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Isolation of enantioselective α-hydroxyacid dehydrogenases based on a high-throughput screening method

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Abstract To isolate enantioselective α-hydroxyacid dehydrogenases (α-HADHs), a high-throughput screening method was established. 2,4-Dinitrophenylhydrazine solution forms a red-brown complex with ketoacid produced during the α-HADH-mediated oxidation of α-hydroxyacid. The complex can be easily quantified by spectrophotometric measurement at 458 nm. The enantioselectivity of α-HADH in each strain can be measured with this colorimetric method using (*R*)- and (*S*)-α-hydroxyacid concurrently as substrates to evaluate the apparent enantioselectivity (E_{app}). The E_{app} closely matches the value of true enantioselectivity (E_{true}) determined by HPLC analysis. With this method, a total of 34 stains harboring enantioselective α-HADHs were selected from 526 potential α-HADH-producing microorganisms. *Pseudomonas aeruginosa* displayed the highest (*S*)-enantioselective α-HADH activity. This strain appears promising for potential application in industry to produce (*R*)-α-hydroxyacids. The method described herein represents a useful tool for the high-throughput isolation of enantioselective α-HADHs.

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Keywords High-throughput screening · α-Hydroxyacid · α-Hydroxyacid dehydrogenase · Enantioselectivity · *Pseudomonas aeruginosa*

Introduction

Many approaches have been developed to produce chiral chemicals for the manufacture of a wide range of intermediates in the pharmaceutical, agrochemical, fine chemical and food industries [1]. In recent years, biocatalysis has emerged as a powerful strategy for the production of enantiomerically pure building blocks that are difficult to be obtained through chemical and fermentation processes. Enantioselective α-hydroxyacid dehydrogenases (α-HADHs) can specifically oxidize one enantiomer of α-hydroxyacid to α-ketoacid, thus leaving the other enantiomer in reaction system. They can be used as catalysts for the production of optically active α-hydroxyacids from their racemic mixtures [2–5]. α-HADHs are promising for industrial application as they can carry out the oxidation at ambient pressure and temperature with high selectivity, activity and broad substrate specificity; most importantly, the isolation of chiral product from the reaction mixture becomes very simple and efficient, which are key characteristics for their development potential as industrial process [6]. Although the theoretical recovery of product is limited to 50 %, a two-step stereoinversion of racemic substrate to one single enantiomer through the asymmetric oxidation with α-HADH to the corresponding ketoacid followed by reduction with a highly stereo-complementary ketoacid reductase can be considered to overcome this disadvantage. The product can be obtained in 100 % yield and with 100 % enantiomeric excess (*ee*) [7–10].

Several microorganisms with enantioselective α-HADHs, including *Alcaligenes bronchisepticus* KU1201 [11],

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Pseudomonas polycolor IFO 3918 [12], *Alcaligenes* sp. ECU0401 [13] and *P. putida* ECU1009 [14], have been reported to catalyze the asymmetric oxidation of racemic α-hydroxyacids for the production of optical pure α-hydroxyacids. However, there is still a need to find strains with α-HADHs that are able to oxidize a broad spectrum of substrates with excellent enantiomeric purity and high activity. Generally employed analytical methods for screening enantioselective α-HADHs are HPLC, GC or LC–MS, which are not readily amenable to high-throughput [12, 14]. All these are very time consuming if the traditional methods of screening are employed. Hence, it is necessary to develop a rapid screening method to identify new biocatalysts with desired activity and selectivity. Up to date, there is no high-throughput screening method for the identification of enantioselective α-HADH, which makes the screening step as rate limiting. Therefore, a direct and sensitive readout of α-HADH activity and enantioselectivity has to be considered.

In this study, we describe a simple and rapid method for the rapid screening of a library of enantioselective α-HADHs based on the analysis of ketoacids. Several methods have been developed for the analysis of ketoacids using certain types of derivatization [15, 16]. Among them, the most widely employed is derivatization via 2,4-dinitrophenylhydrazine (DNPH) [15]. The DNPH derivatives of ketoacids are homogeneously red-brown in alkaline condition and can be analyzed by spectroscopic techniques (Scheme 1). When α-hydroxyacid oxidation reaction caused by α-HADH exists, the color of DNPH derivatives of ketoacids in the samples can be easily quantified by spectrophotometric measurements at 458 nm. Use of optically pure (*R*)- and (*S*)-α-hydroxyacid concurrently as substrates allows the determination of the apparent enantioselectivity (E_{app}). This colorimetric reaction can be applied to screen for active and enantioselective α-HADHs (Fig. 1). With this method, a large number of samples can

