Supporting information

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

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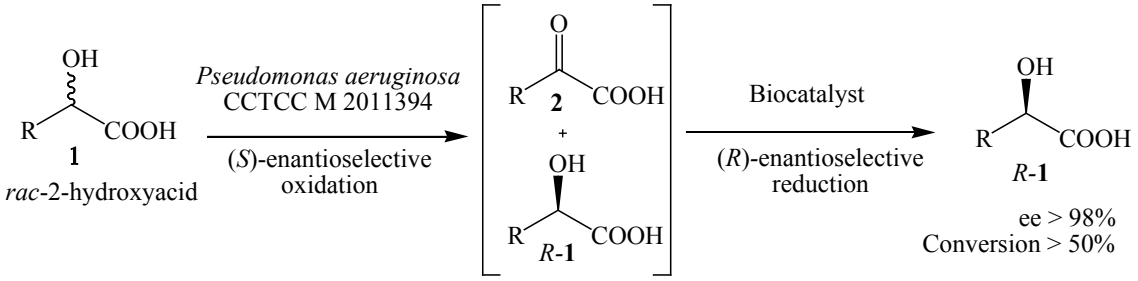

Scheme S1 Deracemization of racemic 2-hydroxyacids through (*S*)-stereoselective oxidation and (*R*)-stereoselective reduction.

1. Chemicals:

1a-1q, (*R*)-**1a-1q**, and (*S*)-**1a-1q** were purchased from J&K Chemical Co., Ltd. (Shanghai, China). All other chemicals were purchased from local suppliers and were of analytical grade.

2. Analytical methods

Direct RP-HPLC was developed for the separation of enantiomers of **1**. The concentrations of the (*R*)-**1**, (*S*)-**1** and **2** were assayed by RP-HPLC (Dionex UltiMate 3000, USA) equipped with a chiral column (Chirobiotic ^{TM} R 250×4.6 mm, particle size 5.0 μm, Sigma, USA) at a flow rate of 1.0 mL/min with a solvent system composed with 0.5% AcOH-CH_3CN (20:80, v/v). The eluate was monitored at 215 nm. Excellent resolutions were achieved for all the investigated compounds in the RP-HPLC analysis. ee=([*R*-**1**]-[*S*-**1**])/([*R*-**1**]+[*S*-**1**])×100%, where [*R*-**1**] and [*S*-**1**] are the concentrations of (*R*)-**1** and **(***S*)-**1**, respectively.

3. Screening of 2-ketoacid reductase-producing microorganisms and identification

One target of the screening procedure was to find a microbe with satisfactory

(*R*)-stereoselective 2-ketoacid reductase activity. Soil samples were collected from chemical plants, farmlands, gardens, and dust heaps. Approximately 0.5 g of each sample was enriched in 50 mL of bean sprouts medium which was prepared as described by Shen et al [1]. The samples were incubated at 30 °C and 150 rpm for 2 days, and an aliquot of the suspension was plated on solid medium (SM) containing: 20 g/L glucose, 15 g/L yeast extract, 2.5 g/L $K_2HPO_4 \cdot 3H_2O$, 2.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7\ H_2O$, 0.03 g/L $FeSO_4 \cdot 7H_2O$, 1.0 g/L NaCl and 20 g/L agar. The plates were incubated at 30 °C until multiple colonies emerged. Representative colonies were selected and incubated in liquid medium (LM), which has the same compositions as SM except agar, at 30 °C and 150 rpm. After 24 h cultivation, **2a** (1.0%, w/v) was added to the culture medium. The reaction mixtures were incubated for another 24 h, centrifuged at 9,000 rpm for 15 min, and the supernatant was analyzed for **2a** and **1a**. Those suspensions which showed obvious activity were inoculated onto plates of SM. Then different strains were isolated and their 2-ketoacid reductase activities and enantioselectivities were assayed. A total of 124 strains of microbes were isolated, of which 16 strains reduced 2-ketoacids to the corresponding (*R*)-2-hydroxyacids. According to the overall performances including conversion, ee of the product and reaction stability, the best strain, marked as ZJB5074, was chosen for further study. Colonies of ZJB5074 are white to cream colored, smooth, glabrous and yeast-like in appearance. Cell morphology of ZJB5074 was examined by an optical microscopy (Leica DM 4000 B, Germany) and a scanning electron microscope (Hitachi H-7650, Japan). Cells are round to ovoid, 5-10 micrometres in diameter (Fig. S1A). The carbon source utilization was examined by a GEN III MicroStation System (Biolog Inc., USA). The results show that strain ZJB5074 can efficiently utilize 10 carbon sources

