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Characterisation of a Recombinant NADP-Dependent Glycerol Dehydrogenase from *Gluconobacter oxydans* and its Application in the Production of L-Glyceraldehyde

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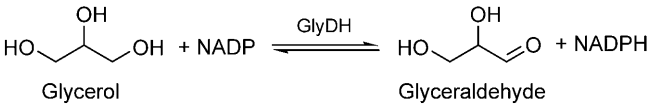
The acetic acid bacterium *Gluconobacter oxydans* has a high potential for oxidoreductases with a variety of different catalytic abilities. One putative oxidoreductase gene codes for an enzyme with a high similarity to the $NADP^+$-dependent glycerol dehydrogenase (GlyDH) from *Hypocrea jecorina*. Due to this homology, the GlyDH (Gox1615) has been cloned, over-expressed in *Escherichia coli*, purified and characterised. Gox1615 shows an apparent native molecular mass of 39 kDa, which corresponds well to the mass of 37.213 kDa calculated from the primary structure. From HPLC measurements, a monomeric structure can be deduced. Kinetic parameters and the dependence of the activity on temperature and pH were determined. The enzyme shows a broad substrate spectrum in the reduction of different aliphatic, branched and aromatic aldehydes. Additionally, the enzyme has been shown to oxidize a variety of different alcohols. The highest activities were observed for the conversion of D-glyceraldehyde in the reductive and L-arabitol in the oxidative direction. Since high enantioselectivities were observed for the reduction of glyceraldehyde, the kinetic resolution of glyceraldehyde was investigated and found to yield enantiopure L-glyceraldehyde on preparative scale.

Introduction

Oxidoreductases (EC 1.1.1.*x*) are cofactor-dependent enzymes that are ubiquitously distributed and catalyse different reactions of biotechnological interest. Alcohol dehydrogenases (ADHs) are members of this enzyme class. Their ability to catalyse the chemo-, regio- and stereoselective reduction of ketones makes them versatile tools for the production of chiral building blocks.[1,2] These chiral building blocks act, for example, as key intermediates in the production of pharmaceuticals, fine chemicals and natural products.[3–5] Due to their stereochemical properties, including the high enantioselectivity, ADHs with a broad substrate spectrum are of primary importance for various technical applications.

Enantiopure glyceraldehyde is an example of a small chiral building block of great interest for further synthesis because of the wide applicability of its structural motif. It has been successfully used in the synthesis of, for example, enantiopure 4-(dihydroxyalkyl)-β-lactams (antibiotic)[6] or 8a-*epi*-swainsoine (anticancer drug).[7] Although the chemical synthesis of both glyceraldehyde enantiomers is possible on laboratory scale,[8,9] the major drawbacks are the extensive and expensive procedures. The chemical synthesis of D-glyceraldehyde starts with D-mannitol, whereas the L-enantiomer is derived from L-arabinose. Up to now, only the synthesis of D-glyceraldehyde was realized on industrial scale. With regard to environmental aspects and cost factors new short enzymatic routes for the preparation to enantiopure glyceraldehyde are desirable. Therefore, enzymes catalysing either the selective oxidation of glycerol or the selective reduction of glyceraldehyde are of great interest. The class of enzymes that catalyse the NADPH-dependent reduction of glyceraldehyde to glycerol are called $NADP^+$ glycerol dehydrogenases (GlyDHs; EC 1.1.1.72; Scheme 1). These enzymes are common in moulds and filamentous fungi and have been isolated from mammalian tissues.[10–14] So far, different bacterial GlyDHs have been reported to catalyse the reduction of dihydroxyacetone (DHA) to glycerol, and can be classified as glycerol:$NADP^+$ 2-oxidoreductases



Scheme 1. General reaction scheme of the NADP(H)-dependent oxidation and reduction catalysed by GlyDHs.

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(EC 1.1.1.156) or glycerol:NAD^+ 2-oxidoreductases (EC 1.1.1.6).[15–20] Among the GlyDHs catalysing the reduction of glyceraldehyde only the GlyDH from *Hypocrea jecorina*[12] has been heterologously expressed, purified and characterised whereas the others have been characterised by using purified enzymes from wild-type stains.

Acetic acid bacteria like *Gluconobacter oxydans* show high activities in the oxidation of a wide range of substrates, such as alcohols, sugar, sugar acids, and sugar alcohols. For such oxidative reactions *G. oxydans* has numerous membrane-bound and cytosolic oxidoreductases, which are of great biotechnological interest, due to their high stereo- and regionselectivity. *G. oxydans* is applied, for example, in the industrial production of 2-keto-L-gulonic acid, which is a vitamin C precursor, 6-amino-L-sorbose, which is a miglitol precursor, and the production of DHA, which is an ingredient in tanning agents.[21] Because of this ability we selected *G. oxydans* for a homology search to identify oxidoreductases for the enzymatic production of enantiopure glyceraldehyde. After homology searching based on the sequence of GlyDH, GDL1 from *H. jecorina*, which is known to catalyse the reduction of glyceraldehyde and DHA to glycerol, we identified an oxidoreductase with the ability to act specifically on glyceraldehyde.

Herein, we report the cloning, heterologous expression and biochemical characterisation of the GlyDH from *G. oxydans*. Furthermore, we show its applicability in the kinetic resolution of glyceraldehyde and the production of enantiopure L-glyceraldehyde on the preparative scale.

