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Graphical Abstract

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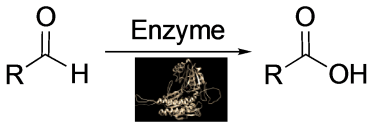

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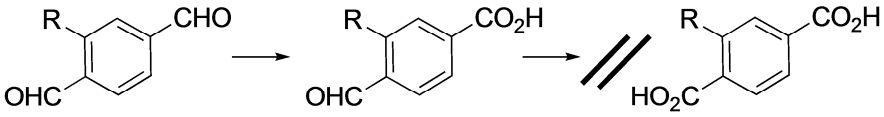

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Declarations of interest: none

Abstract

Oxidation reaction is one of the most important and indispensable organic reactions, so that green and sustainable catalysts for oxidation are necessary to be developed. Herein, biocatalytic oxidation of aldehydes was investigated, resulted in the synthesis of both aromatic and aliphatic carboxylic acids using a *Geotrichum candidum* aldehyde dehydrogenase (*Gc*ALDH). Moreover, selective oxidation of dialdehydes to aldehydic acids by *Gc*ALDH was also successful.

Keywords: Enzyme, Aldehyde dehydrogenase, Oxidation, Aldehyde, Carboxylic acid, Selectivity, Green chemistry

1. Introduction

Oxidation reaction is an indispensable reaction for the chemical and pharmaceutical industry, so that strong and useful oxidative reagents have been developed. However, these reactions may have some drawbacks; these reactive reagents are necessary in a stoichiometric amount, they can be explosive and/or contains hazardous heavy metals, and solvent waste and byproduct are produced. To overcome these issues, the development of environmentally friendly reactions have attracted attentions [1–4]. As a green catalyst, biocatalyst has been well known; the reaction can be conducted in mild conditions, and non-explosive material such as NAD^+ and oxygen in the air can be used as oxidants [5–7]. Therefore, oxidation of alcohols, Baeyer-Villiger oxidation, oxidation of

sulfides, and hydroxylation have been widely studied [8–12], while the oxidation of aldehydes to carboxylic acids have been studied to some extent [13–23]. For example, oxidation of heterocyclic and aromatic aldehydes including 2-furancarboxaldehyde (furfural) and 5-hydroxymethylfurfural to the corresponding carboxylic acids has been studied using *Acetobacter rancens* IFO3297, *A. pasteurianus* IFO13753 and *Serratia liquefaciens* LF14 [13]. Especially, oxidation of biomass-derived 5-hydroxymethylfurfural and related compounds has been extensively examined using *Comamonas testosteroni* SC1588, a carbonyl reductase from *Streptomyces coelicolor* (ScCR), an alcohol dehydrogenase (ADH) from *Synechocystis* sp. (SADH), and horse liver ADH (HLADH), etc [14,18–22]. From 5-hydroxymethylfurfural, both 5-formyl-2-furancarboxylic acid and 2,5-furandicarboxylic acid were obtained with a yield of more than 95% using SADH and HLADH, respectively [20]. The produced 5-formyl-2-furancarboxylic was not over oxidized to the corresponding dicarboxylic acid by SADH. Oxidation of aliphatic and aromatic aldehydes by three recombinant aldehyde dehydrogenases (ALDHs) originated from bovine lens (ALDH-Bov), *Escherichia coli* (ALDH-Ec), and *Pseudomonas putida* (PP-ALDH) has been investigated, and preparative scale oxidation of 5-hydroxymethylfurfural to give the corresponding carboxylic acid in 61% isolated yield (1.37 g) was reported [17]. Recently, α-ketoglutaric semialdehyde dehydrogenase (KGSADH) from *Azospirillum brasilense* has been engineered to produce 3-hydroxypropionic acid by the oxidation of 3-hydroxypropanal, derived from glycerol enzymatically [16]. However, biocatalysts that have been tested for a wide variety of substrates including both aliphatic and aromatic aldehydes with preparative synthetic study [17] is still limited, and the discovery of an aldehyde dehydrogenase is necessary.