(2-keto-D-gluconic acid, D-gluconic acid, gentiobiose, maltose, maltotriose, D-melibiose, D-raffinose, stachyose and sorbitol) among 65 carbon sources tested (Table S1). According to these data mentioned above, the strain was preliminarily confirmed as *Saccharomyces* genus. The 18S rDNA sequence of ZJB5074 was also determined according to the method as described previously [2]. The sequences obtained were compiled and compared with sequences in the GenBank databases using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Sequence analysis was performed using SEQBOOT, NEIGHBOR-JION and DNASENSE of Phylips (version 3.572) software and FITCH, DRAWGRAM and the alignment match was then used to construct the neighbour-joining phylogenetic tree. Evaluation of the MicroSeq database system and comparison with other frequently used systems are from DDBJ/EMBL/GenBank. The analysis of the 18S rDNA gene sequence (GenBank accession No. **KF225479**) revealed that this strain was found to be similar to *S.cerevisiae* (homology, 99%, based on 18S rDNA). Through the alignment and cladistic analysis of homologous nucleotide sequences of known fungi, phylogenetic relationships were inferred and the approximate phylogenetic position of the strain was obtained (Fig.S1B). According to the physiological and biochemical characterization as well as the comparison of 18S rDNA gene sequence, the strain ZJB5074 was identified as *S.cerevisiae*, and named *S.cerevisiae* ZJB5704.