Results

Identification of the GlyDH gene

The genome of *G. oxydans* 621H was recently sequenced by Prust and co-workers.[22] Seventy five genes were annotated to be putative oxidoreductases of unknown function. Therefore, *G. oxydans* was selected for a BLAST search by using the sequence of GLD1 (DQ_422037), which is a NADPH-dependent glycerol dehydrogenase from the mould *H. jecorina*,[12] as template. The named enzyme has previously been described by Liepins et al.[12] and found to catalyse the conversion of D- and L-glyceraldehyde to glycerol. The protein sequence of GLD1 was used to find homologies in the genome of *G. oxydans* 621H and the putative oxidoreductase Gox1615 (YP_192012; Figure 1) was identified. A pairwise alignment between the protein sequences of the two enzymes showed an identity of 17%, a similarity of 32% and 14% gaps. The corresponding gene *gox1615* encodes for a protein with a calculated molecular mass of 37.213 kDa.

Cloning and heterologous over-expression of the GlyDH

In order to improve expression and simplify the purification of the GlyDH, standard cloning techniques were applied to create a recombinant plasmid containing the *glydh* gene with a hexahistidine affinity purification motif (His-tag) fused to the N terminus of the protein. This recombinant protein was expressed in *E. coli* BL21(DE3) at different temperatures and was found to have optimum activity at 25 °C. In the crude extract the protein with the N-terminal His-tag showed a specific activity of 9.2 U mg^{-1} for the reduction of glyceraldehyde. Soluble protein was expressed to a medium level (Figure 2, lane 1). The His-tag allowed the purification of the protein through immobilized metal affinity chromatography (IMAC).

Purification of the GlyDH

The recombinant enzyme with N-terminal His-tag was purified in one chromatographic step by using IMAC with Ni-NTA resin (Table 1). The purification resulted in recovery of 67% of the activity measured in the crude extract. The specific activity of Gox1615 after purification was 36.4 U mg^{-1} corresponding to a fourfold improvement in activity compared to the crude extract. Samples of the crude extract and target fraction after purification were analysed by SDS-PAGE (Figure 2). A single band

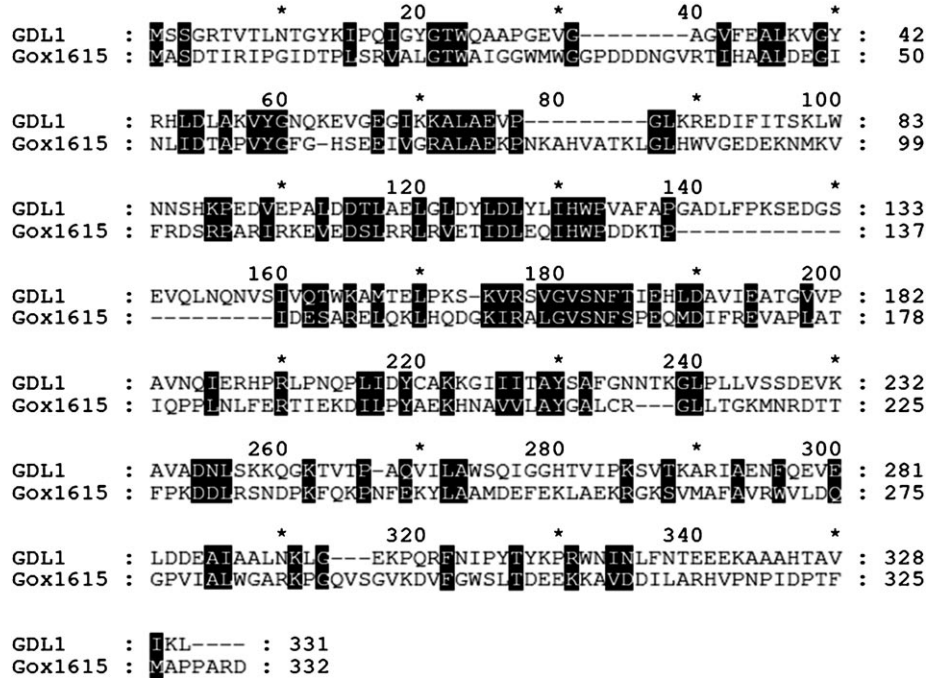

Figure 1. Comparison of amino acid sequences of the GlyDH from the mould *H. jecorina* (GDL1) and the α-proteobacterium *G. oxydans* (Gox1615). The alignment was created with the GeneDoc software. Shaded regions in black indicate conserved or similar amino acids. The two GlyDHs show a homology of 32%.

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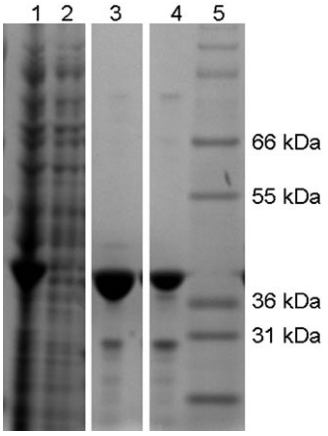

Figure 2. SDS-PAGE analysis of the purified GlyDH from *G. oxydans*. Lane 1: crude extract (25 μg); lane 2: flow through (25 μg); lane 3: purified Gox1615 (12.5 μg); lane 4: purified Gox1615 after desalting (6.25 μg); lane 5: molecular weight marker. Lanes 3 and 4 show a single major band at an apparent molecular weight of 39 kDa.

Table 1. Purification of the recombinant GlyDH from *E. coli* BL21(DE3).