As a source of an enzyme for the oxidation of aldehydes, an organism that produces other valuable enzymes has a high possibility to produce a versatile ALDH, also. Previously, our group has discovered dehydrogenases widely used in organic synthesis from a dimorphic fungus, *Geotrichum candidum* [12,24–32]. One of the enzymes from *G. candidum* NBRC 4597, acetophenone reductase (*Gc*APRD), has been overexpressed in *E. coli* [27], and widely used for enantioselective organic

synthesis [28–32]. *G. candidum* glycerol dehydrogenase [33] and lipases [34,35] have been also used widely. Focusing on the non-aqueous solvent tolerant enzymes from *G. candidum*, and wide applications of its enzymes [12,24–30], an aldehyde dehydrogenase from this organism (*Gc*ALDH) was targeted for the oxidation of aldehydes to carboxylic acids in this research. It was found that the enzyme overexpressed in *E. coli* can catalyze the oxidation of various aromatic and aliphatic aldehydes (Fig. 1). Moreover, the selective oxidation of dialdehydes to aldehydic acids by *Gc*ALDH was also achieved. Preparative scale reactions were performed successfully.

2. Results and discussion

2.1. Preparation and characterization of the enzyme

The gene of *Gc*ALDH was amplified from the genomic DNA of *G. candidum* NBRC 4597 and cloned in an expression vector, pET-21b (+), leading to pET-21b (+)-*Gc*ALDH (the DNA sequence and plasmid map of pET-21b (+)-*Gc*ALDH are shown in Fig. S1 and Fig. S2, respectively). The vector was transformed into *E. coli* Rosetta^{TM}(DE3)pLysS. His-tagged *Gc*ALDH was induced by IPTG and purified by Ni affinity chromatography by 6.5-fold in 33% yield (Table S1). Construction of the overexpression system and purification were successful, as shown in the SDS-PAGE of the purified enzyme (Fig. S3). The size of a single band around 60 kDa in the purified enzyme lane is in accordance with the expected size of 66.2 kDa.

Coenzyme dependency of *Gc*ALDH was investigated. As shown in Table 1 Entry 1, the oxidation activity toward benzaldehyde was observed using NAD^+, but no significant activity was observed using $NADP^+$. Therefore, NAD^+ was used for further studies. Then, the *Gc*ALDH properties, such as the effect of pH, temperature, additives, and substrate concentration, were investigated using the purified enzyme to optimize reaction conditions. First, the effect of pH on *Gc*ALDH activity was examined (Fig. S4). Optimum oxidizing activity was observed in a pH range of around 7.0 -7.5. The enzyme stability was also examined by comparing the activity before the treatment with that after the treatment under pH between 5.0 and 10.5 at 8 °C for 1 week (Fig. S5).

The enzyme was stable under a wide range of pH between 5.0 and 8.5. Then, the effect of temperature on *Gc*ALDH activity was examined (Fig. S6). The temperature with the highest activity was 50 °C, which was 1.4 times that at 37 °C. Thermostability was also examined (Fig. S7). After 24 h, the activity was kept at 4 °C fully, but decreased to 62% at 30 °C and 26% at 40 ^{o}C. However, it was deactivated completely at 45 °C in 6 h and at 50 °C in 15 min. Therefore, the further synthetic study was conducted at 40 ^{o}C. The temperature dependency on the activity and stability of *Gc*ALDH was compared with that of other enzymes reported so far and found to be comparable. The oxidation of aromatic aldehydes by *A. rancens* or *S. liquefaciens* was conducted at 20 ^{o}C or 30 ^{o}C [13]. Knaus *et al* examined temperature profile for the conversion of 2-methylpentanal and benzaldehyde by ALDH-Bov, ALDH-Ec and PP-ALDH at 30 ^{o}C, 40 ^{o}C, and 50 ^{o}C, and the reaction temperature was set to 40 ^{o}C to retain the enzyme stability and to avoid the evaporation of volatile aldehydes [17].

The effect of additives and metal ions on *Gc*ALDH activity was examined (Fig. S8). The enzyme showed slightly higher activity with the addition of dithiothreitol (DTT), but lower activity with metals, especially with Zn^{2+} and Cu^{2+} ions. This is probably due to the binding of Zn^{2+} and Cu^{2+} ion to the sulfur atom in the active site Cys352. The activities of other aldehyde dehydrogenases were also decreased with the addition of Zn^{2+} and Cu^{2+} ions [36–38]. The addition of EDTA did not change the activity which indicates the absence of metal ion in the active site.

