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In vitro biosynthesis of 3-mercaptolactate by lactate dehydrogenases

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ABSTRACT

3-Mercaptolactate (3ML) is an interesting mercapto compound with special regard to the biosynthesis of new polythioesters (PTEs). Unfortunately, this thioester analog of lactic acid is currently not commercially available. For this reason, we developed an *in vitro* biosynthesis pathway to convert cysteine to 3-mercaptopyruvate (3MPy), which is then rapidly and efficiently converted to 3ML by suitable lactate dehydrogenases (LDHs). As liver LDH from *Rattus norvegicus* (LDH_{Rn}) was previously described to Exhibit 3MPy reduction activity, *in silico* studies based on homology to LDH_{Rn} were performed and led to the identification of four potentially suitable bacterial LDH candidates from *Escherichia coli* (LDH_{Ec}), *Corynebacterium glutamicum* (LDH_{Cg}), *Bacillus cereus* (LDH_{Bc}) and *Gloeobacter violaceus* (LDH_{Gv}). After heterologous expression in *E. coli* followed by purification, the enzymes were assessed for their potential to reduce 3MPy to 3ML in comparison to LDH_{Rn}. With 3MPy, LDHs from *E. coli, C. glutamicum* and *B. cereus* showed no or only very low specific activities of 0.23 ± 0.1 U/mg (LDH_{Cg}) and 0.08 ± 0.2 U/mg (LDH_{Bc}), respectively. In contrast, LDH_{Gv} exhibited a remarkable specific activity of 63.6 ± 8.1 U/mg, being even twice as active as the *R. norvegicus* LDH. To verify LDH-catalyzed biosynthesis of 3ML we developed and optimized a detection method allowing qualitative analysis and quantification of 3MPy and 3ML by derivatization with Ellman's reagent and liquid chromatography-mass spectrometry. This study shows once more the impressive versatility of LDHs and presents a rapid and efficient biosynthesis process for 3ML, a biotechnologically interesting, yet hard-to-obtain, compound.

1. Introduction

3-Mercaptolactate (3ML; IUPAC: (2*R*)-2-hydroxy-3-sulfanylpropionate) is an organic sulfur compound and the sulfur-containing structural analog of lactate. In eukaryotes, 3ML naturally occurs when cysteine is degraded via the transamination pathway by cysteine aminotransferase forming 3-mercaptopyruvate (3MPy), which is then further metabolized by 3MPy sulfurtransferase (3MST) or reduced to 3ML by lactate dehydrogenases (LDH) [1–3]. As a result of the rare inheritable disorder mercaptolactate-cysteine disulfiduria caused by MPST deficiency, high concentrations of mercaptolactate-cysteine disulfide are found in the urine [4–6]. In prokaryotes, the cysteine transamination pathway has never been investigated in detail, but it was recently shown that bacterial aminotransferases from several organisms also catalyze transamination of cysteine [7]. While a biotechnical synthesis of 3ML has never been described, there are several reports on the chemical synthesis of 3ML with rather unsatisfactory results [8,9]. Therefore, 3ML is not commercially available and the chemical synthesis on request is paralleled by high costs.

LDHs are ubiquitous in nature and catalyze the interconversion of pyruvate and lactate. There are two distinct enzyme classes within the LDHs each comprising two subclasses: NAD dependent (nLDH) and flavin-containing, NAD-independent (iLDH), each class including members being specific for either L- or D-lactate, respectively (L-nLDH: EC 1.1.1.27, D-nLDH: EC 1.1.1.28 and L-iLDH: EC 1.1.2.3; D-iLDH: EC 1.1.2.4) [10]. All known nLDHs are cytosolic enzymes, while iLDHs are frequently associated with membranes and use cytochrome as an electron acceptor [11]. The LDHs specific for L-isomers share highly homologous sequences, which can also be observed among the subclass of D-specific LDHs. However, there are barely homologies between L- and D-LDHs [12].

nLDHs catalyze the reduction of pyruvate to lactate using the cofactor NADH or *vice versa* the oxidation of lactate to pyruvate with the concomitant reduction of NAD^+. Together with alcohol dehydrogenase

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and NAD(P)H-linked L-malate dehydrogenase, nLDHs are members of the large family of dehydrogenases [13]. As alternative substrates to pyruvate and lactate, LDHs can also convert α-hydroxybutyrate, α-ketobutyrate and also 3MPy [14]. In mammals, LDH is a cytoplasmic protein and present in almost all tissues. A high amount of the enzyme is found in heart, liver, muscle and kidney tissue. Functional LDH enzymes consist of four subunits encoded by two genes (LDH-A and LDH-B), which are assembled in homo- or heterotetramers. While humans and other higher animals as well as plants possess only L-LDH, invertebrates, lower fungi and prokaryotes additionally possess D-LDH [11,12].

Bacterial LDHs are active as dimers or tetramers, and consequently, their molecular weight ranges between 70–140 kDa [10]. In bacteria, LDH is the key enzyme during lactic acid fermentation, the anaerobic version of converting sugars into energy. By reducing pyruvate to lactate, the organism can recycle oxidized NAD^+ for glycolysis. Bacteria that form large amounts of lactate by fermentation are referred to as lactic acid bacteria [15]. Due to the increasing knowledge about metabolic flux, LDH kinetics and optimal cultivation conditions, the biotechnical production of lactic acid has reached high yields of up to 98 % [16].

A simple and cost-effective method for the synthesis of 3ML would not only be of academic interest. The close structural analog 3MP is currently used as one of the few well-established precursor substrates for biosynthesis of polythioesters (PTEs) [17,18] and 3ML as a potential alternative PTE precursor could lead to the synthesis of the new and promising biologically persistent biopolymer poly(3ML) (PML). Furthermore, PML represents the sulfur-containing structural analog to polylactic acid (PLA), which is already widely used to substitute petroleum-derived plastics [19]. Non-biodegradable biopolymers like PTEs combine two important benefits: a sustainable bio-based production as well as potential applications requiring persistent materials [20].

This study focused on finding suitable LDHs to develop a simple and affordable method for the biosynthesis of 3ML from 3MPy. In combination with the previously established synthesis of 3MPy from cysteine [7], this enables biosynthesis of the PML precursor 3ML from the cheap substrate cysteine (Fig. 1). Thus, we present a rapid and efficient biosynthesis process for 3ML, a biotechnologically interesting, yet hard-to-obtain, compound.

2. Material & methods

2.1. Materials

Chemicals were purchased from Merck (Merck KGaA, Darmstadt, Germany), Roth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), or Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA) and enzymes or kits from Thermo Fisher Scientific (Waltham, MA, USA) or VWR (International GmbH, Darmstadt, Germany). Oligonucleotide synthesis and sequencing was performed by Eurofins Genomics (GmbH, Ebersberg, Germany).

2.2. Identification of bacterial LDHs with activity on 3MPy

In order to identify interesting LDH candidates, the amino acid sequence of L-LDH from *Rattus norvegicus* (UniProt No.: P04642) was used as query searching the Swissprot database of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm. nih.gov/Blast.cgi). Selected amino acid sequences (Table 1) were then aligned employing ClustalX algorithm [21] embedded in the Bioedit program [22]. The phylogenetic tree was then constructed with the neighbor joining method using NJplot [23].