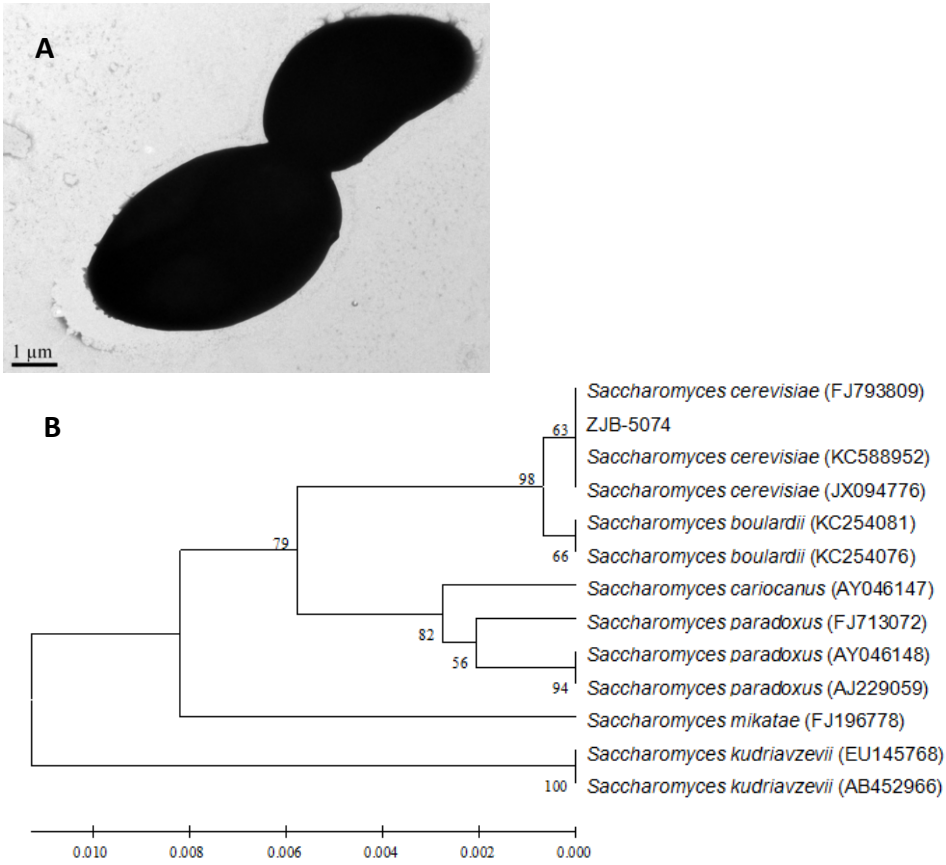

Fig. S1 Morphological characteristics and phylogenetic analysis of strain ZJB5074. (A) Electron micrographs of strain ZJB5074 cells. Strain ZJB5074 cell morphology was observed by a scanning electron microscope (Hitachi H-7650, Japan). (B) The phylogenetic dendrogram for *S.cerevisiae* ZJB5074 and related strains based on the 18S rDNA sequence. Numbers in parentheses are accession numbers of published sequences. Bootstrap values were based on 1000 iterations.

Table S1 Utilization of carbon-substrates by strain 5074 cells using a Biolog YT microplate in a standardized Biolog microstation.

No.	Carbon substrate	ZJB5074	No.	Carbon substrate	ZJB5074
0	Water	0	33	D-Galactose	−
1	Acetic Acid	−	34	D-Psicose	−
2	Formic Acid	−	35	L-Rhamnose	−
3	Propionic Acid	B	36	L-Sorbose	−
4	Succinic Acid	−	37	α-Methyl-D-Glucoside	−
5	L-Aspartic Acid	−	38	ß-Methyl-D-Glucoside	−
6	D-Proline	−	39	Amygdalin	−
7	Fumaric Acid	−	40	Arbutin	−
8	L-Malic Acid	−	41	Salicin	−
9	Succinic Acid Mono-Methyl Ester	−	42	Maltitol	−
10	Bromosuccinic Acid	B	43	D-Mannitol	−
11	L-Glutamic Acid	−	44	Sorbitol	+
12	γ-Aminobutyric Acid	−	45	Adonitol	−
13	α-Ketoglutaric Acid	−	46	D-Arabitol	B
14	2-Keto-D-Gluconic Acid	+	47	Xylitol	−
15	D-Gluconic Acid	+	48	I-Erythritol	−
16	Dextrin	−	49	Glycerol	−
17	Inulin	−	50	Tween 80	−
18	D-Cellobiose	−	51	L-Arabinose	−
19	Gentiobiose	+	52	D-Arabinose	−
20	Maltose	+	53	D-Ribose	−
21	Maltotriose	+	54	D-Xylose	−
22	D-Melezitose	B	55	Succinc Acid Methyl Ester plus D-Xylose	B
23	D-Melibiose	+	56	N-Acetyl-L-Glutamic Acid plus D-Xylose	−
24	Palatinose	−	57	Quinic Acid plus D-Xylose	−
25	D-Raffinose	+	58	D-Glucuronic Acid plus D-Xylose	−
26	Stachyose	+	59	Dextrin plus D-Xylose	B
27	Sucrose	−	60	α-D-Lactose plus D-Xylose	−
28	D-Trehalose	−	61	D-Melibiose plus D-Xylose	−
29	Turanose	−	62	D-Galactose plus D-Xylose	+
30	N-Acetyl-D-Glucosamine	−	63	m-Inositol plus D-Xylose	−
31	D-Glucosamine	−	64	1,2-Propanediol plus D-Xylose	−
32	α-D-Glucose	−	65	Acetoin plus D-Xylose	−

Notes: +, positive; −, negative; B, borderline.