The effect of substrate concentration on *Gc*ALDH activity was examined with benzaldehyde (0.25-50 mM) or pentanal (0.25-80 mM) (Fig. S9). The maximum reaction rate V_{max}, Michaelis-Menten constant K_m, and inhibition constant K_i were determined to be 1.51 μmol/mg protein/min, 3.63 mM, and 22.6 mM for benzaldehyde, and 2.31 μmol/mg protein/min, 6.42 mM, and 92.9 mM for pentanal, respectively. The catalytic parameters of *Gc*ALDH were compared with other enzymes reported so far and found to be comparable in terms of the V_{max} but not for K_m since unnatural substrates are used in this study. For example, for the case of Δ^1-pyrroline-5-carboxylate dehydrogenase (ALDH4A1) from *Saccharomyces cerevisiae,* catalytic parameters, k_{cat} and K_m, were reported to be 1.5 s^{-1} and 104 μM, respectively, using Δ^1-pyrroline-5-carboxylate as the

substrate [39]. For the wild type and mutants of KGSADH, k_{cat} and K_m were reported to be 5-15 s^{-1} and 0.17-1.6 mM, respectively, using 3-hydroxypropanal as the substrate [16]. As a result of the characterization of *Gc*APRD, optimum reaction conditions were determined to be pH 7.2, 40 ^{o}C, and 10 mM of substrate concentration.

2.2. Substrate specificity

Substrate specificity was investigated using the purified enzyme. It was revealed that *Gc*ALDH has broad substrate specificity for the oxidation of a wide range of aromatic and aliphatic aldehydes (Table 1), but it does not catalyze the oxidation of primary alcohols (Entries 33 and 34) or reduction of carboxylic acids (Entries 35 and 36). Thus, the oxidation of aldehydes was successfully conducted under mild conditions without any potentially explosive chemicals. Moreover, the large difference in the relative activities between aldehydes and alcohols indicates the possibility of chemoselective reactions. The excellent chemoselectivity was also demonstrated by the transformation of 5-hydroxymethylfurfural to 5-hydroxymethl-2-furancarboxylic acid by *Comamonas testosteroni* SC1588 [14].

Among substituted benzaldehyde analogs, substitutions at the *ortho* position decreased the activities (Entries 2-6) while those at the *para* position with electron-withdrawing groups increased the activities (Entries 7, 8, 10 and 11). The nucleophilic attack to the carbonyl carbon by Cysteine 352 (Fig. S10 and S11) can be improved by the electron-withdrawing effects at the *para* position, while it can be hindered sterically by the *ortho* substitutions. Similar results were also observed for the reaction by ALDH-Bov and ALDH-Ec; the yields of *para*-substituted benzaldehydes were much higher than those of *ortho*-substituted analogs, especially when the substituents are large [17].

The activities toward benzyl aldehydes and propanals with an aromatic ring (Entries 15-19) largely depend on the structures. The activities toward two benzyl aldehydes (Entries 15 and 16) were lower than that toward benzaldehyde (Entry 1), while activities toward two out of three

3-arylpropanals (Entries 18 and 19) were higher than that toward benzaldehyde (Entry 1). Similarly, the yields of oxidation of 2-phenylpropanal and (2*E*)-3-phenylprop-2-enal (cinnamaldehyde) by ALDH-Bov, ALDH-Ec and PP-ALDH were low (less than 30%) while those of 3-phenylpropanal are around 79-85% [17].