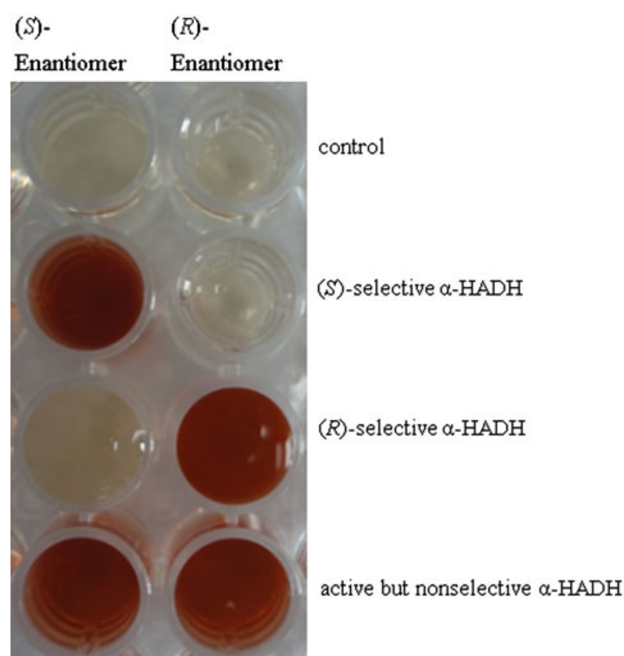
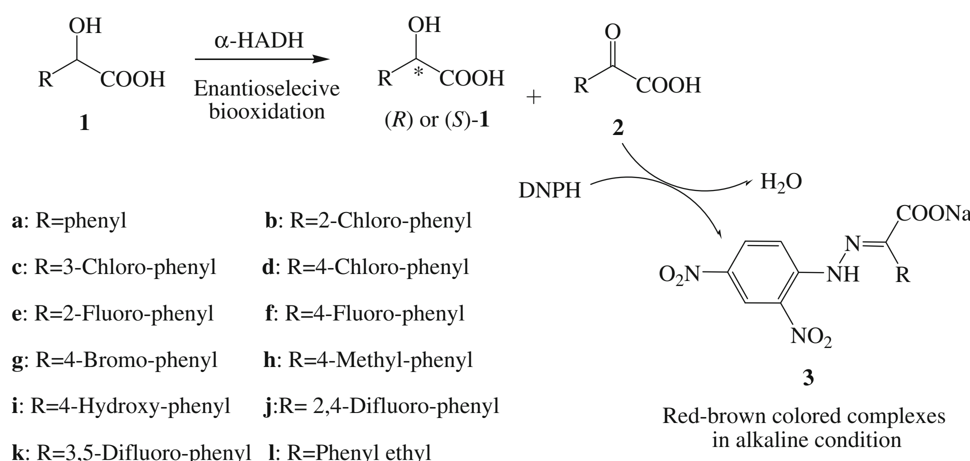


Fig. 1 Examples of the selective colorimetric screen for enantioselective α-HADH-producing microorganisms. If the positive reaction occurred, the color of DNPH derivatives of ketoacids in the sample will change to red-brown after adding DNPH solution and NaOH. The color change is visible to the naked eye. The strain will be rejected if neither enantiomer reacts or if both enantiomers react at similar rates. If one enantiomer reacts significantly faster than the other, the strain will be tested further



Scheme 1 Asymmetric oxidation of α-hydroxyacids with enantioselective α-HADH, yielding optically active α-hydroxy acids. The ketoacids released during the bioconversion can react with DNPH to produce a bright yellow hydrazones, which can be easily quantified by spectrophotometric measurements at 458 nm in alkaline condition. The initial rates of ketoacids formation can thus be determined. Use of optically pure (*R*)- and (*S*)-α-hydroxyacid concurrently as substrates allows the determination of the apparent enantioselectivity (E_{app})

be examined and (*R*)- or (*S*)-α-HADHs can be obtained much faster and easier than with traditional GC or HPLC-based methods.

Materials and methods

Chemicals

1a–1l, (*R*)-**1a–1l**, (*S*)-**1a–1l** and **2a–2l** (Scheme 1) were purchased from J&K Chemical Co., Ltd. (Shanghai, China). *Sinorhizobium* sp. CCTCC M 2011391 and *Serratia marcescens* CCTCC M 2011392, which are known producers of enantioselective α-HADH, were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China). All other chemicals were of reagent grade and obtained commercially. DNPH solution was prepared by dissolving 198 mg of DNPH and 83.3 mL of hydrochloric acid (38 %) in distilled water to make 1 L.