4. Microorganisms and culture conductions

P. aeruginosa CCTCC M 2011394 was cultured in a medium containing 10 g/L glucose, 10 g/L yeast extract, 2.5 g/L $K_2HPO_4 \cdot 3H_2O$, 2.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7\ H_2O$, 0.03 g/L $FeSO_4 \cdot 7\ H_2O$, 1.0 g/L NaCl, 2.0 g/L 2-chloromandelic acid. After adjusting to pH 7.0 using 2.0 M NaOH solution, 50 mL of the medium was placed in a 250-mL flask, sterilized (121 °C, 20 min) and inoculated with the preincubated seed culture, shaken at 30 °C, 150 rpm for 48 h. *S.cerevisiae* ZJB5074 was cultured in a medium containing 20 g/L glucose, 15 g/L yeast extract, 2.5 g/L $K_2HPO_4 \cdot 3H_2O$, 2.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7\ H_2O$, 0.03 g/L $FeSO_4 \cdot 7H_2O$, 1.0 g/L NaCl. After adjusting to pH 7.0 using 2.0 M NaOH solution, 50 mL of the medium was placed in a 250-mL flask, sterilized (121 °C, 20 min) and inoculated with the preincubated culture, shaken at 30 °C, 150 rpm for 24 h. Cultivated *P. aeruginosa* CCTCC M 2011394 and *S.cerevisiae* ZJB5074 cells were harvested by centrifugation (12,000 × g, 4 °C, 10 min). The harvested cells were washed with 100 mM phosphate buffer (pH 7.5) and stored at 4 °C for further use.

5. Optimization of bioreduction conditions by *S. cerevisiae* ZJB5074

Application of the whole-cell biocatalysts for biotransformation brings about more advantages compared to using purified enzyme. During the whole-cell biocatalysis, the enzymes are often more stable due to the presence of their natural environment inside the cell and the cost is much lower because of no requirements for tedious separation of enzymes and the regeneration of expensive cofactors. Therefore, *S. cerevisiae* ZJB5074 cells were used to transformer 2-ketoacid to 2-hydroxyacid. The reaction conditions of the whole-cell bioreduction were optimized. The effect of pH on the bioreduction was

determined at 30 °C for 36 h. The reaction mixture (10 mL) consisted of 20 gdcw/L of *S. cerevisiae* ZJB5074 cells in 100 mM of buffer at various pH values (pH 6.0-8.0, phosphate buffer; pH 8.0-9.0, Tris-HCl buffer), 10 g/L of glucose and 30 mM of **2a**. The results are shown in Fig.S2A. The pH of the reaction mixture had a significant effect on enzyme activity. The maximum conversion was observed at pH 8.0. When the pH was below 7.0 or over 8.0, the enzyme activity decreased dramatically. The enantioselectivity was not affected by pH of the reaction system. In all experiments, the ee of the product remained above 99 %. The effect of temperature on the bioreduction was determined for 36 h at various temperatures (25-50 °C). The reaction mixture (10 mL) consisted of 20 gdcw/L of *S. cerevisiae* ZJB5074 cells, 10 g/L of glucose and 30 mM of **2a** in 100 mM phosphate buffer (7.5). The maximum activity was observed at 35 °C, and at higher temperatures the activity began to decrease significantly. The conversion at 50 °C was only 32 % of that at 35 °C. The ee (%) values of the product were all kept above 99 % and showed little variation in temperature range from 25 to 50 °C (Fig.S2B). Many saccharides and alcohols such as glycerol, fructose, isopropanol, lactose and glucose, can be used as sacrificial co-substrates to supply redox equivalents for biosynthetic processes [3]. The effect of co-substrate on the bioreduction was determined for 36 h at 35 °C. The reaction mixture (10 mL) consisted of 20 gdcw/L of *S. cerevisiae* ZJB5074 cells, 10 g/L of co-substrate and 30 mM of **2a** in 100 mM phosphate buffer (7.5). As shown in Fig.S2C, compared with the systems without co-substrate, the addition of co-substrates to the reaction media remarkably enhanced the conversion efficiency except for isopropanol, which exhibited no significant promotion to reductase activity. Glycerol was the most suitable co-substrate for *S. cerevisiae* ZJB5074 among all the tested compounds.