2.3. Bacterial strains and cultivation conditions

All bacterial strains used in the present study are described in Table 2. The donor strains for *ldh* were cultivated at 30 °C using lysogeny broth (LB) medium [24]. *E. coli* Top10 strains were cultivated in LB medium at 30 °C under aerobic conditions on a rotary shaker at 120 rpm. Media for plasmid-harboring strains were supplemented with 100 µg/ml ampicillin. Cultures were harvested by centrifugation (10 min, 3500 × *g*, 4 °C), and cells from ampicillin-containing cultures were additionally washed with 0.9% (w/v) sodium chloride before the pellets were stored at −20 °C.

2.4. Expression of recombinant lactate dehydrogenases

The selected genes encoding bacterial LDHs were PCR-amplified from genomic DNA of *B. cereus*, *C. glutamicum*, *E. coli* and *G. violaceus* using Phusion High-Fidelity polymerase (Thermo Fisher Scientific) and oligonucleotides listed in Table 2. The approximately 1000 kb PCR products were subcloned into pJET1.2/blunt (Thermo Fisher Scientific), verified by sequencing and excised with *Nde*I and *Xho*I to ligate the purified insert fragments with *Nde*I-*Xho*I-digested pET19b expression vectors. The resulting pET19b:ldh_x plasmids encode the respective LDH with N-terminal His_{10}-tag to facilitate purification. In contrast, *ldh* of *G. violaceus* was directly introduced into the pET19b vector via the Gibson Assembly method [25]. For this purpose, the pET19b vector was restricted with *Nde*I and *Xho*I and incubated with the amplified *ldh* of *G. violaceus* (containing overlapping sequences to pET19b) at 50 °C for 20 min. The mixture containing the recombinant vector was directly used for transformation of *E. coli* Top10.

The eukaryotic LDH encoding gene from *R. norvegicus, ldha,* contains exons. Therefore, the amino acid sequence of LDH_{Rn} (UniProt No. P04642) was used as the template for *in silico* reverse translation. The sequence was codon optimized for expression in *E. coli* and a stop codon as well as restriction sites (*Nde*I & *Xho*I) were added. This artificial and optimized gene sequence was then synthesized by Gene Art (Thermo Fisher Scientific). The supplied pMA-T vector was treated with *Nde*I and *Xho*I, and the purified insert fragment was ligated with the *Nde*I-*Xho*I-digested pET19b expression vector.

For expression of LDHs from *B. cereus*, *C. glutamicum*, and *E. coli*, the expression host *E. coli* C43 (DE3) harboring the respective expression vector (Table 2) was cultivated in LB medium supplemented with 100 µg/ml ampicillin. The main cultures, 1 l baffled flasks containing 250 ml LB, were inoculated with 1 % (v/v) of the final volume of an overnight grown pre-culture and cultivated at 30 °C until reaching an optical density at 600 nm (OD_{600}) of 0.6. They were then induced with 1 mM IPTG and further cultivated for 20 h at 20 °C. For heterologous expression of LDH from *G. violaceus* and *R. norvegicus* the expression host *E. coli* BL21 (DE3) was chosen, and cultivation was performed for 20 h at 20 °C in ZYP-5052 autoinduction medium [26].

2.5. Purification of recombinant LDHs

To purify recombinant LDHs, *E. coli* cells were resuspended in 4 ml

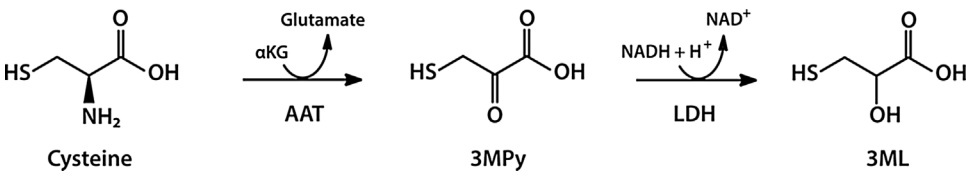


Fig. 1. Schematic reaction of cysteine transamination by AAT to 3MPy, which then serves as substrate for LDH. Abbreviations: αKG, α-ketoglutarate, AAT, aspartate aminotransferase; LDH, lactate dehydrogenase; 3ML, 3-mercaptolactate; 3MPy, 3-mercaptopyuruvate; NADH, nicotinamide adenine dinucleotide.

Table 1
Molecular characteristics of four selected gene sequences encoding bacterial LDHs with high similarity to LDH of *Rattus norvegicus*.

	Organism	NCBI Acc. no.	Sequence comparison		Size		MW[b]
			Identity[a]	Coverage	bp	aa	[kDa]
LDH_{Rn}	*Rattus norvegicus*	NP_058721.1	reference	reference	999	333	39
LDH_{Gv}	*Gloeobacter violaceus*	NP_926270	47 (68) %	97%	993	331	39
LDH_{Cg}	*Corynebacterium glutamicum*	WP_003857765.1	41 (63) %	93%	945	315	38
LDH_{Bc}	*Bacillus cereus*	NP_834568	41 (62) %	91%	945	315	38
LDH_{Ec}	*Escherichia coli*	NP_415898	n.d.	20%	990	330	39

[a] Percentage of identical amino acids compared to LDH_{Rn}; percentage of similar amino acids is given in parentheses; for LDH_{Ec} identities are not given due to the low query coverage.
[b] Molecular weight of recombinant His-tagged proteins.

Table 2
Applied and generated strains, plasmids and oligonucleotides.

Strains, plasmids, oligonucleotides	Description or sequence	Source or reference
Strains		
Bacillus cereus DSM31	Type strain	[37]
Corynebacterium glutamicum ATCC13022	Type strain	[38]
Escherichia coli MG1655	F^-, λ^-, *ilv*G^-, *rfb*-50, *rph*-1	[39]
E. coli BL21 (DE3)	F^- *dcm*, *omp*T, *gal*, *hsdS* (r_B- m_B), (DE3)	Novagen, Madison, WI
E. coli C43 (DE3)	F^- *dcm*, *omp*T, *gal*, $hsdS_B$ (r_B- m_B), (DE3)	Novagen, Madison, WI
E. coli Top10	F^- *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), *rps*L, *nup*G, Φ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*) 7697, *aal*U, *gal*K, *end*A1	Invitrogen, Carlsbad, CA
Plasmids		
pJET1.2/blunt	Amp^R, *eco*47IR, T7 promoter	Thermo Scientific
pET19b	Amp^R, N-terminal His_{10}-tag	Novagen
pET19b:$aspC_{Ec}$	*aspC* from *E. coli* with N-terminal His_{10}-tag	[7]
pET19b:ldh_{Bc}	*ldh* from *B. cereus* DSM31 with N-terminal His_{10}-tag	This study
pET19b:ldh_{Cg}	*ldh* from *C. glutamicum* ATCC 13022with N-terminal His_{10}-tag	This study
pET19b:ldh_{Gv}	*ldh* from *Gloeobacter violaceus* PCC 7421with N-terminal His_{10}-tag	This study
pET19b:ldh_{Rn}	*ldh* from *R. norvegicus*, optimized codon usage, N-terminal His_{10}-tag	This study
pMA-T-ldh_{Rn}	Synthesized *ldh* of *R. norvegicus* optimized for *E. coli* codon usage	Thermo Scientific
Oligonucleotides	Sequence 5′ − 3′[a]	Purpose
bc_ldh_fw	aaacatATGAAAAAAGGTATTAACCGTGTTG	Amplification of ldh_{Bc}
bc_ldh_rv	aaactcgagTTAAAGAACCGGAGCCATTG	
cgl_ldh_fw	aaacatATGAAAGAAACCGTCGGTAAC	Amplification of ldh_{Cg}
cgl_ldh_rv	aaactcgagTTAGAAGAACTGCTTCTGAATTTC	
Gibson_ldh_Gvi_fw	CGACGACGACGACAAGcatatgCAAGATCGACTGTTCG	Amplification of ldh_{Gv}
Gibson_ldh_Gvi_rv	CTTTGTTAGCAGCCGGATCctcgagTTAAAACTGGATCCCGTCG	
ldhA_fw	aaacatATGAAACTCGCCGTTTATAGCACAAAACAG	Amplification of ldh_{Rn}
ldhA_rv	aaactcgagTTAAACCAGTTCGTTCGGGCAGGTTTC	