Among linear aliphatic aldehydes tested, high activities were exhibited especially with substrates with a chain length from 5 to 8 (Entry 22-25, and 28). This is in good agreement with the fact that the predicted natural substrate found by the blast search of the protein sequence of this enzyme, δ-1-pyrroline-5-carboxylate, has also the chain length of 5 [39,40]. However, the activities were lower toward branched aliphatic aldehydes with the same chain length (Entries 20-23 vs. 29-32). These might be explained by the steric hindrance by the two phenyl-groups of phenylalanine 214 and phenylalanine 458 at the entrance of the substrate in the enzyme structure constructed by modeling (Fig. S10). For the case of the oxidation of aliphatic aldehydes by ALDH-Bov, ALDH-Ec, and PP-ALDH, the yield of the oxidation of pentanal, 4-pentenal and 2-methylpentanal were >99%, while the oxidation of longer aliphatic aldehydes resulted in moderate yields [17]. Therefore, the substrate specificity of *Gc*ALDH is unique, so that *Gc*ALDH is useful for the reaction of aliphatic substrates, similar to *Gc*APRD which is an excellent catalyst for the asymmetric reduction of challenging aliphatic ketones [28,29].

To sum up the substrate specificity of *Gc*ALDH, wider varieties of substrates, including both aromatic and aliphatic aldehydes, were tested and found to be accepted than those tested in most of the previous publications which concentrated on the furfural and furan related compounds [13,14] or 3-hydroxypropanal, a glycerol derived compound [16], except for the study by Knaus *et al* [17]. It was also found that the activities largely depend on the detail of the substrate structure, so that further studies such as determination of enzyme structure as well as investigation of docking poses of the substrate in the enzyme active site are necessary to predict the substrate specificity and its substrate recognition mechanism.

Encouraged by the high activity and wide substrate specificity of *Gc*ALDH, preparative scale reactions were conducted for the representative substrates. The reaction proceeded smoothly, and the products were successfully isolated, purified, and identified by the ^{1}H NMR and ^{13}C NMR (Appendix section in the Supplementary Material).

2.3. Selective oxidation of dialdehydes to aldehydic acids

Selective oxidation of dialdehydes to aldehydic acids is challenging since the high reactivity of the aldehyde group generally makes it difficult to oxidize only one of the aldehyde groups selectively using strong chemical reagents [13]. To investigate the selectivity of *Gc*ALDH, relative activities toward dialdehydes **1a**-**5a** and the corresponding aldehydic acids **1b**-**5b** were determined (Table 2). High activities were observed for **2a**-**5a** while there was no activity toward **1a**. Activity toward an aldehydic acid **1b** was not observed, either. The reactivities of **1a** and **1b** are possibly due to the steric hindrance by the *ortho* substitutions. The yield of the oxidation of **1a** by *S. liquefaciens* is also much lower than those of **2a** and **3a** [13].

Interestingly, the activities toward dialdehydes were much higher than those toward the corresponding aldehydic acids for the case of terephthalaldehyde **3a** and **3b** (Entry 3), 4-bromoisophthalaldehyde **4a** and **4b** (Entry 4), and 2-bromoterephthalaldehyde **5a** and **5b** (Entry 5), but not for the case of isophthalaldehyde **2a** and **2b** (Entry 2). To investigate the selectivity difference between the **2a**/**2b** and **3a**/**3b** (Entry 2 vs. Entry 3), their kinetic parameters were examined (Table S2). For the case of **3a** and **3b**, there was a large favorable difference in V_{max} and small unfavorable difference in K_m. For the case of **2a** and **2b**, there was a large unfavorable difference in K_m and small favorable differences in V_{max}. Therefore, the selective reaction is possible for the oxidation of **3a** as the reaction rate of **3a** is much faster than that of **3b** (Fig 2), while for the case of **2a**, **2b** is oxidized further to the corresponding *m*-dicarboxylic acid as soon as formed in a small concentration due to the smaller K_m for **2b** than **2a**. The selectivity difference between **2a**/**2b** and **3a**/**3b** was compared with other enzymes (Table S3), while the biocatalytic selective oxidation of **4a** and **5a** was reported

for the first time to the best of our knowledge. Similar results were reported for ScCR and SADH [20]; the selectivity between **3a** and **3b** was higher than that between **2a** and **2b**. However, the opposite trend was also reported for *S. liquefaciens* [13]; the selectivity between **2a** and **2b** is high while that between **3a** and **3b** is not. The preferential formation of aldehydic acid **2b** might be ascribed to the difference of membrane permeability or the affinities of aldehydic acid and dialdehyde for the enzyme. On the other hand, for the case of the whole-cell biocatalytic oxidation of **3a** using *Deinococcus wulumuqiensis* R12, **3c** was obtained in >99% yield [22].