Microbial cultures and cultivation conditions

The rich medium for fermentation of α-HADH consisted (g/L): glucose 10, yeast extract 10, K_2HPO_4 2.5, KH_2PO_4 2.5, $MgSO_4$ 0.2, $FeSO_4$ 0.03, and (*R,S*)-**1a** 2. (pH 7.0). *Sinorhizobium* sp. CCTCC M 2011391 and *S. marcescens* CCTCC M 2011392 were grown at 30 °C for 24 h in the rich medium. The preculture (3 mL) was transferred to a 50-mL medium of the same composition and was allowed to grow for 48 h at 30 °C in an incubator shaker (150 rpm).

Potential enantioselective α-HADH-producing microorganisms were isolated using enrichment culture technique. Each isolate was cultivated in rich medium at 30 °C. After 24 h of cultivation, 3 mL of this culture was transferred to 50 mL rich medium in a 250 mL-flask and incubated at 30 °C for 48 h (150 rpm). Cells were harvested by centrifugation at 12,000×*g* for 8 min at 4 °C. The cell pellets were washed thoroughly with 100 mM phosphate buffer (pH 8.0), and then they were stored at 4 °C for further use. α-HADH enantioselectivity of each strain was measured using (*R*)- and (*S*)-α-hydroxyacid concurrently as substrates to evaluate the E_{app}.

Screening procedure

Resting cells (0.2 g) of different microorganisms were resuspended in 10.0 mL phosphate buffer (100 mM, pH 8.0) containing 20 mM (*R*)-isomer or (*S*)-isomer. The mixtures were shaken at 30 °C and 150 rpm. Samples (10 μl) were taken at regular intervals and transferred to a 96-well microtiter. 100 μl of DNPH (1.0 mM) was added to the plate for the derivatization with ketoacid produced during the bioconversion. The colorimetric reactions were carried out for 30 min at room temperature and then 890 μl of NaOH (0.4 M) was added. The absorbance of the homogeneous red-brown solution in the plate was determined at 458 nm by an absorbance microplate reader (SpectraMAX puls 384, MD, USA). The initial reaction rates for each enantiomer separately were calculated. The quotient of these rates gives the E_{app}. The enantioselectivity of α-HADH from these selected microorganisms was further confirmed by HPLC in the kinetic resolution of **1**. Reactions were carried out at 30 °C and 150 rpm for 3 h in 10 mL phosphate buffer (100 mM, pH 8.0) with a wet cell concentration of 20 g/L and 20 mM substrate.

Asymmetric oxidation of different substrates with the newly isolated strain

Resting cells of *P. aeruginosa* were resuspended to a cell concentration of 50 g/L in 10 mL phosphate buffer (100 mM, pH 8.0) containing 20 mM **1a–1l**. The mixtures were shaken at 30 °C and 150 rpm. Samples were taken at regular intervals and the reaction was terminated through centrifugation (10,000×*g*, 5 min). The yield and *ee* (%) were determined by chiral HPLC.

HPLC analysis

The concentrations of **1a–1l** and **2a–2l** were assayed by HPLC (LC-10AS, Shimadzu, Japan) equipped with a octadecylsilica column (250 × 4.6 mm) (Elite Analytical instruments Co., Ltd, China) at a flow rate of 1.0 mL/min with a solvent system composed of 10 mM $NH_4H_2PO_4$ and methanol (4:1, v/v). Detection wavelength was set at 228 nm.

The optical purities of products were assayed by HPLC (Dionex UltiMate 3000, USA) equipped with a chiral column (Chirobiotic^{TM} R 250 × 4.6 mm, 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. Enantioselectivities (E_{true} values) were calculated from conversion and enantiomeric excess (% *ee*) according to the equation $E_{true} = \ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$ as described by Chen et al. [17].