[a] restriction sites and added terminal nucleotides are indicated by underlined lower case letters.

binding buffer (100 mM MOPS buffer, pH 7.4, containing 500 mM NaCl and 20 mM imidazole) and disrupted by three passages through a precooled French Pressure cell at 1000 MPa. The crude extract was centrifuged (45 min, 3500 × *g*, 4 °C) to obtain the soluble fraction and to subsequently purify the His-tagged proteins using the His SpinTrap Kit (VWR International). To obtain pure protein of LDH_{Rn} ultracentrifugation for 1 h at 4 °C and 100,000 x *g* was performed.

All purification steps were carried out at 0–4 °C. After applying the soluble fraction, the matrix was washed with buffer containing increasing concentrations of imidazole; finally, the target proteins were eluted with 500 mM imidazole. Optimal washing protocols were determined for all five recombinant LDHs and the applied imidazole concentrations for three subsequent washing steps were as follows: 20, 50, 75 mM for LDH_{Bc}; 50, 75, 100 mM for LDH_{Cg}, 20, 50, 120 mM for LDH_{Ec} and 75, 100, 300 mM for LDH_{Gv} and LDH_{Rn}. Protein concentrations and purities were assessed using Bradford assays and SDS-polyacrylamide gels. Purified protein was directly applied in enzyme activity assays, as described below, or stored overnight in an ice-bath.

2.6. Purification of recombinant aminotransferase

Purification of aspartate aminotransferase (AAT) from *E. coli* was performed as described recently [7].

2.7. Enzyme activity assays with purified LDH

In vitro enzyme activities of purified LDH were determined in a spectrophotometer (V-750 UV–vis/NIR Spectrophotometer, JASCO Labor und Datentechnik GmbH, Gross-Umstadt, Germany) at 340 nm and 30 °C. For initial determination of enzyme activity, pyruvate was used as substrate. By default, the reaction mixture with a total volume of 1 ml was premixed in a cuvette and contained 30 mM pyruvate, 0.2 mM NADH and 2.5–20 µg purified LDH in 20 mM MOPS buffer, pH 7.4.

2.8. Coupled activity assays with aminotransferase and LDH

For measurement of LDH activity with 3MPy as substrate, a two-step enzyme assay was performed. The reaction mixture for the first step, the transamination of cysteine catalyzed by AAT, contained 30 mM cysteine, 5 mM α-ketoglutarate and 100 µg purified AAT supplemented with 2 mM of the cofactor pyridoxal phosphate (PLP) in 20 mM MOPS buffer, pH 7.4 and was incubated at 30 °C for 30 min. Subsequently, AAT was inactivated by incubation at 95 °C for 60 s and separated by centrifugation (10 min, 13 000 *x g*). The supernatant containing the substrate 3MPy for the second reaction was transferred to a cuvette and placed into the spectrophotometer. To start the second reaction step,

the reduction of 3MPy to yield 3ML, 0.2 mM NADH and 2.5–20 µg LDH were added, and the reaction was assessed at 30 °C by following the decrease in NADH concentration at 340 nm.

2.9. Enzyme activity assays with cytoplasmic fractions

For a first activity assay of heterologously expressed LDHs with 3MPy, cytoplasmic fractions from expression cultures were obtained by cell disruption via French press and subsequent ultracentrifugation at 100,000 x *g* for 1 h at 4 °C. Protein concentrations of the soluble protein fractions were assessed using Bradford assays and 100 µg were applied for the activity assay containing 0.2 mM NADH and 0.5 mM 3MPy in a total volume of 1 ml 20 mM MOPS buffer. 3MPy reduction was measured in a spectrophotometer at 30 °C by following the decrease in NADH concentration at 340 nm.

2.10. Determination of kinetic characteristics

Specific activities were measured employing a continuous enzyme activity assay. By default, the reaction mixture with a total volume of 1 ml was premixed in a cuvette and contained either 30 mM pyruvate or 3MPy as the substrate and 0.2 mM NADH as the cofactor (20 mM MOPS buffer, pH 7.4). The cuvette was placed in a spectrophotometer and pre-incubated until reaching a stable baseline. Then the reaction was started with 2.5–20 µg of the respective purified LDH.

To determine enzyme kinetics of LDHs, their activity was measured in presence of different concentrations of pyruvate and 3MPy ranging from 0.005–90 mM and 0.01-1 mM, respectively, with a constant concentration of 5 mM NADH. Apparent V_{max} and K_m values were determined by fitting the obtained data to the Michaelis-Menten equation. All measurements were done in triplicate at 30 °C.

2.11. Derivatization of 3MPy and 3ML with ellman's reagent

In order to verify conversion of 3MPy and 3ML by LDHs, the thiol group of the two mercapto compounds was derivatized using Ellman's reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [27]. Calibration was performed for pure 3MPy from 50 to 300 µM. To derivatize samples from enzyme assays, samples of 200–300 µl were withdrawn after the enzymatic reaction was completed, the enzyme was heat-inactivated at 98 °C for 2 min, and denatured protein was removed by centrifugation for 20 min at 17,000 x *g*. Prior to derivatization, dithiothreitol was added to the standard samples as well as samples obtained from enzyme assays to a final concentration of 0.2 mM followed by an incubation at 20 °C for 10 min to reduce potential disulfides. Derivatization was then accomplished by mixing the sample with one volume of a 2 mM DTNB solution (final concentration of 1 mM) and incubated for 5 min 20 °C. For stabilization of the derivative, 7% (v/v) of 1 M HCl were added and the sample was mixed thoroughly. Subsequently, the derivatized sample was subjected to LC–MS analysis.

2.12. Detection and quantification of 3MPy and 3ML via LC–MS

For detection of 3MPy and 3ML, a HPLC system consisting of a LC-20AD XR pump, a Sil20AC XR autosampler a CTO-10AS VP column compartment and a SPD-M30A DAD detector (all Shimadzu, Duisburg, Germany) was coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) operated in the negative ionization mode. Chromatographic separation was achieved after injection of 5 µl on a 50 × 2 mm, 3 µm Reprosil C18AQ column (Dr. Maisch GmbH, Ammerbuch) at 40 °C using a binary system of water + 1 % formic acid (A) and methanol + 1 % formic acid (B) at a flow rate of 450 µl/min. The following gradient was applied: 0 min 100 % A, 1 min 100 % A, 10 min 70 % A; 11 min 0 % A; 15 min 0 % A, 15.1 min 100 % A, 20 min 100 % A. Peaks were detected by their UV-spectra with additional identification by HRMS data recorded as a full scan between *m/z* 90 and 500 with a resolving power of 30000. As no reference for 3ML was available, identification of 3ML was done via its exact mass as well as by its fragmentation spectrum. Quantification of 3MPy and 3ML was performed via measurement of the peak area recorded at 316 nm, corresponding to the maximum of the UV absorption of the DTNB group, using a calibration curve of the 3MPy-DTNB derivative. The limit of detection (LOD) and limit of quantification (LOQ) were determined from the signal-to-noise-ratios of the UV-signal of 3MPy-DTNB in buffer, and were 15 µM and 50 µM for signal-to-noise ratios of 3:1 (LOD) and 9:1 (LOQ), respectively.