Purification step	Total activity [U mL^{-1}]	Specific activity [U mg^{-1}]	Purification factor	Yield [%]
crude extract	10323	9.0	1	100
purified GlyDH	6867	36.4	4	66.5

with an apparent molecular size of 39 kDa corresponds to the enzyme (Figure 2, lane 4); this confirms the calculated molecular mass of 37.213 kDa derived from the amino acid sequence. It was therefore possible to purify the recombinant GlyDH almost to homogeneity (>95%) by applying a single purification step (Figure 2).

Molecular characteristics of the GlyDH

The calculated molecular mass of 37 kDa of one subunit of the GlyDH was confirmed by SDS-PAGE (39 kDa; Figure 2). Additionally, the native molecular mass was determined by using size-exclusion chromatography. The apparent molecular mass of the GlyDH was estimated to be 31 kDa; this suggests a monomeric structure.

Studies concerning cofactor dependency showed that Gox1615 prefers NADPH over NADH. Using NADPH, we observed a 200-fold increase in activity compared to the activity achieved with the same concentration of NADH (data not shown).

Characterisation of the kinetic parameters

The kinetic properties of the GlyDH during reduction and oxidation were characterised based on the standard substrates glyceraldehyde and L-arabitol. Respective kinetic constants for the reduction of glyceraldehyde and oxidation of L-arabitol were determined. Additionally, kinetic parameters for NADPH or rather $NADP^+$ were estimated by using the purified enzyme. Michaelis–Menten constants determined during reduction of glyceraldehyde were 2.69 ± 0.35 mM (K_M) and 77.9 ± 3.5 U mg^{-1} (V_{max}). For the cofactor NADPH, inhibition at concentrations above 0.15 mM was observed. Therefore, an equation for substrate-excess inhibition was used ($v = V_{max} \times s/(K_M + s(1 + s/K_i))$) for fitting to the Michaelis–Menten equation. The estimated kinetic constants are a K_M value of 0.04 ± 0.009 mM, a V_{max} of 101 ± 13 U mg^{-1} and a K_i value of 0.4 ± 0.1 mM. For the substrate L-arabitol the enzyme showed a hyperbolic reaction velocity in a plot versus substrate concentration, with a K_M value of 193 ± 11 mM and a V_{max} of 8.67 ± 0.2 U mg^{-1}. The oxidized cofactor $NADP^+$ follows a typical Michaelis–Menten equation with a K_M value of 0.02 ± 0.001 mM and a V_{max} of 2.1 ± 0.02 U mg^{-1}.

Dependence of GlyDH activity on temperature and pH

Since the influence of temperature on enzyme activity and stability is often a limiting parameter in technical applications, studies concerning the temperature optimum and stability were performed. Figure 3A illustrates that the purified enzyme has optimum activity at 55 °C, whereas the activity is approximately 50% lower when the temperature is changed to 57 or 37 °C. The pH dependence during the reduction of glyceraldehyde was measured in the range of pH 3 to 10. As demonstrated in Figure 3B the optimal activity was determined to be at pH 5.5. The pH dependence of the enzyme was examined by using TEA (pH 6.8 to 10.0) and citrate phosphate buffers (pH 3 to 7.2). The best activities at the same pH values (pH 7.0) were observed by using citrate phosphate buffer.

For the determination of the pH optimum in the oxidation of L-arabitol, TEA buffer (pH 7.0 to 9.2) and potassium carbonate buffer (pH 9.2 to 11) were used. The optimum activity was rather broad with a maximum at pH 10.0 (Figure 3C). At pH 9.2 GlyDH showed an activity of 3.1 U mg^{-1} in TEA buffer and 3.2 U mg^{-1} in potassium carbonate buffer. These results indicate a similar activity in both buffers.

Enzyme stability is also a very crucial parameter for technical applications and processes; hence temperature-dependent inactivation over time and pH and the thermostability of the GlyDH were investigated. Studies concerning the thermostability of the purified enzyme were performed at different temperatures (4, 20 and 30 °C) over 48 h, and at 50 °C over 8 min. As shown in Figure 4A the enzyme is stable at 4, 20 and 30 °C during the investigated period, but at 50 °C a rapid inactivation of the enzyme was found (Figure 4B). After incubation for 4 min only 50% activity was observed and after 8 min no residual activity was detected. In a second study the midpoint thermal inactivation (T_M) of Gox1615 was determined to be 47.2 °C (Figure 4C).

The pH stability was investigated at three different pH values (pH 5.5, 7.0 and 9.7) over a period of 43 h by using the standard activity assay for the reduction of glyceraldehyde, determining the residual activity. At all pH values the enzyme was stable and showed a residual activity of 93% (pH 7), 84%