Results

Full wavelength scanning for the colored complexes 3

To develop a simple colorimetric assay for rapid identification of active and enantioselective α-HADH based on the reaction of ketoacids with DNPH to form stable hydrazones, it is very important to choose the suitable detection wavelength. We selected **1a–1d** as the model substrates,

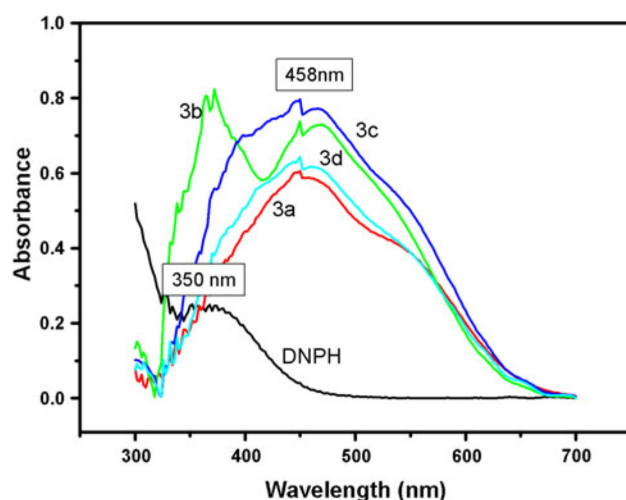


Fig. 2 Absorption spectrums of DNPH and colored complexes **3a–3d**

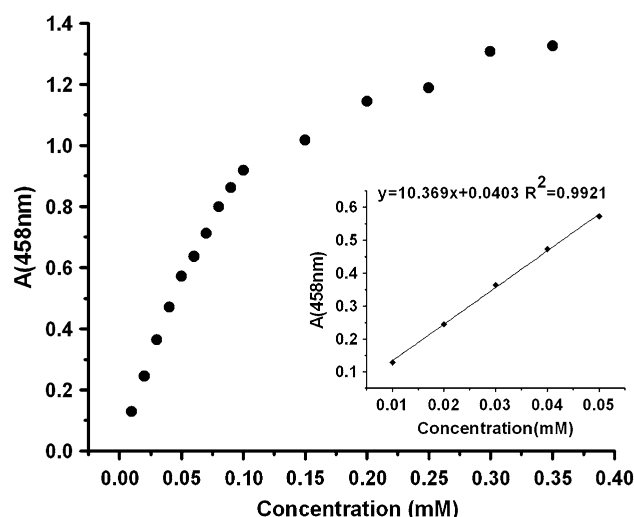


Fig. 3 The relationship between the concentration of ketoacid **2a** and the absorbance at 458 nm. The *insert* shows the linear range of the measurement

the colored complexes **3a–3d** were prepared by reactions of ketoacids **2a–2d** with DNPH, respectively [18]. The reactions were carried out for 30 min at room temperature. After adding the NaOH solution, the reaction mixtures become homogeneously red-brown. To find out the ideal detection wavelength, the colored complexes solutions were taken full wavelength scanning with a Beckman DU800 spectrophotometer (Beckman Coulter, USA) from 300 to 700 nm. The results are shown in Fig. 2. The maximum absorbance of DNPH and **3a–3d** were 350 and 458 nm, respectively. It was suitable to choose the 458 nm as the detection wavelength, which can accurately determine the amount of the ketoacids **2a–2d** and exclude the interference of DNPH.

Linearity of calibration curves

To quantify the ketoacids **2** formed in the biotransformation by α-HADH, according to the Beer–Lambert equation, it is necessary to find the relationship between the concentrations of **2** and the absorbances. Different concentrations of **2a–2d** which were enzymatically synthesized from **1a–1d** by *Sinorhizobium* sp.CCTCC M 2011391 were derivatized with DNPH. The relationship between the concentration of **2a** and the absorbance at 458 nm is shown in Fig. 3. The insert showed the linear range of the measurement. The linearity parameters of spectrophotometry between the concentrations of **2b–2d** and the absorbance at 458 nm are all shown in Table 1. OD_{458} were linear with the concentrations of **2b–2d**. Thus, it is possible to calculate the conversion for each reaction after determining the standard cures. Lower limit of quantifications of **2a–2d** in the solution was 10 μM. Using this method, the ketoacids could be determined sensitively in the μM concentration range by spectrophotometry. Moreover, this assay could be performed either in test tubes with a UV–Vis spectrophotometer or on a 96-well plate with a universal microplate spectrophotometer requiring only a simple testing apparatus at a room temperature, indicating that this method is simple, easy to use and sensitive.