Encouraged by the relative activity differences between the dialdehydes (**3a**, **4a**, and **5a**) and aldehydic acids (**3b**, **4b**, and **5b**), preparative scale synthesis of **3b**, **4b** and **5b** was conducted (Table 3). Interestingly, the reaction proceeded selectively, and the dicarboxylic acids were not detected. The selectivities in a preparative scale were higher than the relative activity differences because in the presence of dialdehydes, the reaction of aldehydic acids, competing to get into the substrate-binding site with dialdehydes, is inhibited. Furthermore, regioselectivities for oxidation of **4a** and **5a** giving only one isomer of aldehydic acids, **4b** and **5b,** were also excellent.

3. Conclusions

It was found that *Gc*ALDH has a broad substrate specificity and can selectively oxidize only one aldehyde group of dialdehydes. The oxidation of aldehydes under mild conditions using a green catalyst, *Gc*ALDH, was successfully demonstrated. In the future, further studies such as mutagenesis are required to develop this enzyme for industrial use.

4. Experimental section

4.1. General materials and methods

All chemicals, except **4a** and **5a** which were prepared according to the literature methods [41], were purchased from commercial suppliers and used without further purification. Enzyme activity assays were performed using UV-1600-UV-Visible spectrophotometer Shimadzu (Kyoto, Japan). The assay was performed in duplicate, and the average value was taken. ^{1}H NMR and ^{13}C NMR analyses were performed on a Bruker Biospin AVANCE III 400 spectrometer at 400 MHz.

4.2. Substrate specificity

0.10 M HEPES-NaOH buffer pH 7.2 (470 µl) and 10 mM benzaldehyde solution in 0.10 M HEPES-NaOH buffer pH 7.2 (500 µl) were added to a UV cell, and incubated at 37 ^{o}C for 20 min. Then, an enzyme solution (20 µl) and 10 mM NAD^+ solution (10 µl) were added to start the reaction. Thus, the final concentration is 5.0 mM substrate and 0.10 mM NAD^+. For the assay of the reduction activity toward carboxylic acids, NADH was used in substitution for NAD^+. The absorbance at 340 nm was monitored after 10 sec to 132 sec. One unit of enzyme is defined as µmol of NADH produced in 1 min under the above conditions using benzaldehyde as a substrate. Relative activity was based on benzaldehyde activity as 100% (12 mU).

4.3. Preparative scale reactions

Reactions were performed in 0.10 M HEPES-NaOH buffer (20 ml) pH 7.2 containing 10 mM substrate, 10 mM NAD^+, and 0.125 U/ml *Gc*ALDH cell-free extract for 3 h at 40 °C with a rotational speed of 160 rpm. The reaction was quenched by thermal treatment at 95 °C for 10 min. The reaction mixture was centrifuged at 12000 G for 5 min, and the supernatant was recovered. NaOH (solid) was added to adjust pH >14, and extracted with diethyl ether (30 ml x 2), and the aqueous layer was recovered. 12 N HCl (aq) was added to adjust pH < 1. The product was extracted with diethyl ether (30 ml), dried over $MgSO_4$, evaporated under reduced pressure, purified by silica

gel column chromatography (hexane:ethyl acetate, 1:1) and characterized by ^{1}H-NMR and ^{13}C-NMR analysis using $CDCl_3$ or DMSO-d_6 as a solvent. The yields are shown in Table 1 and Table 3. The NMR spectrum is in the Appendix section of the supplementary material.

Acknowledgment

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Appendix A. Supplementary Data

Supplementary data related to this article can be found.