3. Results & discussion

Previous studies revealed that mammalian LDHs are capable of using 3MPy as an alternative substrate, and especially the LDH of *R. norvegicus* (LDH_{Rn}) was investigated in the context of L-cysteine catabolism [1,28–30]. In contrast, this reduction activity could never been shown for bacterial LDHs, so far. Therefore, an *in silico* comparison of bacterial sequences with LDH_{Rn} was performed, and four bacterial LDHs were selected for a more detailed characterization.

3.1. In silico analysis and selection of LDH candidates

In order to identify bacterial LDHs, capable of converting 3MPy to 3ML, the amino acid sequences of liver LDH from *R. norvegicus* was applied as template to search for similar enzymes among prokaryotes. 15 enzymes showing the highest sequence similarity to LDH_{Rn} were selected. To generate a sequence alignment and the corresponding phylogenetic tree (Fig. 2) LDHs of well characterized genera (*Advenella, Cupriavidus, Escherichia, Klebsiella* and *Rhodococcus*) and all three LDHs of *E. coli* were included to visualize the fact that LDHs from *E. coli* are highly diverse when compared to LDH_{Rn}. LDH of *Variovorax paradoxus* was chosen as the outgroup.

Based on the sequence alignment with LDH_{Rn} four bacterial LDHs were selected for subsequent *in vitro* analyses (Table 1). However, LDHs from strictly anaerobic and highly pathogenic organisms, such as *Moorella* or *Clostridium* spp., were excluded, even though some of them showed high similarities to LDH_{Rn} (see Table 1 for characteristics). LDH from the cyanobacterium *Gloeobacter violaceus* (LDH_{Gv}) was chosen as the most interesting candidate among bacterial LDHs because it has the highest sequence identity of 47 % in comparison to LDH_{Rn}. The LDHs from *Corynebacterium glutamicum* (LDH_{Cg}) and *Bacillus cereus* (LDH_{Bc}), both sharing 41 % identical amino acids with LDH_{Rn}, were also chosen because these genera play key roles in numerous biotechnical processes [31,32] and, thus, it was considered of high interest to investigate these LDHs regarding 3MPy reduction potential. Although D-LDH (UniProt No.: P52643) of *E. coli* was not amongst the first 100 LDHs most similar to LDH_{Rn}, this enzyme was additionally chosen because it is the only cytosolic LDH in *E. coli* and nothing is known about the substrate specificity of D-LDHs towards 3MPy. Moreover, as *E. coli* could be a potential production strain for *in vivo* 3ML synthesis, it seemed reasonable to analyze its native capacity regarding 3MPy reduction. Beside the cytosolic LDH, *E. coli* possesses two flavin mononucleotide (FMN) dependent LDHs, D-LDH and L-LDH, which are both membrane bound and were thus not chosen for heterologous expression and *in vitro* tests. As these iLDHs do not utilize NAD/$NADH^+$ and remain in the membrane fraction, they cannot influence the activity measurements of the selected, recombinant LDHs in *E. coli* soluble cell extracts.

A multiple sequence alignment was generated including the amino acid sequences of the selected bacterial LDHs and LDH_{Rn}. Additionally, the sequences of human heart and liver LDH were included, because important analyses regarding active residues and catalytic amino acids of LDHs have been conducted with these enzymes [33,34]. The eukaryotic LDHs of rat and human show several highly conserved regions throughout the sequence, whereas clear deviations can be recognized even for the most similar bacterial LDH of *G. violaceus* (Fig. 3).