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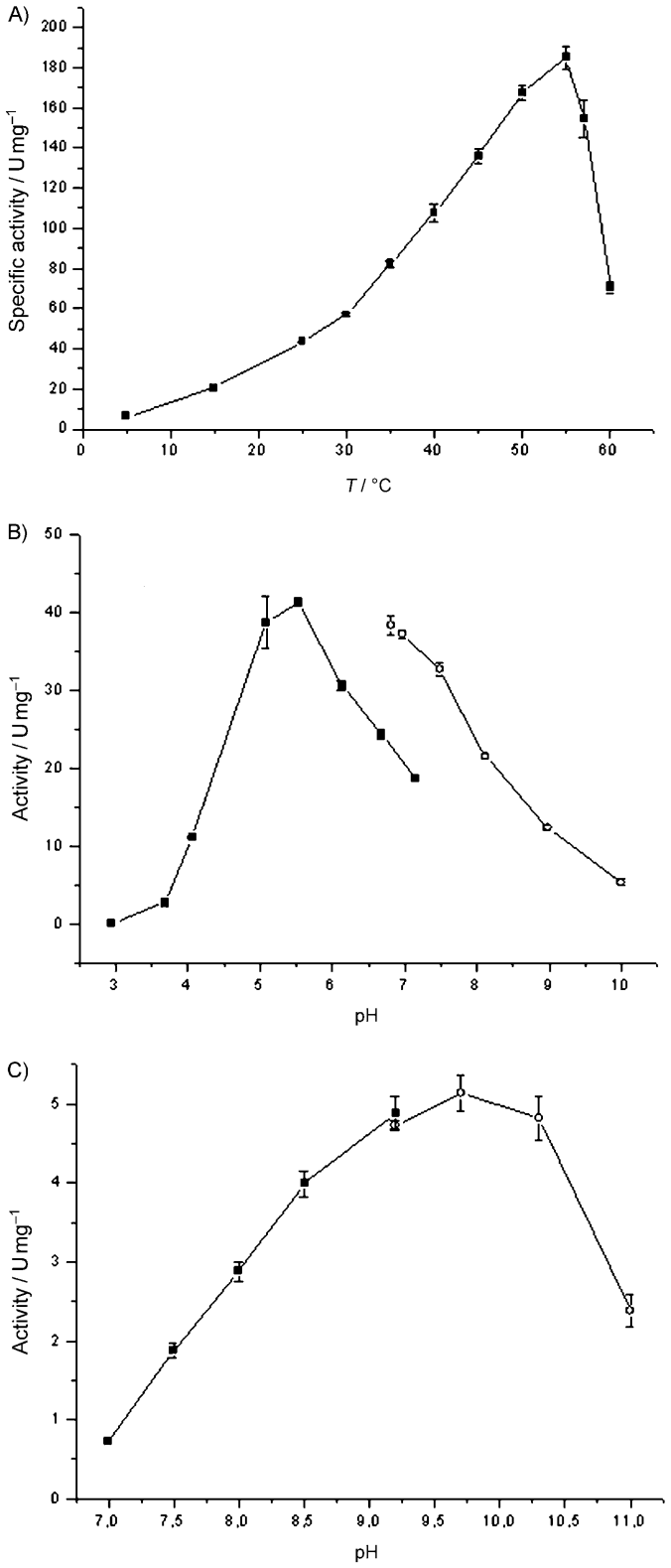

Figure 3. Determination of optimum: A) temperature, and pH for the B) reduction and C) oxidation reactions of the GlyDH. The effects of temperature and pH on enzyme activity were measured by using the standard spectrophotometric assays for reduction and oxidation. For the determination of the optimum pH in the reduction of glyceraldehyde two different buffers, citrate phosphate buffer (■; pH 3.0 to 7.2) and TEA (○; pH 6.8 to 10.0) were used. The pH optimum for the oxidation reaction was determined by using TEA (■; pH 7.0 to 9.2) and potassium carbonate buffer (○; pH 7.0 to 11.0).