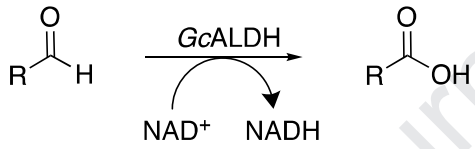


Fig. 1 Oxidation of aldehydes by *Geotrichum candidum* aldehyde dehydrogenase (*Gc*ALDH)

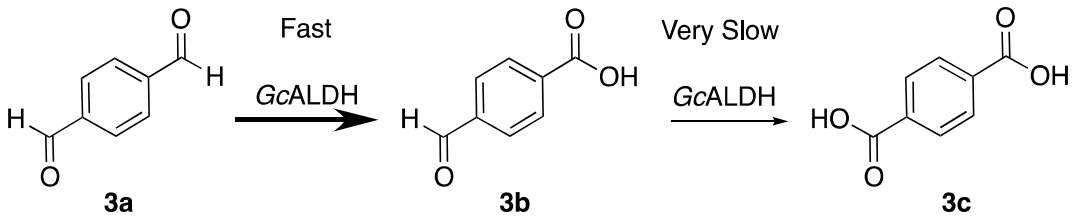


Fig. 2 Selective oxidation of terephthalaldehyde **3a** by *Gc*ALDH

Table 1. Substrate specificity of *Gc*ALDH

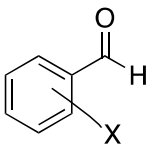
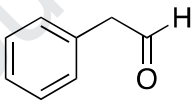
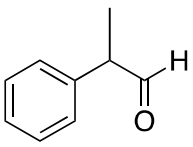
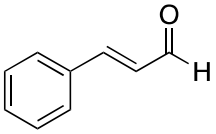
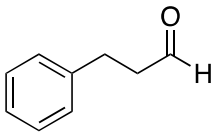
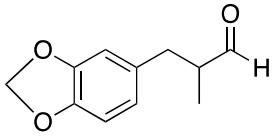
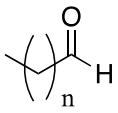
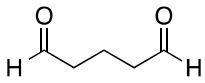
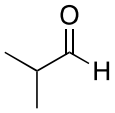
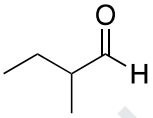
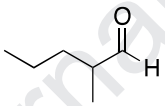
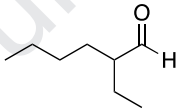
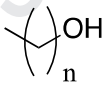
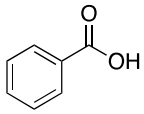
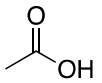
Entry	Substrate		Relative activity (%)[a]
1		X= H	100
			2[b]
2		*o*-F	75
3		*o*-Cl	19
4		*o*-Br	8
5		*o*-CF_3	<1
6		*o*-CH_3	10
7		*p*-F	178
8		*p*-Cl	122
9		*p*-Br	98
10		*p*-$COOCH_3$	123
11		*p*-CF_3	151
12		*p*-CH_3	42
13		*p*-$C(CH_3)_3$	8
14		*p*-OCH_3	17
15			10
16			9
17			16
18			310
19			168

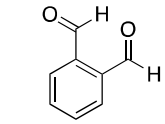
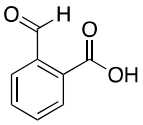
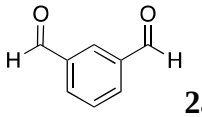
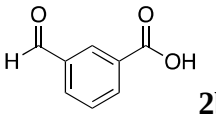
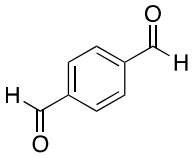
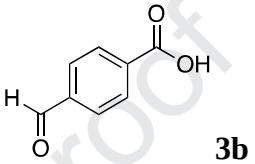
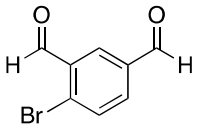
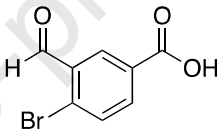
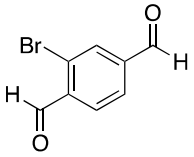
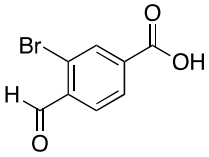
Table 1. continued

Entry	Substrate		Relative activity (%)[a]
20		n= 1	123
21		2	208
22		3	302
23		4	436
24		5	434
25		6	313
26		7	197
27		8	50
28			413
29			78
30			85
31			101
32			4
33		n= 6	<1
34		7	<1
35			<1[c] <1[d]
36			<1[c]

