activity. *S. cerevisiae* ZJB5074 exhibited a relatively low activity on substrates **2c** and **2d** (Table 1, entries 3 and 4) and substrates with a longer carbon chain between the hydroxy group and the benzene ring (Table 1, entries 16 and 17), the relative activities on **2p** and **2q** were only 75% and 59% of that on **2a**, respectively.

The unique characteristics of *S. cerevisiae* ZJB5074 provide a solid basis for combining with *P. aeruginosa* CCTCC M 2011394 to deracemize 2-HAs *via* a one-pot process. The biocatalytic deracemization of **1b** (20 mM) was performed by an oxidation–reduction one-pot, two-step strategy and a one-pot, single-step strategy. Fig. 1A shows a typical time course for production of (*R*)-**1b** from racemic **1b** by *P. aeruginosa* CCTCC M 2011394 and *S. cerevisiae* ZJB5074. After 6 h of oxidation, (*S*)-**1b** was almost completely converted to its corresponding ketoacid by *P. aeruginosa* CCTCC M 2011394 in the first step. The (*R*)-isomer of **1b** was not oxidized during the entire period examined. After removal of resting cells of *P. aeruginosa* CCTCC M 2011394 by centrifugation, the second step reaction was started by adding resting cells of *S. cerevisiae* ZJB5074 and glycerol. **2b**, the oxidized by-product of (*S*)-**1b**, was gradually reduced to (*R*)-**1b**. After 28 h of reduction, the yield of (*R*)-**1b** formed from its racemate was 92.28%. To develop a more simplified and practical process, deracemization of racemic **1b** to (*R*)-**1b** was conducted using two types of resting cells simultaneously (one-pot, single-step). The reaction yield of (*R*)-**1b** reached as high as 98.67% in a short period of reaction time (24 h) (Fig. 1B). Oxidation was the faster step of the overall reaction because **2b** was detected. The results indicated that the oxidation and reduction reactions can be run simultaneously in one pot in parallel without compartmental separation. The two microorganisms show high compatibility with each other and are suitable for single-step deracemization *via* an oxidation–reduction concurrent reaction in the same reactor. Compared with the two-step reaction, the single-step reaction for deracemization has a simpler procedure, shorter reaction time and higher productivity. Although oxidoreductase needs a cofactor to take part in the reaction, each of the resting-cell biocatalysts has its own *in vivo* cofactor regeneration system. (*S*)-2-HA dehydrogenase in *P. aeruginosa* ZJB1125 is a FMN-dependent flavoprotein that oxidizes (*S*)-2-HAs to 2-KAs, resulting in the reduction of FMN.[12] Flavoproteins are known to modulate the reoxidation of reduced flavin by molecular oxygen to a great extent.[16] In the case of *S. cerevisiae* ZJB5074, NADPH was regenerated by addition of glycerol. So, there is no need to add the expensive cofactor and/or the oxidoreductase for cofactor regeneration in the tandem oxidation and reduction reactions.

Table 1 Substrate specificity of *S. cerevisiae* ZJB5074[a]



2 (*R*)-**1**

Entry	Substrate	X	*n*	Relative activity[b]	ee[c] (%)
1	**2a**	H	0	1.00	99.9
2	**2b**	2-Cl	0	1.53	99.9
3	**2c**	3-Cl	0	0.56	99.9
4	**2d**	4-Cl	0	0.71	99.9
5	**2e**	2-F	0	1.46	99.9
6	**2f**	4-F	0	1.23	99.9
7	**2g**	2,4-F	0	1.42	99.9
8	**2h**	3,5-F	0	1.57	99.9
9	**2i**	4-Br	0	1.57	99.9
10	**2j**	3-OH	0	1.13	99.8
11	**2k**	4-OH	0	1.27	99.9
12	**2l**	4-CH_3	0	1.06	99.9
13	**2m**	4-OCH_3	0	1.21	99.9
14	**2n**	3-OCH_3-4-OH	0	1.25	99.9
15	**2o**	4-CF_3	0	1.40	99.4
16	**2p**	H	1	0.75	99.5
17	**2q**	H	2	0.59	99.3

[a] Reaction conditions: 5 gdcw per L *S. cerevisiae* ZJB5074, 10 mM **2a–2q**, 10 g L^{-1} glycerol, pH 7.5 (potassium phosphate, 100 mM), 30 °C, 180 rpm. [b] Determined by chiral RP-HPLC. [c] The ee value of (*R*)-**1**.

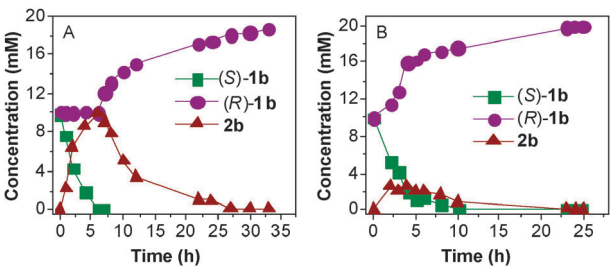


Fig. 1 Deracemization of racemic **1b** in one-pot biocatalysis by combination of resting cells of *P. aeruginosa* ZJB1125 and *S. cerevisiae* ZJB5074. (A) A one-pot, two-step procedure; (B) a one-pot, single-step procedure.