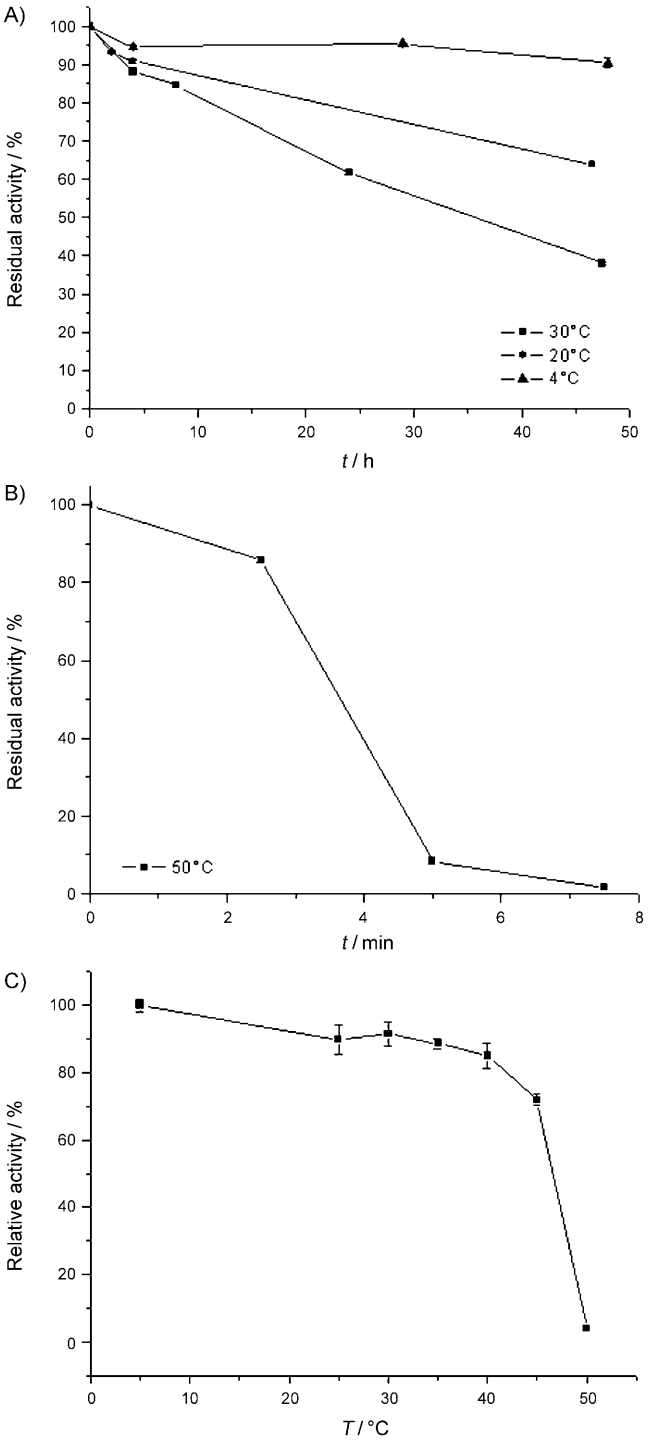

Figure 4. Thermal stability of Gox1615. The effect of temperature over a period of 48 h at: A) 4, 20 and 30 °C, and B) 50 °C was investigated. For the determination of the residual activity the standard spectrophotometric assay for reduction was used. C) For the determination of the midpoint thermal inactivation (T_M) the different temperatures were maintained for 10 min before samples were withdrawn for measuring the residual activity in the soluble protein content. The standard deviations are indicated in the diagram.

(pH 5.5) and 82% (pH 9.7); the results indicate the enzyme's applicability at these pH values.

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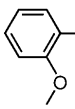
Substrate specificity of the GlyDH

Substrate specificity of the enzyme was assessed by using spectrophotometric assays for either the reduction or oxidation reaction. The specific activities of the purified enzyme in the reduction of different aliphatic, branched and aromatic aldehydes and ketones and oxidation of a variety of alcohols were determined. For the reduction reaction activities were compared to the standard substrate glyceraldehyde (entry 9, specific activity 39.1 U mg^{-1}), and for the oxidation reaction L-arabitol (entry 44) was used as standard substrate (2.46 U mg^{-1}). By comparing the reduction and oxidation reactions, it became obvious that the GlyDH favours the reduction reaction: the observed specific activities were up to 19-times higher than those observed for the oxidation. In the reduction the best activity was obtained with D-glyceraldehyde as substrate; activities towards other substrates are shown in Table 2. In general, aliphatic, branched and aromatic aldehyde substrates are accepted by the GlyDH.

In the oxidation the highest activity was achieved by using L-arabitol as substrate. Various other substrates with up to six alcohol groups were oxidized with different specific activities (Table 3).

Table 2. Substrate spectrum in the reduction catalysed by the GlyDH from *G. oxydans*. The activity was obtained by using the standard spectrophotometric assay.

	Substrate	Specific activity [U mg^{-1}]
Aldehydes		
1		0.1
2		1.1
3		23.3
4		31.9
5		4.9
6		35.5
7		15.9
8		19.3
9		39.1
9a		46.6
9b		2.0
10		6.7
11		26.8
12		25.7
13		13.8
14		19.6
15		0
16		0.3
Ketones		
17		0.02
18		0.04
19		0.03
20		0.03
21		0.5

Kinetic resolution of glyceraldehyde

L-Glyceraldehyde is a versatile building block and has been successfully applied in a broad range of syntheses.[6,7,23,24] Enantiopure glyceraldehyde can be obtained by regio- and stereoselective oxidation of glycerol or the stereoselective reduction of racemic glyceraldehyde. Thus, preparative scale kinetic resolution of glyceraldehyde was performed with simultaneous regeneration of NADPH by using glucose and glucose dehydrogenase (GDH; Scheme 2). The conversion and enantiomeric excess (*ee*) were determined by means of HPLC and GC.

Figure 5 illustrates a typical time curve for the kinetic resolution of glyceraldehyde using crude extract of GlyDH from *G. oxydans*. The figure shows the development of glyceraldehyde and glycerol concentrations, and additionally the *ee* value of L-glyceraldehyde, measured over a period of 26 h. A conversion of 50% and an *ee* > 99% was achieved by using Gox1615 in the kinetic resolution of glyceraldehyde on preparative scale.

Discussion

In the present study, a GlyDH from *G. oxydans* (Gox1615) was successfully cloned, expressed, purified, and biochemically characterised. Gox1615 was annotated as a putative oxidoreductase and showed 32% homology to the protein sequence of the GlyDH GLD1 from *H. jecorina*.[12] Both enzymes favour the reduction of glyceraldehyde compared to DHA. Therefore, Gox1615 belongs to the glycerol:NADP$^+$ oxidoreductases (EC 1.1.1.72). Other purified and characterised enzymes belonging to this class are, for example, the GlyDH from *Neurospora crassa*[10] and the GlyDH from rabbit skeletal muscle.[13] Among these known members of this enzyme class Gox1615 is the only GlyDH of bacterial origin.