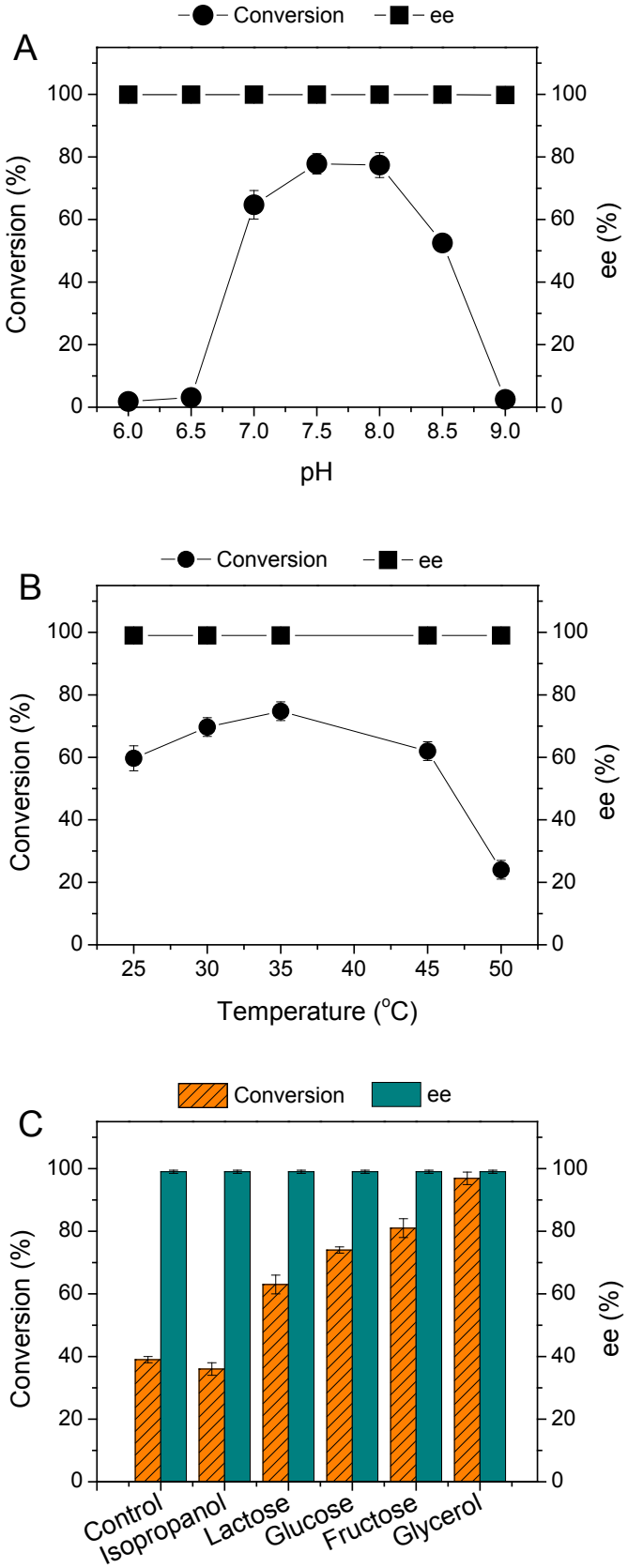

Fig.S2 Optimization of bioreduction conditions by *S. cerevisiae* ZJB5074. (A) Effect of

pH on the bioreduction; (B) Effect of temperature on the bioreduction; (C) Effect of co-substrate on the bioreduction. After 36 h biotransformation, the reaction mixtures were centrifuged and the concentrations of (*R*)-**1a** and **2a** in the supernatant were analyzed by RP-HPLC. Results are average of three parallel replicates.

6. Cofactor requirement

Microbial cells can be made permeable for the cofactor NAD(P)H and $NAD(P)^+$ by controlled treatment with an organic solvent and/or a detergent, while keeping the necessary enzymes inside cells [4]. Toluene was used to permeabilize the yeast cells. After 10 min treatment at room temperature in 2.5% (v/v) toluene:ethanol (1:4) solution, cells were collected by centrifugation at 10,000 rpm for 5 min. The cell pellet was washed three times with potassium phosphate buffer (100 mM, pH 7.5). The washed cells were then resuspended and 2-ketoacid reductase activity was analyzed by adding different cofactors. The reaction mixture (10 mL) consisted of 20 gdcw/L of permeabilized *S. cerevisiae* ZJB5074 cells, 10 g/L of co-substrate and 30 mM of **2a** in 100 mM phosphate buffer (7.5) without or with the addition of cofactor. The reactions were carried out at 30 °C, pH 7.5, and 180 rpm for 36 h. The results are shown in Fig.S3. Permeabilized cells without addition of cofactor showed a lower activity most likely due to the loss of most intracellular cofactors. The addition of NADH or NAD^+ did not improve the conversion. However, when NADPH was added to the reaction system, the conversion was increased by 100%. The results demonstrated that the 2-ketoacid reductase in *S. cerevisiae* ZJB5074 is NADPH-dependent. The permeabilization had no effect on the cofactor regeneration because addition of $NADP^+$ can also improve the conversion with the same level.