Quantitative validation of the assay

To confirm that the color change accurately measured the enzyme activity, the progresses of *Sinorhizobium* sp. CCTCC M 2011391 [a known producer of (*S*)-α-HADH] catalyzed asymmetric oxidation of **1a–1d** were determined both by the spectrophotometry and HPLC. Typical graphs of **2a–2d** concentrations versus reaction time are given in Fig. 4. Comparisons of both data showed that the concentrations of ketoacids **2a–2d** based on the spectrophotometry were in close agreement with the values obtained from HPLC. These results indicated that this colorimetric method can be used to determine the α-HADH activity. To demonstrate the efficiency and accuracy of this method for the determination of α-HADH enantioselectivity, we both determined the E_{app} and true enantioselectivity (E_{true}) through colorimetry and HPLC analysis method in the kinetic resolution of **1a–1d** by *Sinorhizobium* sp. CCTCC M 2011391 and *S. marcescens* CCTCC M 2011392. E_{app} values are the ratio of initial rates determined from the oxidation of optically pure (*S*) and (*R*)-enantiomers. E_{true} values are derived from resolutions of racemates. Measuring initial rates of the ketoacids generated from (*S*)- and (*R*)-substrates (20 mM) at a wet cell concentration of 20 g/L gives the E_{app}. E_{true} values are determined according to the equation of Chen et al. [17] using racemic substrates

Table 1 Linearity parameters of spectrophotometry

Ketoacid	Extinction coefficient (ε), L/(mol cm)	Linear range, mM	Linear regression equation	Correlation coefficient
2a	1.04×10^4	0.01–0.05	$y = 10.369x + 0.0403$	0.9921
2b	1.11×10^4	0.01–0.05	$y = 11.12x + 0.0134$	0.9967
2c	1.67×10^4	0.01–0.05	$y = 16.74x + 0.0378$	0.9939
2d	1.03×10^4	0.01–0.06	$y = 10.263x + 0.0558$	0.9904

All the experiments were carried out in triplicate and the mean values are taken. The standard deviations of the experiments were <15 %

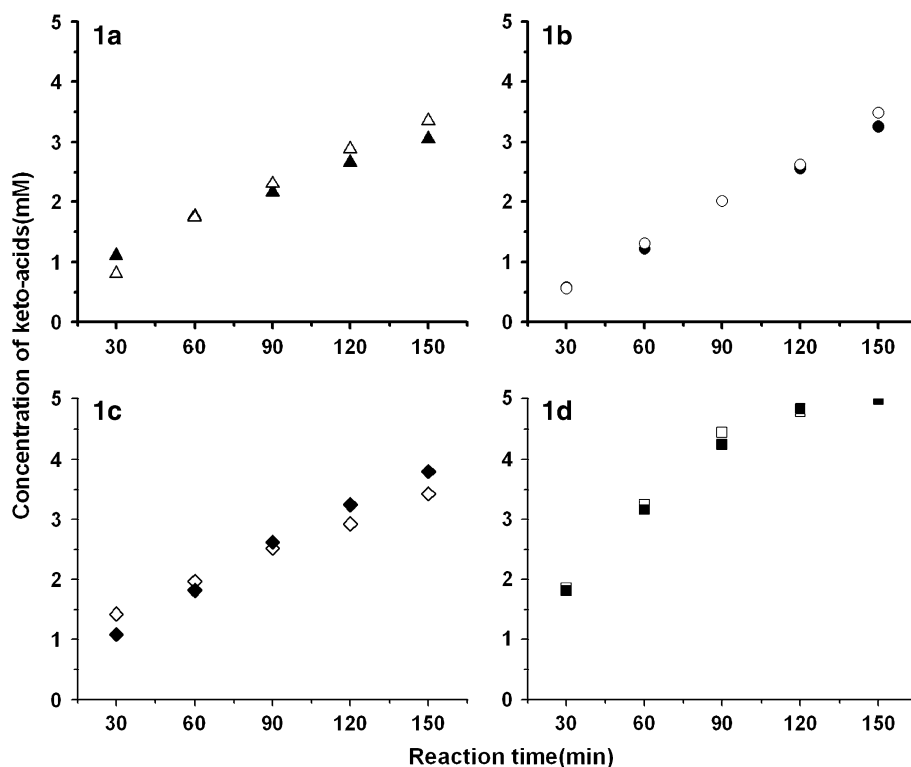


Fig. 4 Time course of *Sinorhizobium* sp. CCTCC M 2011391 catalyzed the oxidation of **1a** (*open triangles*, *filled triangles*), **1b** (*open circles*, *filled circles*), **1c** (*open diamonds*, *filled diamonds*) and **1d** (*open squares*, *filled squares*). The concentrations of ketoacids were determined by the colorimetric method (*open symbols*) and HPLC (*filled symbols*). The experiments were carried out in triplicate and the mean values are taken. The standard deviations of the experiments were <15 %

(20 mM) for conversion reaction. The bioconversions of (*R*) and (*S*)-α-hydroxyacids by *Sinorhizobium* sp. CCTCC M 2011391 are shown in Fig. 5. The results revealed an almost sole increase in absorption for the (*S*)-enantiomer reflecting the high enantioselectivity of α-HADH from *Sinorhizobium* sp. CCTCC M 2011391. The E_{app} determined by the colorimetric method closely matches the value of true enantioselectivity (E_{true}) determined by HPLC analysis (Table 2). These results disclosed that this colorimetric method was sensitive enough to screen the enantioselective α-HADH-producing microorganisms.