Moreover, quaternary structure comparison of different GlyDHs showed a considerable difference between the native conformations of Gox1615 and the GlyDH from *N. crassa*. Our results indicate that Gox1615, much like the GlyDH from rabbit

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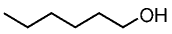
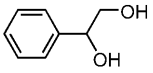
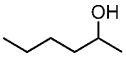
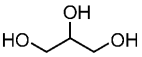
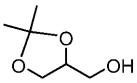
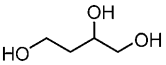
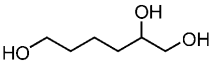
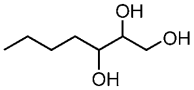
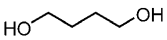
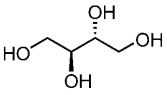
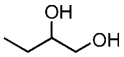
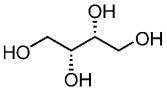
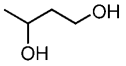
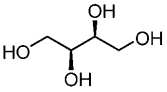
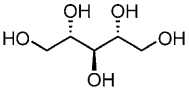
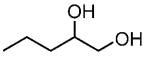
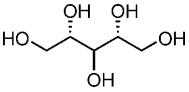
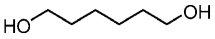
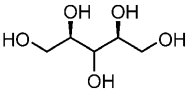
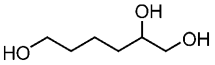
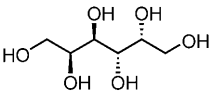
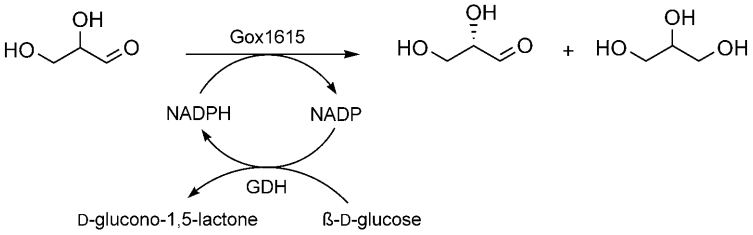
Table 3. Substrate spectrum in the oxidation catalysed by the GlyDH from *G. oxydans*. The activity was obtained by using the standard spectrophotometric assay.

	Substrate	Specific activity [U mg^{-1}]		Substrate	Specific activity [U mg^{-1}]
22		0.74	34		0.77
23		0.22	35		0.17
24		0.03	36		1.21
25		0.05	37		0.45
26		0.5	38		0.76
27		0.21	39		0.75
28		0.66	40		0
29		0.04	41		0.90
30		0.59	42		0.25
31		0.39	43		0
32		0.87	44		2.46
33		0.30	45		1.08



Scheme 2. Scheme of the reduction of glyceraldehyde by using Gox1615.

muscle, has a monomeric structure whereas the GlyDH from *N. crassa* is a tetrameric protein. A biochemical comparison of the described GlyDHs and Gox1615 revealed a number of similarities with respect to substrate specificity, but also a number of differences concerning selectivity.

Regarding substrate specificity, Gox1615 acts on a broad substrate range in the reduction as well as in the oxidation reaction, with the reduction being the favoured reaction as evident from a 19-fold higher activity for D-glyceraldehyde compared to L-arabitol, which is the best substrate in the oxidation.

With respect to the oxidative potential, Gox1615 showed a broad acceptance of alcohols with up to six alcohol groups. The best specific activity was reached with the oxidation of L-arabitol, for which Gox1615 oxidized the L enantiomer with a specific activity of 2.5 U mg^{-1}, whereas no conversion was detected with the D enantiomer. These results lead to the suggestion that as with the reduction the enzyme shows stereo- and regioselectivity in the oxidation.

This broad substrate range together with the assumed stereo- and regioselectivity in the oxidation make Gox1615 a versatile tool for enzymatic oxidation in the production of chiral hydroxyaldehydes. This enzymatic oxidation opens an environmentally-friendly process of great interest[25,26] as an al-

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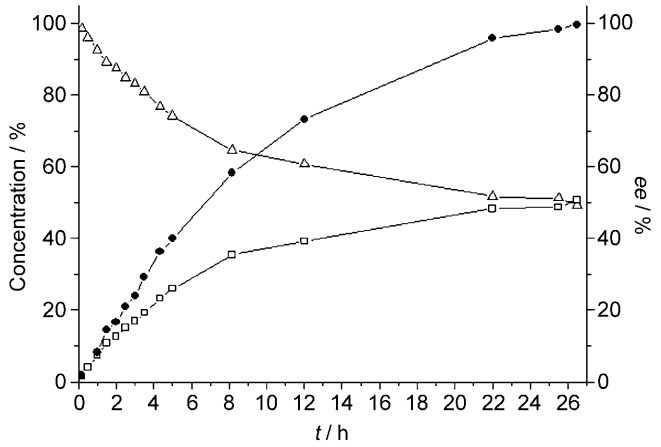

Figure 5. Kinetic resolution of glyceraldehyde by Gox1615. The figure shows the development of glycerol (□) and *rac*-glyceraldehyde (△) concentration over a period of 26 h. Concentrations were determined by HPLC. The determination of the *ee* for L-glyceraldehyde (●) was carried out by using GC.

ternative to the metal-catalysed chemical oxidations. Additionally, the chemical oxidations often suffer from a lack of regio- and stereoselectivity—a complicated protecting group strategy is necessary to obtain the desired products.[27–30] Therefore, the oxidative potential of Gox1615 is currently under investigation.

Due to the named preference for the reduction, the main focus of this work was the investigation of the substrate specificity and selectivity in this reaction. We could show that Gox1615 reduces different aliphatic aldehydes and showed the best activity with a medium chain length of C_4 to C_8 (entries 3, 4, 6), which is similar to the GlyDH from rabbit skeletal muscle. A comparison of the unsaturated aldehyde *trans*-hex-2-enal (entry 5) with hexanal (entry 4) showed significant variations in activity; this indicates the preference for saturated substrates. Branched-chained and aromatic aldehydes (entries 13 and 14) have also been accepted by the enzyme; this emphasises the broad substrate range of Gox1615 in the reduction of different aldehydes.