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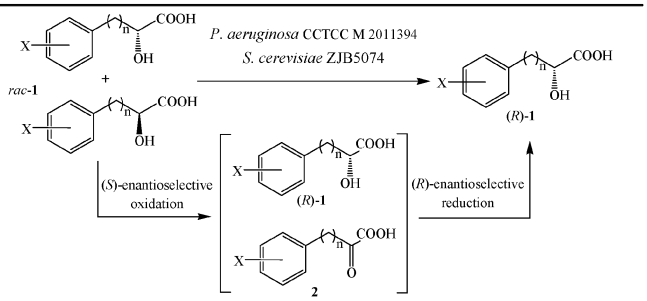
Table 2 Biocatalytic deracemization of various racemic 2-HAs in one-pot concurrent catalysis[a]



Entry	Substrate	X	*n*	Time (h)	(*R*)-**1**[b] (%)	ee[c] (%)
1	*rac*-**1a**	H	0	25	92.67	99.9
2	*rac*-**1b**	2-Cl	0	24	98.67	99.5
3	*rac*-**1c**	3-Cl	0	33	90.94	99.9
4	*rac*-**1d**	4-Cl	0	33	92.65	99.9
5	*rac*-**1e**	2-F	0	25	92.46	99.9
6	*rac*-**1f**	4-F	0	24	92.15	99.9
7	*rac*-**1g**	2,4-F	0	24	98.53	99.9
8	*rac*-**1h**	3,5-F	0	24	93.43	99.9
9	*rac*-**1i**	4-Br	0	33	85.29	99.9
10	*rac*-**1j**	3-OH	0	21	62.24	99.9
11	*rac*-**1k**	4-OH	0	24	65.59	99.9
12	*rac*-**1l**	4-CH_3	0	27	75.09	99.9
13	*rac*-**1m**	4-OCH_3	0	30	67.65	99.9
14	*rac*-**1n**	3-OCH_3-4-OH	0	21	55.91	98.7
15	*rac*-**1o**	4-CF_3	0	27	79.64	99.9
16	*rac*-**1p**	H	1	24	60.95	99.5
17	*rac*-**1q**	H	2	33	66.69	99.3

[a] Reaction conditions: 10 gdcw per L *P. aeruginosa* CCTCC M 2011394, 5 gdcw per L *S. cerevisiae* ZJB5074, 20 mM racemic **1a–1q**, 10 g L^{-1} glycerol, pH 7.5 (potassium phosphate, 100 mM), 30 °C, 180 rpm. [b] Conversion of (*R*)-**1**, determined by chiral RP-HPLC. [c] The ee value of (*R*)-**1**.

As the process of concurrent catalysis was quite efficient, biocatalytic deracemization of a range of 2-HAs was performed in a one-pot, single-step procedure. The results are given in Table 2. For deracemization of **1a–1i** (Table 2, entries 1–9), the enantiomeric (*R*)-2-HAs were obtained with high stereo-selectivities (>99% ee) and high yields (85.29–98.67%) within 24–33 h. In the case of **1j–1q** (Table 2, entries 10–17), (*R*)-2-HAs were obtained with high enantioselectivities (>99% ee) and moderate yields (55.91–79.64%) within 21–33 h. Overall, a variety of enantiopure (*R*)-2-HAs were successfully prepared in a one-pot and single-step procedure with good yield and excellent ee. Deracemization by stereoinversion in one-pot concurrent catalysis thus overcomes the limitation of the maximum theoretical yield of 50% encountered during kinetic resolution of racemic 2-HAs. This method represents an easy, cheap and environmentally benign way for the biocatalytic synthesis of chiral 2-HAs from their racemates.

To further evaluate the feasibility of the bioprocess for production of (*R*)-HAs for practical application, a 1.0 L scale one-pot concurrent reaction system for the deracemization of **1b** under the above-mentioned conditions was performed in a 2.0 L stirred-tank reactor. (*R*)-**1b** is one of the most preferred chiral building block for the industrial synthesis of the anti-thrombotic agent, (*S*)-clopidogrel, which has been commercialized with a brand name, Plavix.[17] The process of deracemization was monitored by RP-HPLC, and it was stopped at 24 h. The conversion and ee of (*R*)-**1b** were 94.7% and 99.5%, respectively. The resting cells in the reaction mixture were removed by centrifugation. The pH of the supernatant was adjusted to 1.5 with 6.0 M HCl and the desired product was extracted with 1.0 L of ethylacetate. The extract obtained was concentrated under reduced pressure and yielded 3.18 g of (*R*)-**1b** (99.5% ee, 90% isolated yield). The results confirmed that deracemization of racemic 2-HAs in a one-pot, single-step oxidation–reduction process by combination of resting cells of *P. aeruginosa* CCTCC M 2011394 and *S. cerevisiae* ZJB5074 for production of (*R*)-2-HAs was feasible in practical applications.

Deracemization of racemic 2-HAs to (*R*)-2-HAs was successfully realized *via* concurrent asymmetric oxidation and stereoselective reduction employing two resting-cell biocatalysts. One biocatalyst was *P. aeruginosa* CCTCC M 2011394 which catalyzed the stereoselective oxidation of (*S*)-2-HAs to corresponding 2-ketoacids and another was *S. cerevisiae* ZJB5074 which reduced the 2-ketoacids to (*R*)-2-HAs. This proposed one-pot, single-step deracemization method leads to the development of a new tool for the preparation of (*R*)-2-HAs.

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