Isolation of enantioselective α-HADHs by the high-throughput screening method

The potential α-HADH-producing microorganisms were evaluated with the high-throughput screening method employing (*R*) and (*S*)-α-hydroxyacid as substrates concurrently for the identification of α-HADH enantioselectivity. We selected (*R*) and (*S*)-**1a** as substrates, 34 isolates including bacteria and yeasts, able to produce enantioselective α-HADH, were selected from 526 potential α-HADH-producing microorganisms. Twenty-four strains preferentially produce (*S*)-α-HADHs. Ten strains preferentially produce (*R*)-α-HADHs. Microorganisms that prefer the (*S*)-isomer are widespread in the nature, while those with α-HADHs selective for the (*R*)-isomer are relatively rare. The enantioselectivity of α-HADHs from these selected microorganisms was confirmed by HPLC in the kinetic resolution of **1a**. The results are shown in Fig. 6. HPLC results substantiate the findings from the high-throughput screening method and established beyond doubt that this method can be used for high-throughput enantioselective screening of α-HADH-producing microorganisms. One of the isolated strains ZJB1125 which have the relatively high activity and enantioselectivity of α-HADH

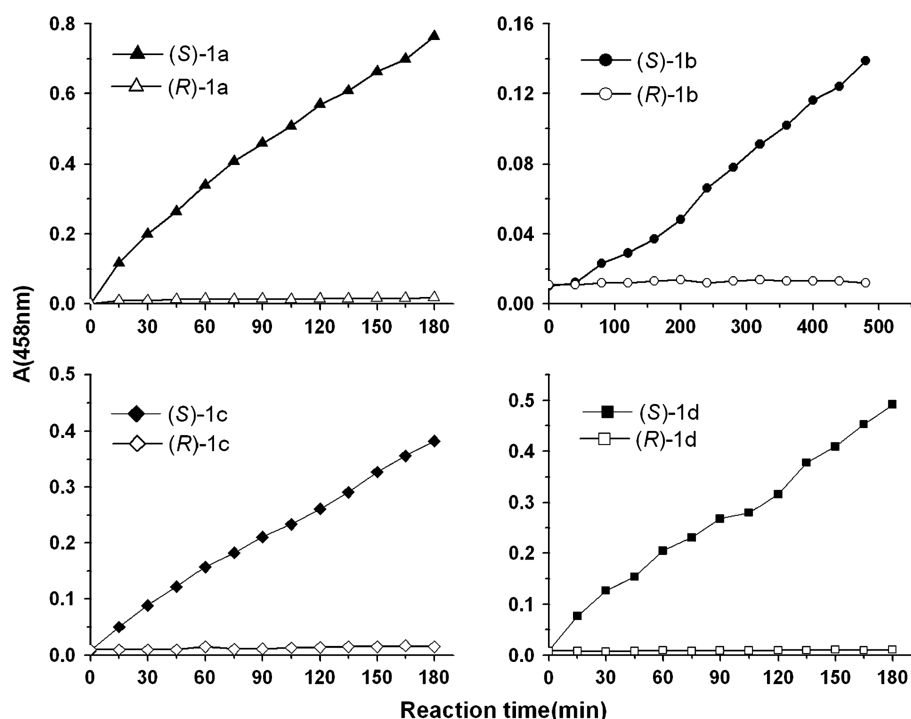


Fig. 5 Time course of *Sinorhizobium* sp. CCTCC M 2011391 catalyzed the oxidation of (*R*)-**1a**–**1d** (*open symbols*) and (*S*)-**1a**–**1d** (*filled symbols*). *open triangles* (*R*)-**1a**, *filled triangles* (*S*)-**1a**, *open circles* (*R*)-**1b**, *filled circles* (*S*)-**1b**, *open triangles* (*R*)-**1c**, *filled diamonds* (*S*)-**1c**, *open squares* (*R*)-**1d**, *filled squares* (*S*)-**1d**. Reactions were carried out at 30 °C and 150 rpm in 10 mL phosphate buffer (100 mM, pH 8.0) with a cell concentration of 20 g/L and 20 mM (*R*)-or (*S*)-**1a**–**1d**, respectively. Measuring initial rates of the ketoacids generated from pure enantiomers of (*S*)- and (*R*)-substrates as the E_{app}. The experiments were carried out in triplicate and the mean values are taken. The standard deviations of the experiments were <15 %