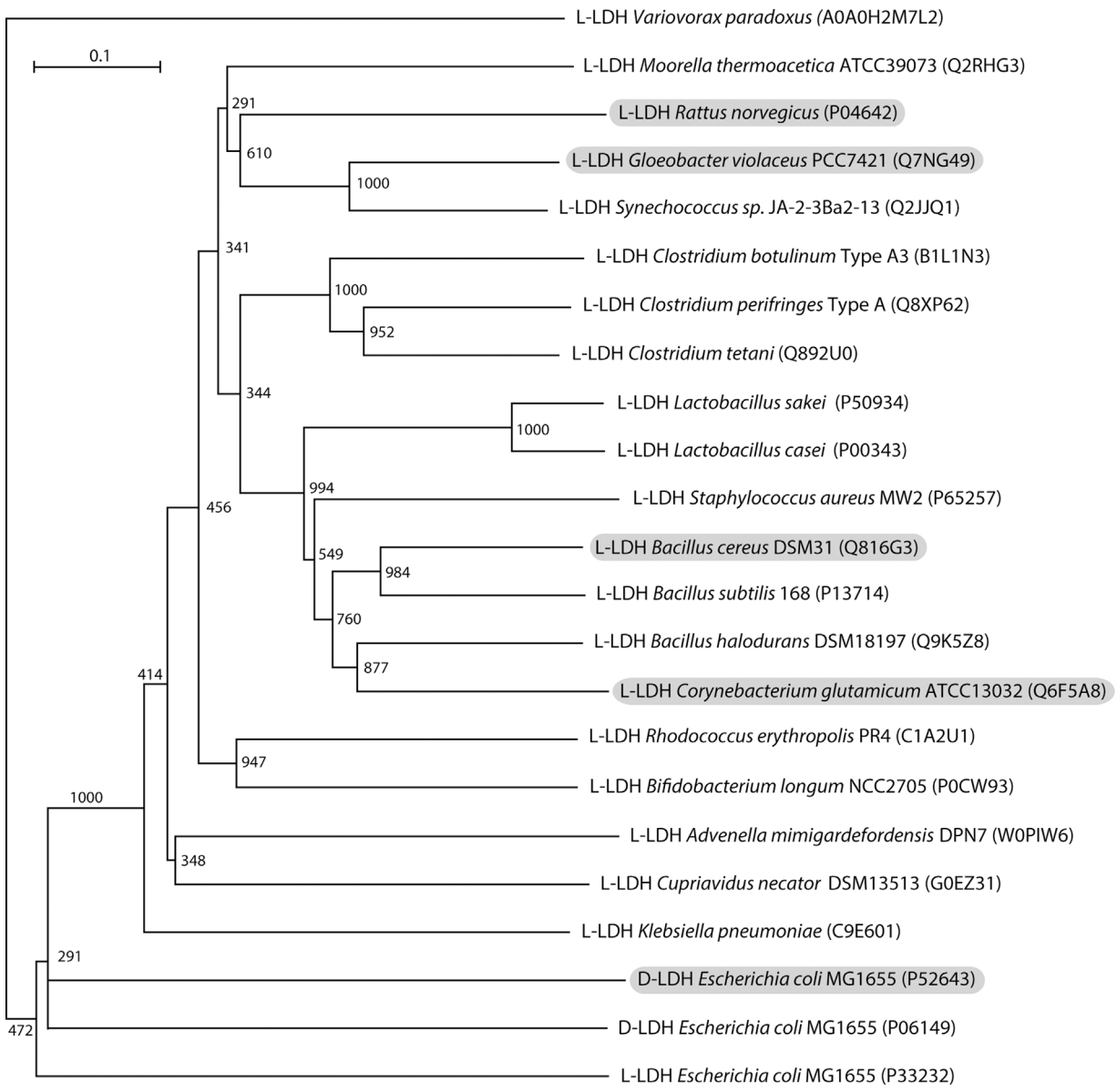


Fig. 2. Phylogenetic tree of different bacterial LDHs and the LDH_{Rn}. The multiple sequence alignment was accomplished with ClustalX2 and visualization of the phylogenetic tree was performed with NJplot. LDH of *Variovorax paradoxus* was set as outgroup. Uniprot codes for each enzyme are indicated in brackets. LDHs highlighted with grey background represent the chosen enzymes for the described *in vitro* experiments. Abbreviations: LDH, lactate dehydrogenases.

However, areas conserved in six of the seven sequences can also be found among the bacterial L-LDHs and LDH of human and rat (for example positions 25–70, 136–155, 164–184, 196–210, and 245–262). A closer look on these regions, which represent common motifs typical for L-LDHs [35,36], reveals that among the bacterial LDHs especially LDH_{Gv} shares many similar or identical amino acids with LDH_{Rn} besides the highly conserved catalytical residues. Hardly any similar residues were found between the D-LDH of *E. coli* and the other L-LDH sequences, as expected from the poor coverage during direct comparison with LDH_{Rn}. D- and L-LDHs are known to be very different from each other, which is why no significant activity with 3MPy was expected for D-LDH [12]. As activity can partially be deduced from analysis of primary protein sequences, activities with 3MPy were assumed to be highest for LDH_{Gv}, lower for LDH_{Cg} and LDH_{Bc} and only very low or not at all present for LDH_{Ec}.

3.2. Heterologous expression of LDHs and determination of 3MPy reduction activity in soluble cell fractions

As a first approach, soluble cell fractions of *E. coli* C43 (DE3) expressing the selected LDH-encoding genes in the vector pET19b were tested for activity with pyruvate and 3MPy. Overexpression in the soluble cell fraction was successful in case of LDH from *B. cereus*, *C. glutamicum* and *E. coli*, whereas LDHs from *G. violaceus* and *R. norvegicus* were mostly detectable in the crude extract (Fig. 4A). However, activities measured with 3MPy as the substrate of interest were highest in soluble fractions containing LDH_{Gv} and LDH_{Rn} with 0.23 ± 0.04 U/ml and 0.26 ± 0.08 U/ml, respectively (Fig. 4B–C). The very high amounts of LDH_{Ec}, LDH_{Cg} and LDH_{Bc} showed only very low or almost no detectable activity with 3MPy.

3.3. Purification of LDHs and activity with pyruvate and 3MPy

The selected, heterologously expressed LDHs were purified to electrophoretic homogeneity. After optimizing the purification protocol

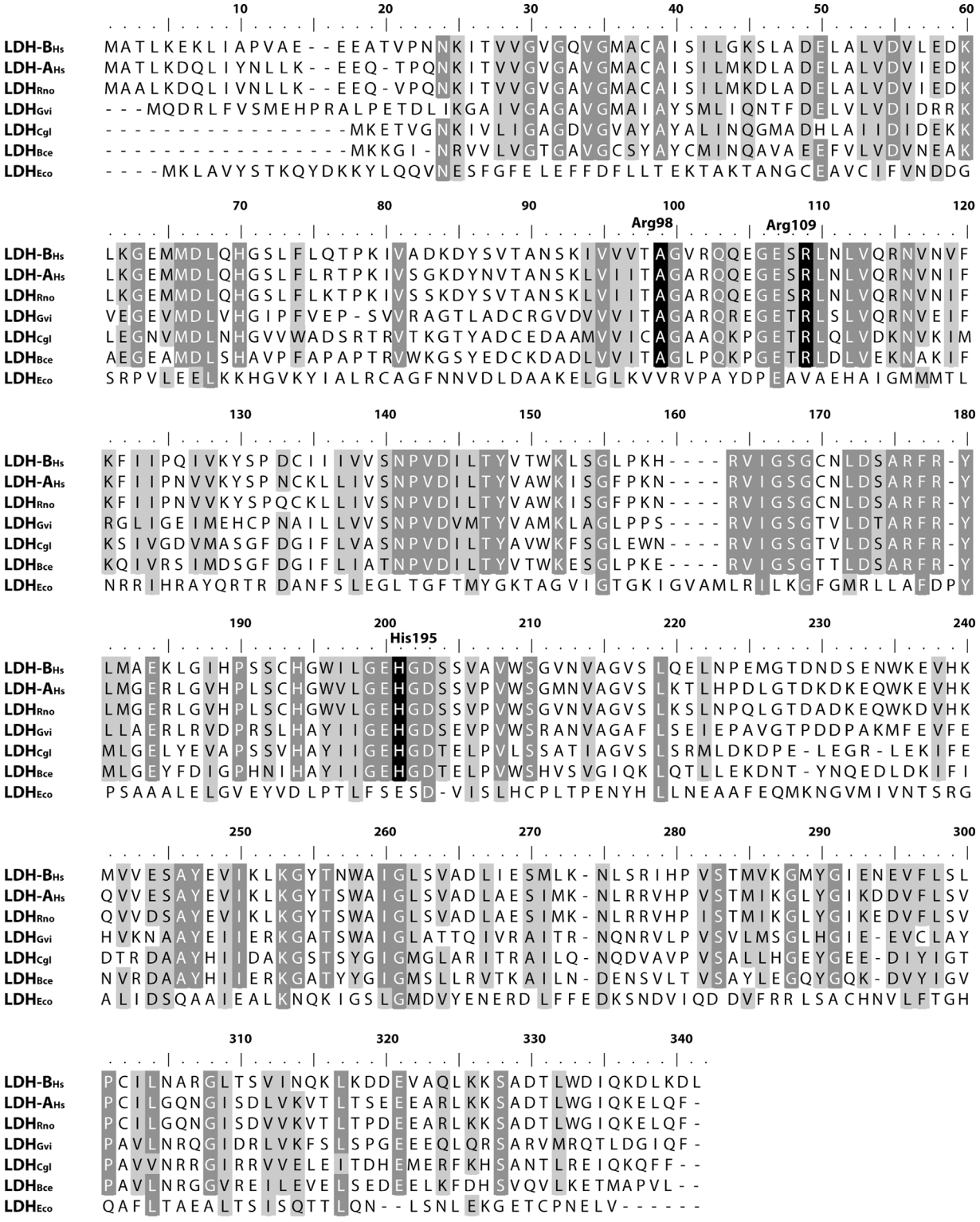
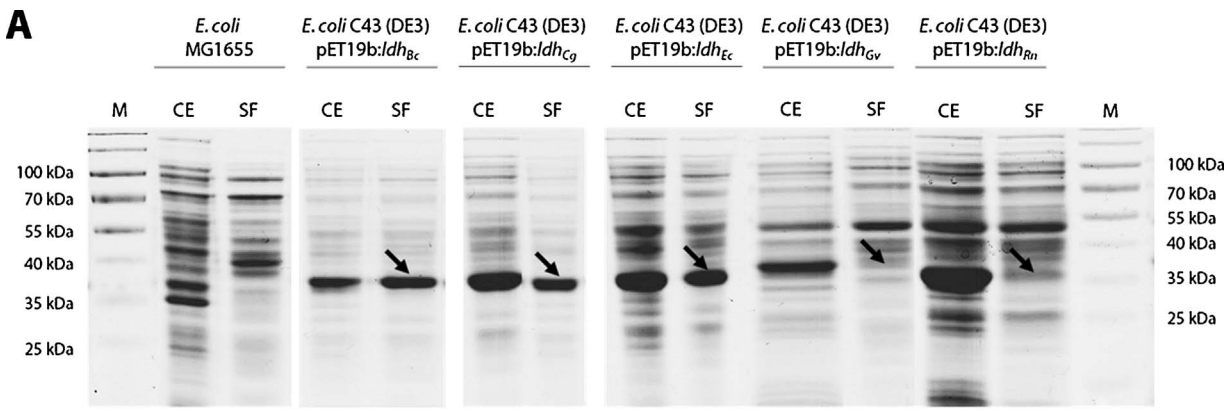
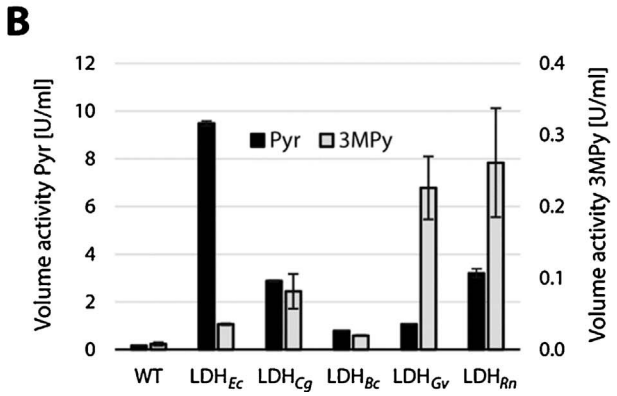