Interestingly, Gox1615 showed almost no activity in the reduction of ketones. The other GlyDHs so far known from the literature show an inferior selectivity in the discrimination of ketones and aldehydes compared to that observed for Gox1615. All four compared enzymes show the highest activity in the reduction of glyceraldehyde (entry 9), but differ in their ability to reduce DHA (entry 21). The GlyDH from *N. crassa* reduces DHA with 43% activity relative to the activity obtained for D-glyceraldehyde, GLD1 with 20%, the GlyDH from rabbit skeletal muscle with 4%. Gox1615 showed the lowest side activity towards DHA in the range of 1%; this means a quite high regioselective reaction and a good discrimination of aldehydes and ketones by Gox1615 compared with the other enzymes.

Comparison of the enantiopreference of the different GlyDHs showed that while GLD1 has no enantiopreference in the reduction of glyceraldehyde, the other GlyDHs displayed a preference for the reduction of the D enantiomer. As a result a lower activity in the reduction of L-glyceraldehyde compared to that of the D enantiomer was observed. Gox1615 reduced L-glyceraldehyde with only 4% of the activity reached for the D enantiomer, compared to the higher activity (36%) obtained for the GlyDH from *N. crassa* and rabbit skeletal muscle (Table 4). Therefore, Gox1615 showed the best stereoselectivity

Table 4. Comparisson of the biochemical properties of Gox1615, GLD1 and the GlyDHs from *N. crassa* and rabbit skeletal muscle.

	Gox1615 (*G. oxydans*)	GLD1 (*H. jecorina*)	GlyDH (*N. crassa*)	GlyDH (rabbit skeletal muscle)
DHA acceptance (D-Ga[a]/DHA)	100:1	100:20	100:43	100:4
stereopreference	D-specific	no preference	D-specific	D-specific
(D-Ga[a]/L-Ga*)	100:4	100:93	100:36	100:36
molecular weight	39000 (monomer)	– –	43000 (tetramer)	34000 (monomer)
pH optimum	reduction: 5.5 oxidation: 10	– –	reduction: 6.5 oxidation: 9.5	reduction: 5.0–7.0

[a] Ga = glyceraldehyde.

as evident from a ninefold lower activity with L-glyceraldehyde compared to the other GlyDHs. The enantiopreference was proven by GC analysis, with which Gox1615 showed an *ee* > 99% for L-glyceraldehyde.

Due to this excellent enantioselectivity a kinetic resolution of racemic D,L-glyceraldehyde was our primary focus. In the literature, several reports can be found on kinetic resolution by using oxidoreductases in the oxidative direction, starting, for example, from racemic alcohols.[26,31–33] But only few examples are described that employ reductive resolution of racemates, such as the kinetic resolution of racemic bicyclo[3.3.1]nonane-2,6-dione[34] by using whole genetically engineered cells of *Saccharomyces cerevisiae* as the catalyst. More common are kinetic resolutions with hydrolases[34–36] due to their easy applications without the need for coupled cofactor regeneration systems. The advantage of using oxidoreductases for kinetic resolutions are the excellent stereoselectivity of these enzymes for most of the substrates, compared to hydrolases, which often show moderate stereoselectivity, especially towards primary alcohols.[37]

Additionally, L-glyceraldehyde is a valuable chiral building block that has to be synthesised by using expensive L-arabinose as starting material. Consequently, L-glyceraldehyde has not been easily accessible at industrial scale. Furthermore, our results show that by using Gox1615 in the kinetic resolution of glyceraldehyde the optimal conversion of 50% was reached with an *ee* > 99%.[36] The kinetic resolution was also performed on preparative scale (4.05 g, 45 mmol) with a high *ee* value. Therefore, the present direct enzymatic one-step preparation of enantiopure L-glyceraldehyde represents an important milestone towards a viable industrial bioprocess.

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Experimental Section

General: If not stated otherwise all chemicals were purchased from Sigma–Aldrich. All columns and instruments for chromatography (FPLC, Äkta-Explorer) were purchased from GE Healthcare. Centrifugations were carried out by using the centrifuges RC5BPlus (Sorvall), Mikro22 and Rotina 35R (Hettich). For analytical methods GC-17A and LC-20AHT (both Shimadzu) were used. Restriction enzymes were purchased from Fermentas. The studied GlyDH is commercially available at evocatal (evo-1.1.190).

Molecular cloning of the GlyDH gene: Genomic DNA from *G. oxydans* (DSM 2323) was used as template for the amplification of the *glydh* gene. For the following cloning steps primers with recognition sites for the restriction enzymes NdeI and SalI were used for the construction of the enzyme with N-terminal His-tag in pET-28a (5′-ATA TAT ACA TAT GGC ATC CGA CAC CAT CCG CAT CCC C-3′ and 5′-ATA TGT CGA CTC AGT CCC GTG CCG GGG GC-3′). The PCR product was cloned into pET-28b (Novagen) between the NdeI and SalI restriction sites by using standard techniques.

Heterologous expression of the GlyDH: *E. coli* BL21(DE3) cells carrying the recombinant plasmid were cultivated in LB medium (5 mL)[38] containing ampicillin (100 μg mL^{-1}), overnight, at 37 °C. These cultures were used to inoculate different amounts of LB medium containing ampicillin (100 μg mL^{-1}) for expression in shaking flasks at a final concentration of 0.05 optical density at 600 nm (OD_{600}). The cultures were grown at 37 °C. When the OD_{600} reached 0.5 to 0.7, production of the recombinant GlyDH was induced by addition of isopropyl thio-β-D-galactoside (IPTG; 0.3 mM). For the determination of the optimal growth conditions cultures were grown at 25, 30 and 37 °C (after induction) and assayed after a period of 20 h.