Table 2 Comparison of enantioselectivity determined by the high-throughput screening method (E_{app}) and by HPLC analysis (E_{true}) in the kinetic resolution of **1a**–**1d** by *Sinorhizobium* sp. CCTCC M 2011391 and *S. marcescens* CCTCC M 2011392

Strain	Substrate	E_{app}	E_{true}	Selectivity
Sinorhizobium sp. CCTCC M 2011391	**1a**	>200	>200	*S*
	1b	>200	>200	*S*
	1c	>200	>200	*S*
	1d	>200	>200	*S*
S. marcescens CCTCC M 2011392	**1a**	23.2	34.8	*R*
	1b	ND	ND	ND
	1c	3.1	2.3	*R*
	1d	2.8	1.9	*R*

All the experiments were carried out in triplicate and the mean values are taken. The standard deviations of the experiments were <15 %

ND not detectable

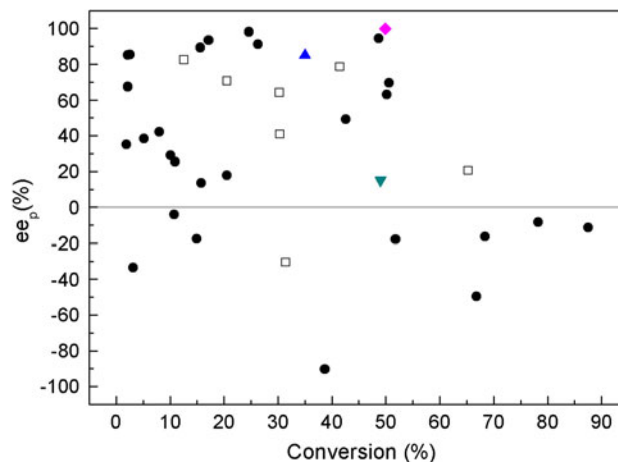


Fig. 6 Confirmation of activity and enantioselectivity of the α-HADHs from the selected microorganisms and positive control strains by HPLC in the kinetic resolution of **1a**. Selected strains: *filled circles* bacteria, *open squares* yeasts, *filled diamond* ZJB1125. Positive control strains: *filled triangle Sinorhizobium* sp. CCTCC M 2011391, *filled inverted triangle Serratia marcescens* CCTCC M 2011392. Reactions were carried out at 30 °C and 150 rpm for 3 h in 10 mL phosphate buffer (100 mM, pH 8.0) with a cell concentration of 20 g/L and 20 mM **1a**. *Minus values* of *ee* stand for (*S*) absolute configuration of product

was identified as *P. aeruginosa* by 16S rDNA sequence (GenBank accession no. JQ927220) analysis, chemotaxonomical analysis and biochemical test. This strain was deposited at China Center for Type Culture Collection as CCTCC M 2011394.

Asymmetric oxidation of different substrates with the newly isolated strain

Reactions were carried out to investigate the asymmetric oxidation of **1a**–**1l** to prepare (*R*)-**1a**–**1l**. The yields and *ees* (%) of the products in the reactions were monitored by chiral HPLC. The results are shown in Table 3. It can be seen that *P. aeruginosa* was highly (*S*)-selective for **1a**–**1l**. Using the resting cell as a biocatalyst for enantioselective oxidation **1a**–**1l**, the optically pure α-hydroxyacids (*R*)-**1a**–**1l** were obtained in 42.3–49.9 % yield with *ee* of 92.5–100 % within 3–10 h. The position of the substituent

Table 3 Preparations of (*R*)-**1a**–**1l** by asymmetric oxidation of **1a**–**1l** with newly isolated *P. aeruginosa*