Fig. 3. Alignment of seven pro- and eukaryotic LDHs. The alignment was performed with the BioEdit Sequence Alignment Editor using the ClustalW algorithm (default settings) and the Blossum62 matrix. Regions with high similarities are framed in a black box and matches of amino acids between human, rat and LDH of *Gloeobacter* are framed in grey box. Amino acids were shaded in light grey if at least six of seven aligned proteins share amino acid which are similar at the particular position. Framed and dark grey shaded amino acids indicate that at least six of the seven proteins have the same amino acid at the particular position. Abbreviations: *Bc*, *Bacillus cereus*; *Cg*, *Corynebacterium glutamicum*; *Ec*, *Escherichia coli*; *Gv*, *Gloeobacter violaceus*; *Hs*, *Homo sapiens*; LDH, lactate dehydrogenase; *Rn*, *Rattus norvegicus*.

for each LDH, total yields of 3–4 mg purified protein were obtained for LDH_{Ec}, LDH_{Cg} and LDH_{Bc}, while only 1.2 mg and 0.7 mg of pure LDH_{Gv} and LDH_{Rn}, respectively, were obtained from a 50 ml expression culture.

In order to analyze and compare specific activities with 3MPy a coupled enzyme assay with AAT from *E. coli* (AAT_{Ec}), which transaminates cysteine to 3MPy, was developed providing the substrate of interest for the purified LDHs (Fig. 1) [7]. When AAT_{Ec} and LDH_{Cg} were simply incubated together with the required substrates, the specific activity of LDH_{Cg} was only 0.11 U/mg (Fig. 5B). Therefore, an





C

***E. coli* strain** [100 µg SF]	Volume activity 3MPy [U/ml]	Volume activity 3MPy/Pyr [%]
MG1655	0.01 ± 0.00	-
C43 pET19b::ldh$_{Ec}$	0.04 ± 0.00	0.4
C43 pET19b::ldh$_{Cg}$	0.08 ± 0.02	2.8
C43 pET19b::ldh$_{Bc}$	0.02 ± 0.00	2.5
C43 pET19b::ldh$_{Gv}$	0.23 ± 0.04	21.1
C43 pET19b::ldh$_{Rn}$	0.26 ± 0.08	8.1

Fig. 4. Enzyme activity assay with cytosolic fractions of *E. coli* strains overexpressing different LDHs. (**A**) SDS gels showing crude cell extracts and soluble cell fractions of the respective strains overexpressing *ldh* genes from different organisms. The parent strain *E. coli* MG1655 serves as a control. (**B**) Volume activity of 100 µg soluble cell fraction with 0.5 mM pyruvate or 3MPy as substrate and 0.2 mM NADH. (**C**) Volume activities of soluble cell fractions with 3MPy and relative activity in comparison with the natural substrate pyruvate. Abbreviations: LDH, lactate dehydrogenase; 3MPy, 3-mercaptopyruvate; NADH, nicotinamide adenine dinucleotide; Pyr, pyruvate.

optimization of the coupled assay was performed by pre-incubation of the AAT reaction, adjustment of the amounts of AAT and LDH and inactivation of AAT prior to addition of LDH in order to increase the specific activity of LDH (see Fig. S1 in Supplemental Material for an overview of all variations of the coupled reaction). Regarding the pre-incubation of the first reaction step catalyzed by AAT$_{Ec}$, an increase of the incubation time from 10 to 50 min led to a doubling of LDH$_{Cg}$ activity to 0.21 U/mg in the second step (Fig. 5B). Then, the amounts of AAT and LDH were adapted, resulting in LDH$_{Cg}$ activity of 0.80 U/mg (Fig. 5B). Another modification was the complete inactivation of AAT$_{Ec}$