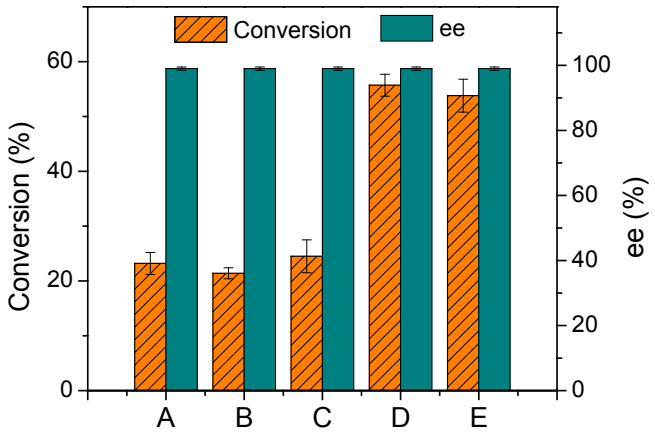

Fig.S3 Bioreduction of **2a** by permeabilized whole cells of *S. cerevisiae* ZJB5074 at 30 °C, pH 7.5, and 180 rpm for 36 h without (A) or with (B-E) the addition of cofactors. A: without cofactor addition; B: 1.0 mM NAD^+; C: 1.0 mM NADH; D: 1.0 mM NADPH; E: 1.0 mM $NADP^+$. Results are average of three parallel replicates.

7. Substrate specificity of *S. cerevisiae* ZJB5074

Resting cells of *S. cerevisiae* ZJB5074 were resuspended to a cell density of 5 g dcw/L in 5.0 mL potassium phosphate buffer (100 mM, pH 7.5) containing 10 g/L of glucose and 30 mM **2a-2q**. The mixture was incubated at 30 °C and 150 rpm. Enzymatic activity and ee of products were determined by RP-HPLC analysis. For the determination of enzymatic activity, the reaction was carried out for 3 h. One unit of enzyme activity was defined as the amount of enzyme catalyzing the bioreduction of 1.0 μmol 2-ketoacids per minute. For the determination of ee of (*R*)-**1**, the reaction was carried out for 24 h.

8. Deracemization of various 2-hydroxyacids by combination of resting cells of *P. aeruginosa* CCTCC M 2011394 and *S.cerevisiae* ZJB5074

The biocatalytic deracemization of racemic **1b** was performed by the following two methods. One-pot, two-step strategy: a reaction mixture (5.0 mL) containing 20 mM **1b**, 100 mM potassium phosphate buffer (pH 7.5) and resting cells of *P. aeruginosa* CCTCC M 2011394 (10 g dcw/L) was placed in a 50 mL-flask. The first step of the **1b** oxidation reaction was performed at 150 rpm and 30 °C for 6 h. After the reaction, resting cells of *P. aeruginosa* CCTCC M 2011394 were removed by centrifugation. The second step of **2b** reducing reaction was performed using the supernatant from the first step reaction. Glycerol (10 g/L) and resting cells of *S.cerevisiae* ZJB5074 (5 g dcw/L) were added to the supernatant. The second step reaction was performed at 30 °C and 150 rpm for 28 h. One-pot, single-step strategy: a reaction mixture (5.0 mL) containing 20 mM **1b**, resting cells of *P. aeruginosa* CCTCC M 2011394 (10 g dcw/L) and *S.cerevisiae* ZJB5074 (5 g dcw/L) and glycerol (10 g/L) was placed in a 50 ml-flask. The reaction was performed at 30°C and 150 rpm for 24 h. For the biocatalytic deracemization of other 2-hydroxyacids, the reactions were carried out by concurrent reaction as described above by using different racemic 2-hydroxyacids as substrates.

References

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