Substrate	Product	Reaction time (h)	*ee* (%)	Recovery (%)
Mandelic acid (**1a**)	(*R*)-**1a**	3	99.5	49.2
2-Chloromandelic acid (**1b**)	(*R*)-**1b**	6	>99.9	49.9
3-Chloromandelic acid (**1c**)	(*R*)-**1c**	3	>99.9	45.8
4-Chloromandelic acid (**1d**)	(*R*)-**1d**	3	98.7	47.8
2-Floromandelic acid (**1e**)	(*R*)-**1e**	3	>99.9	49.8
4-Fluoromandelic acid (**1f**)	(*R*)-**1f**	3	>99.9	49.1
4-Brolomandelic acid (**1g**)	(*R*)-**1j**	3	98.5	49.9
4-Methylmandelic acid (**1h**)	(*R*)-**1h**	3	>99.9	47.5
4-Hydroxymandelic acid (**1i**)	(*R*)-**1i**	3	92.5	42.3
2,4-Difluoromandelic acid (**1j**)	(*R*)-**1j**	3	>99.9	49.5
3,5-Difluoromandelic acid (**1 k**)	(*R*)-**1k**	3	>99.9	48.7
Phenyllactic acid (**1l**)	(*R*)-**1l**	10	>99.9	49.6

Reactions were carried out at 30 °C and 150 rpm in 10 mL phosphate buffer (100 mM, pH 8.0) with a cell concentration of 50 g/L and 20 mM **1a–1l**. Samples were taken at regular intervals. The recovery and *ee* (%) were determined by HPLC

and the pattern of the substitution on the benzene ring had some effect on the enantioselectivity. Comparing the specific activity and substrate specificity, the newly isolated *P. aeruginosa* was superior to all other α-HADH-producing strains reported [11–14]. It can be concluded that the isolated microorganism is a suitable candidate for the production of optically active α-hydroxyacids from their racemic mixtures. This strain appears promising for potential applications in industry. Because of the encouraging results described above, work is in progress in our laboratory to optimize the different physico-chemical parameters for the higher enzyme productivity and characterization of the enantioselective α-HADH produced by *P. aeruginosa*.

Discussion

The application of biocatalysis for the production of key chiral intermediates in the pharmaceutical and fine chemicals industries is rapidly growing [19]. A major problem in the development of a specific biocatalysis is to find the appropriate biocatalyst. If there are no commercially available enzyme preparations, the desired activities will be found either by screening of strains from culture collections or by isolation of new microorganisms. Screening these biocatalysts for activity and enantioselectivity against target molecules becomes a major bottleneck in the process-development timeline. Implementation of a rapid enantioselective screening method is very important [20–22]. One of the most convenient ways to assay an enzyme is through a method that allows the development of color and thus can be used in qualitative as well as quantitative measurements [23, 24]. In this work, a novel and simple colorimetric assay for the identification of active and enantioselective α-HADH based on the reaction of keto-acids with DNPH to form stable hydrazones was established. Using the convenient and rapid screening method described here, one can analyze large numbers of enantioselective α-HADH-producing microorganisms or mutants simultaneously in a short period, thus reducing the number of samples to be analyzed in a more quantitative detail (HPLC analysis). Although the use of DNPH to precolumn derivatization reaction for HPLC determination has been reported [16, 25, 26], this is the first report on a rapid colorimetric assay method for the identification of enantioselective α-HADH using whole cells.

This new method offers some advantages over the conventional screening method. Firstly, the assay format contained a very broader substrate spectrum. Besides model compounds, other hydroxyacids including the different substituent groups on benzene or different length of side chain are also suitable for this screening strategy. Therefore, we are able to screen satisfactory enantioselective α-HADHs by applying different substrates for specific biotransformation. Secondly, the color change from light yellow to red-brown is readily apparent and sensitive when a positive reaction occurs. This characteristic is very useful when a primary screening is needed. Moreover, this method is quantitative. Use of optically pure (*R*)- or (*S*)-enantiomers allows the determination of the E_{app}. Thirdly, this assay can be both performed in microplates and cuvettes with a conventional UV–vis spectrophotometer under room temperature. As GC, HPLC, or NMRS is avoided, the screening process is dramatically faster and the requirement for equipment is very low. Lastly, the DNPH reagent/solution is stable under ambient conditions and does not need to be freshly prepared before each use. Therefore, this method is particularly useful for the screening of large numbers of microorganisms or mutants, allowing quick identification of organisms with desired enantioselectivity, discarding poor to moderate enantioselective organisms or mutants that will not be acceptable for the development of a

biocatalytic resolution process. This fits the strategy of a hierarchical screen for the identification of best biocatalyst as one of the earliest steps and eliminates the weakest candidates for a more streamlined process viability study [24, 27, 28]. The screen was designed in such a way as to obtain α-HADHs with high activity and enantioselectivity for the production of optically active hydroxyacids from their racemic mixtures, which will have practical applications.

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