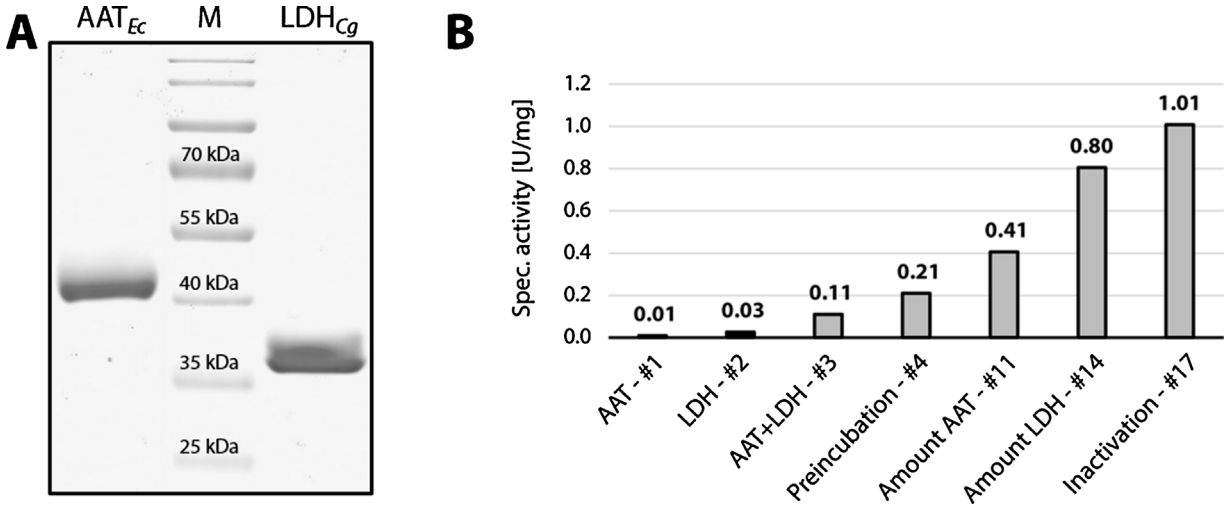
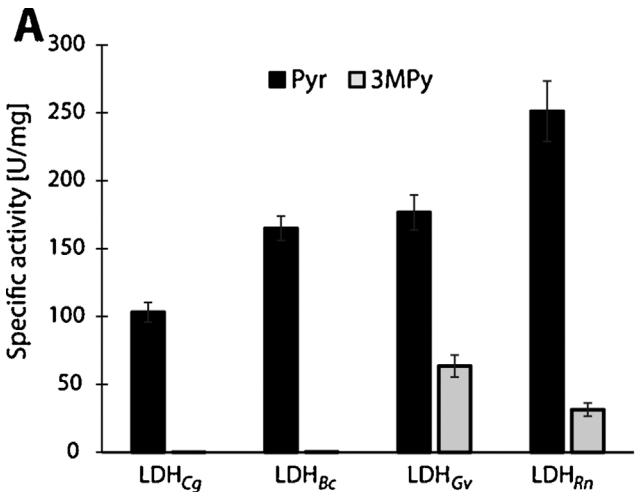


Fig. 5. Optimization of coupled enzyme assay with AAT from *E. coli* (AAT$_{Ec}$) and purified LDH from *C. glutamicum* (LDH$_{Cg}$). (**A**) SDS gel showing purified AAT$_{Ec}$ and LDH$_{Cg}$. (**B**) Specific activities of pure LDH$_{Cg}$ with 3MPy as substrate during different stages (#) of the optimization process for the coupled enzyme assay. By default, the reaction mixture with a total volume of 1 ml was premixed in a cuvette containing 30 mM cysteine, 5 mM α-ketoglutarate, 0.2 mM NADH, 50–200 µg AAT$_{Ec}$ and 10–20 µg LDH$_{Cg}$ in 20 mM MOPS buffer (pH 7.4). #1, 50 µg AAT, no LDH; #2, 10 µg LDH, no AAT; #3, 50 µg AAT and 10 µg LDH; #4, 50 µg AAT were pre-incubated for 10 min, before NADH and LDH were added; #11, 100 µg AAT were pre-incubated for 5 min; #14, 200 µg AAT were pre-incubated for 12 min, before NADH and 20 µg LDH were added; #17, pre-incubation of the first reaction with 200 µg AAT for 25 min and subsequent inactivation of AAT for 1 min at 95 °C, before NADH and 20 µg LDH were added to the mixture. Abbreviations: AAT, aspartate aminotransferase; LDH, lactate dehydrogenase; 3MPy, 3-mercaptopyruvate; NADH, nicotinamide adenine dinucleotide.



B

LDH	Specific activities		
	Pyr [U/mg]	3MPy [U/mg]	3MPy/Pyr [%]
C. glutamicum	103.1 ± 9.0	0.23 ± 0.01	0.22
B. cereus	165.0 ± 7.2	0.08 ± 0.02	0.05
G. violaceus	176.7 ± 12.9	63.6 ± 8.1	35.97
R. norvegicus	251.2 ± 22.3	31.5 ± 4.9	12.52

Fig. 6. Specific activities of purified LDHs from *C. glutamicum, B. cereus, G. violaceus* and *R. norvegicus* with pyruvate or 3MPy as the substrate. By default, the reaction mixture with a total volume of 1 ml was premixed in a cuvette containing 30 mM pyruvate or 1 mM 3MPy, 0.2 mM NADH and the respective LDH in 20 mM MOPS buffer (pH 7.4). For activity with pyruvate 2.5 µg LDH was added to the mixture to start the reaction, while for 3MPy 80 µg were added in case of LDH$_{Cg}$ and LDH$_{Bc}$, and 2.5 µg in case of LDH$_{Gv}$ and LDH$_{Rn}$. Abbreviations: *Bc*, *Bacillus cereus*; *Cg*, *Corynebacterium glutamicum*; *Gv*, *Gloeobacter violaceus*; LDH, lactate dehydrogenase; 3MPy, 3-mercaptopyruvate; NADH, nicotinamide adenine dinucleotide; Pyr, pyruvate; *Rn*, *Rattus norvegicus*.

after the first reaction was completed by incubating the mixture for 1 min at 95 °C, thus leading to an LDH$_{Cg}$ activity of 1.01 U/mg in the subsequent step (Fig. 5B). To ensure that 3MPy synthesized during the first reaction was not degraded by incubation at 95 °C and lost as substrate for the second reaction, a control without heat inactivation was performed. Eventually, the specific activity of LDH$_{Cg}$ was improved by the factor 10, thus providing a suitable protocol for biosynthesis of 3ML from cysteine as the precursor substrate − instead of the more expensive 3MPy − with the described coupled enzyme assay.

Specific activities of purified LDHs were measured in assays applying pure 3MPy as substrate. As LDH$_{Ec}$ showed no significant reduction activity with 3MPy, it was excluded from further analyses. LDH$_{Cg}$ and LDH$_{Bc}$ showed only very low activities of 0.23 ± 0.01 U/mg and 0.08 ± 0.02 U/mg, respectively (Fig. 6). Probably because of low similarities of LDH$_{Cg}$ and LDH$_{Bc}$ with LDH$_{Rn}$, significant structural differences of the enzymes are responsible that 3MPy is not converted efficiently by these enzymes, as was already predicted from the sequence alignment. In strong contrast to all other bacterial LDHs, LDH$_{Gv}$ exhibited the highest 3MPy reduction activity of 63.6 ± 8.1 U/mg, which was even twice as high as the activity of LDH$_{Rn}$ towards 3MPy. Based on these results, LDH from *G. violaceus* is considered the most interesting bacterial LDH regarding conversion of 3MPy to the highly valuable product 3ML.

3.4. Kinetic characterization of LDH$_{Gv}$ and LDH$_{Rn}$ regarding 3MPy reduction

Several kinetic parameters were determined for the two most active enzymes, LDH$_{Gv}$ and LDH$_{Rn}$ using either pyruvate or 3MPy as substrates (Table 3). A direct comparison of the two enzymes with pyruvate as substrate revealed that LDH$_{Gv}$ shows lower affinity (0.38 vs. 0.14 mM) as well as a slower turnover number with 160.9 s^{-1} compared to 174.9 s^{-1} of LDH$_{Rn}$. Using 3MPy as substrate, LDH$_{Gv}$ exhibited a slightly lower substrate affinity as well (K_m value of 0.3 mM in comparison to 0.2 mM of LDH$_{Rn}$). However, due to a more than twofold higher turnover number of LDH$_{Gv}$, its catalytic efficiency is considerably higher than that of LDH$_{Rn}$ (179 mM^{-1} s^{-1} in comparison to 128 mM^{-1} s^{-1}). The more comprehensive results from kinetic studies supported the finding that LDH$_{Gv}$ exhibits highest specific activity for 3MPy among the selected LDHs. Accordingly, LDH$_{Gv}$ is not only as efficient in reducing 3MPy to 3ML as LDH$_{Rn}$, but even faster in catalyzing the desired reaction and is therefore the more interesting candidate for further applications regarding *in vitro* synthesis of 3ML.