Purification of the GlyDH: The bacterial culture was harvested by centrifugation at 17000*g* for 20 min at 4 °C. A cell suspension (10%) was prepared in triethanolamine buffer (TEA, 100 mM) pH 7. Cells were disrupted by five sonication cycles of 5 min (40% power output) with cooling periods in between. The lysed cells were centrifuged at 17000*g* for 30 min at 4 °C, and the supernatant was used for further purification steps. The enzyme was purified from the crude extract by using immobilised metal affinity chromatography IMAC (Ni-NTA, Qiagen). The column was equilibrated with TEA buffer (50 mM, pH 7). The GlyDH was eluted by using a linear gradient of imidazole (0 to 500 mM) in TEA buffer. After a desalting step by gel filtration on Sephadex G25 (Pharmacia) the enzyme was freeze dried and stored at −20 °C until use.

Activity assay: GlyDH activities for the reduction or oxidation reactions were determined by measuring the initial velocity change in absorbance at 340 nm. For the enzymatic reduction racemic glyceraldehyde was used as standard substrate. One unit of activity was defined as the amount of enzyme that catalyses the oxidation of 1 μmol NADPH per minute under standard conditions (30 °C, pH 7). The reduction assay mixture contained substrate solution (970 μL of 10 mM substrate in 100 mM TEA buffer, pH 7), NADPH (20 μL, 12.5 mM) in distilled water and enzyme solution (10 μL). For the oxidation the standard conditions were 37 °C and pH 9.7 with L-arabitol as substrate. The assay mixture contained assay solution (990 μL of 1 mM NADP, 30 mM ammonium sulfate, 100 mM substrate in 100 mM potassium carbonate buffer, pH 9.7) and enzyme solution (10 μL). Reactions were started by addition of the enzyme solution and measured over 1 min. All measurements were carried out at least in triplicate.

Protein analysis: Protein concentrations were determined according to the Bradford by using BSA as a standard.[39] SDS-PAGE was performed by using Bis-Tris gels (4–12%) in MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer (Invitrogen); Mark 12 protein standard (Invitrogen) was used for molecular weight estimation of proteins. The molecular mass of the GlyDH was determined by using size-exclusion chromatography. Therefore, a BioSep SEC S2000-HPLC column (300×7.8 mm, Phenomenex) connected to a Shimadzu LC-20AHT system with Tris-HCl buffer (200 mM, pH 7.5) as mobile phase (1 mL min^{-1}) was used. The lyophilized protein and the protein standard (AQUEOUS SEC 1, Phenomenex) were dissolved in Tris-HCl buffer (200 mM, pH 7.5) and analysed by HPLC.

Determination of pH and temperature optima: In order to obtain the temperature optimum of the GlyDH the standard reduction enzyme assay was performed at different temperatures. The pH optimum was determined by using both standard assays at different pH values (reduction and oxidation). For the investigation of the pH optimum for the reduction reaction the standard activity buffer TEA (pH 6.8 to 10.0) as well as citrate phosphate buffer (pH 3.0 to 7.0) were used. For the oxidation reaction potassium carbonate buffer (pH 9.2 to 11.0) and TEA buffer (pH 7.0 to 9.2) were used.

Stability investigations: For the investigation of temperature and pH stability the enzyme was incubated at different temperatures and pH values. At appropriate time intervals samples were taken and the residual activity was assayed by using the standard reduction assay. For the determination of the midpoint of thermal inactivation (T_M) each temperature was maintained for 10 min before samples were withdrawn for the assessment of the residual activity.

Determination of kinetic constants: To determine the kinetic constants (K_M und V_{max}) the standard activity assays of reduction and oxidation were used. All parameters were kept constant, only the investigated parameter, cofactor or substrate concentration was modified. For the determination of the kinetic parameters the Michaelis–Menten equation was used with the exception of the cofactor NADPH (see the Results section).

Preparative kinetic resolution of glyceraldehyde: The reaction mixture was prepared by dissolving racemic glyceraldehyde (4.05 g, 0.045 mol, 150 mM), glucose (8.92 g, 0.045 mol, 150 mM), ammonium sulfate (7.93 g, 0.06 mol, 200 mM) and $NADP^+$ (0.12 g, 0.15 mmol, 0.5 mM) in potassium phosphate buffer (250 mL, 100 mM, pH 7). The reaction was started by addition of Gox1615 crude extract (50 mL, 700 U) and glucose dehydrogenase from *Thermoplasma acidophilum* (300 μL, 460 U; Sigma: G5909). Samples (50 μL) were taken periodically and diluted either with trifluoroacetic acid (300 μL, 0.5 M) for HPLC analysis or with ethyl acetate (300 μL) for GC analysis. GC samples were derivatized for 30 min with acetic anhydride (100 μL) and pyridine (10 μL). Concentrations of glyceraldehyde and glycerol were determined by using Agilent 1100 HPLC equipped with a CS organic acid resin column (300×8 mm) with trifluoroacetic acid (5 mM, 0.7 mL min^{-1}) at 5 °C. The enantiomeric ratio for glyceraldehyde was determined by using a HP 6890 GC with a Varian CP-Chirasil-Dex CB column (25 m×0.32 mm).

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