The activity was measured at 37 °C in 0.10 M HEPES-NaOH buffer pH 7.2, containing 5.0 mM substrate and 0.1 mM NAD^+ by monitoring the absorbance at 340 nm. Relative activity was based on benzaldehyde (entry 1) activity as 100% (12 mU). One unit of enzyme activity was defined as the micromoles of NADH released by the oxidation of benzaldehyde by enzyme per minute at 37 °C. [a]For preparative scale oxidation of aldehydes to carboxylic acids, the reaction was conducted at 40 °C for 3 h in HEPES-NaOH buffer (20 mL 0.10 M pH 7.2), containing an aldehyde (0.20 mmol), NAD^+ (0.20 mmol) and *Gc*ALDH cell-free extract (2.5 U). Benzoic acid derivatives, X=H (21 mg, 85%), *o*-F (24

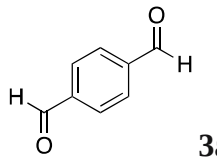
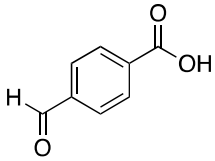
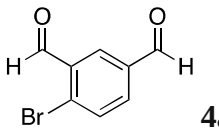
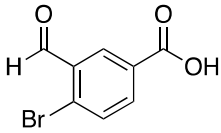
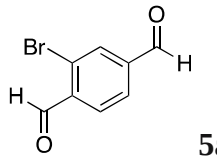
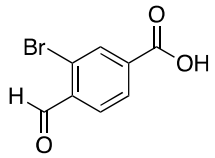
mg, 85%), *o*-Cl (10 mg, 32%), *p*-F (24 mg, 86%), *p*-Cl (25 mg, 80%), *p*-Br (31 mg, 77%), *p*-CF_3 (27 mg, 72%), *p*-CH_3 (20 mg, 74%), *p*-OCH_3 (20 mg, 64%), cinnamic acid (16 mg, 54%), 3-phenylpropanoic acid (22 mg, 74%), heptanoic acid (14 mg, 54%), nonanoic acid (14 mg, 43%). [b]$NADP^+$ was used in substitution for NAD^+. [c]NADH was used in substitution for NAD^+. [d]NADPH was used in substitution for NAD^+.

Table 2. Differences in *Gc*ALDH activities toward dialdehydes and aldehydic acids

Entry	Substrate dialdehyde	Relative activity (%)	Substrate aldehydic acids	Relative activity (%)
1	 **1a**	< 1	 **1b**	< 1
2	 **2a**	313	 **2b**	78
3	 **3a**	357	 **3b**	7
4	 **4a**	113	 **4b**	9
5	 **5a**	115	 **5b**	9

The activity was measured at 37 ^{o}C, in 0.10 M HEPES-NaOH buffer pH 7.2, containing 5.0 mM substrate and 0.1 mM NAD^{+}. Relative activity was based on benzaldehyde activity as 100% as in Table 1.

Table 3. Preparative scale selective oxidation of dialdehydes to aldehydic acids

Substrate	Product	Isolated yield (%)[a]	Regioselectivity (%)
 **3a**	 **3b**	90[b]	-
 **4a**	 **4b**	71[c]	>99 %
 **5a**	 **5b**	62[d]	>99 %

The reaction was conducted at 40 ^{o}C and 160 rpm for 4 h in HEPES-NaOH buffer (20 mL, 0.10 M, pH 7.2), containing aldehydes (0.20 mmol), NAD^{+} (0.20 mmol) and purified *Gc*ALDH (1.0 U). [a]By-products including dicarboxylic acids were not detected based on TLC analysis using UV (254 nm) lamp. [b]**3b**: 27 mg, [c]**4b**: 33 mg, [d]**5b**: 29 mg.

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Highlight

- An enzyme, *Geotrichum candidum* aldehyde dehydrogenase (*Gc*ALDH), was overexpressed.
- *Gc*ALDH was used for oxidation of aldehydes to carboxylic acids for the first time.
- Dialdehydes were oxidized to aldehydic acids without further oxidation to diacids.
- Regioselectivities for oxidation of dialdehydes to aldehydic acids were excellent.
- Preparative scale reactions were performed successfully.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Declaration of interest: none