3.5. Detection and quantification of 3ML as reaction product

To further verify the formation of 3ML from 3MPy by LDH, a derivatization method to detect and quantify both compounds was established (see sections 2.11 and 2.12 for a detailed description). After reduction of potential disulfides with DTT, the Ellman's reagent DTNB was used to derivatize the thiol group of 3MPy and 3ML, respectively. The derivatized samples were then subjected to LC-DAD-MS analysis. Identification of both compounds was achieved via the 3MPy reference and comparison of the recorded HRMS full MS and product ion spectra of 3ML with those of 3MPy. For the 3ML signal, besides the characteristic UV absorption of the DTNB group, two additional hydrogen atoms could be assigned via a mass increase of +2.0156 Da. A shift of the retention time from 4.01 min to 3.11 min additionally confirmed the conversion of the carbonyl function of 3MPy to an alcohol. Based on a calibration with 3MPy, 3MPy and 3ML, formed during enzymatic reaction by LDH$_{Gv}$ and LDH$_{Rn}$ were quantified via UV absorption of the DTNB group at 316 nm. The formation of 0.3 mM 3ML from 1 mM 3MPy and 0.3 mM NADH during enzymatic reaction by LDH$_{Gv}$ and LDH$_{Rn}$ could be confirmed, while 3ML was not detectable in samples without enzyme or heat-inactivated LDHs (Fig. 7). The newly developed method had a limit of detection of 15 µM and a limit of quantitation of

Table 3
Kinetic characteristics of LDH$_{Gv}$ and LDH$_{Rn}$ with pyruvate and 3MPy as substrates.

	Pyruvate				3-Mercaptopyruvate			
	K_m [mM]	V_{max} [mM/min]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1}s^{-1}$]	K_m [mM]	V_{max} [mM/min]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1}s^{-1}$]
LDH$_{Gv}$	0.38	61.9	160.9	423.3	0.30	20.4	53.0	178.9
LDH$_{Rn}$	0.14	66.9	174.9	1249.3	0.20	9.8	25.6	128.1

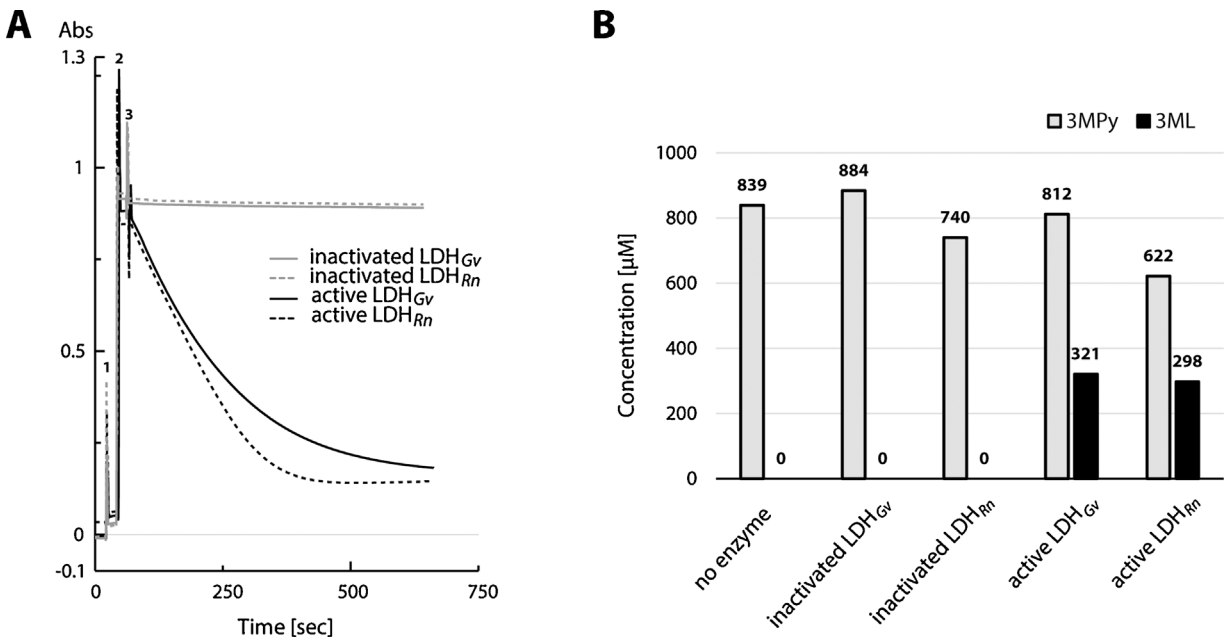


Fig. 7. Formation of 3ML from 3MPy by LDH from *G. violaceus* and *R. norvegicus*. (**A**) Enzymatic conversion of 3MPy by LDH$_{Gv}$ and LDH$_{Rn}$. The reaction mixture with a total volume of 1 ml was premixed in a cuvette containing 1 mM 3MPy, 0.3 mM NADH and 5 µg LDH in 20 mM MOPS buffer (pH 7.4). Controls were performed without addition of enzyme and with inactivated LDHs (achieved by pre-incubation of LDH$_{Gv}$ and LDH$_{Rn}$ at 98 °C for 15 min). The enzymatic reactions were performed at 30 °C for 10 min. (**B**) Quantification of 3MPy and 3ML in enzyme assays with purified LDH$_{Gv}$ and LDH$_{Rn}$. Samples of the enzyme assay were derivatized with DTNB after adding the reducing agent DTT and subsequently analyzed via LC–MS. Abbreviations: *Gv*, *Gloeobacter violaceus*; LDH, lactate dehydrogenase; 3ML, 3-mercaptolactate; 3MPy, 3-mercaptopyruvate; NADH, nicotinamide adenine dinucleotide; Pyr, pyruvate; *Rn*, *Rattus norvegicus*.

50 µM, based on signal-to-noise ratios of 3:1 and 9:1, respectively. Due to the selective reaction with Ellman's reagent, a chromophore was coupled to both analytes, allowing a convenient and sensitive quantification despite a missing reference of 3ML.

4. Conclusion

For the first time, a bacterial LDH was shown to catalyze the *in vitro* biosynthesis of 3ML from 3MPy. From our selection of bacterial LDHs, only the enzyme from the cyanobacterium *G. violaceus* exhibited significant activity and a high catalytic efficiency of 179 $mM^{-1}s^{-1}$ with this artificial substrate. LDH$_{Gv}$ not only exceeded the activity of the mammalian control enzyme LDH$_{Rn}$ two-fold, but could also be overexpressed and purified with higher yields in *E. coli*, which makes LDH$_{Gv}$ a better candidate for future biosynthesis of 3ML and also for PTE production. By establishing an efficient *in vitro* biosynthesis of 3ML as well as robust detection methods for 3MPy and 3ML, important milestones on the way towards the synthesis of a new PTE polymer could be achieved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2017.08.005.

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