Synthesis of α-hydroxy ketones and vicinal diols with the *Bacillus licheniformis* DSM 13^T butane-2,3-diol dehydrogenase

Lukas Muschallik[a], Carina Ronja Kipp[a], Inga Recker[a], Johannes Bongaerts[a], Martina Pohl[b], Melanie Gellissen[a], Michael J. Schöning[a], Thorsten Selmer[a]†, Petra Siegert[*a]

[a] *Institute of Nano- and Biotechnologies, Aachen University of Applied Sciences, 52428 Jülich, Germany*

[b] *IBG-1: Biotechnology, Forschungszentrum Jülich, 52425 Jülich, Germany*

† *deceased*

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Abbreviations: BlBDH, SDR

*Corresponding author at: Heinrich-Mussmannstr. 1, 52428 Jülich, Germany. *E-mail address:* siegert@fh-aachen.de

Abstract

The enantioselective synthesis of α-hydroxy ketones and vicinal diols is an intriguing field because of the broad applicability of these molecules. Although, butandiol dehydrogenases are known to play a key role in the production of 2,3-butandiol, their potential as biocatalysts is still not well studied. Here, we investigate the biocatalytic properties of the *meso*-butanediol dehydrogenase from *Bacillus licheniformis* DSM 13^T (BlBDH). The encoding gene was cloned with an N-terminal StrepII-tag and recombinantly overexpressed in *E. coli*. BlBDH is highly active towards several non-physiological diketones and α-hydroxyketones with varying aliphatic chain lengths or even containing phenyl moieties. By adjusting the reaction parameters in biotransformations the formation of either the α-hydroxyketone intermediate or the diol can be controlled.

1 Introduction

Acetoin reductases (also known as 2,3-butanediol dehydrogenases) belong to the class of oxidoreductases (EC 1.1.1.4) and are involved in the microbial production of acetoin, diacetyl and 2,3-butanediol. These compounds are by-products of the carbohydrate metabolism with α-acetolactate, acetoin and 2,3-butanediol as the main intermediates [1,2]. Especially, 2,3-butanediol is an important platform chemical for several industrial applications such as printing inks, perfumes,

fumigants, moistening and softening agents, explosives, plasticizers, foods, and pharmaceuticals [3–5]. Besides these natural substrates, butanediol dehydrogenases catalyze the stereoselective reduction of different diketones to α-hydroxy ketones and vicinal diols, which are valuable products due to their broad application range, for example as flavouring compounds, pheromones or as precursors for fine chemicals [3,6–8]. For instance, beetles from the family of *Cerambycidae* (longhorn beetles) highly react on mixtures of 3-hydroxy-2-hexanone and 2,3-hexanediols [9,10]. Furthermore, longer chain α-hydroxy ketones and diols like 3-hydroxy-2-octanone or 2,3-octanediols, proofed to be efficient as pheromones and can be used in traps for pest control [11–13]. To access these molecules different chemical approaches were reported, such as asymmetric α-aminoxylation of aldehydes to generate enantiopure 1,2-diols or the use of cyclic ruthenates for regioselective oxidation of vicinal diols to α-hydroxy ketones [14,15]. Chemoenzymatic routes reported the synthesis of α-hydroxy ketones by CalB lipase[16]. Moreover, enantioselective biocatalytic synthesis of α-hydroxy ketones and vicinal diols by thiamine diphosphate-dependent lyases (ThDP lyases) that catalyze the carboligation of aldehydes to α-hydroxy ketones [17–19] and oxidoreductases [20–25] proofed to be efficient.

The butanediol dehydrogenase from *Bacillus licheniformis* DSM 13^T catalyzes the NADH-dependent enantioselective reduction of diacetyl via acetoin to the corresponding *meso*- and (*S*,*S*)-2,3-butanediol stereoisomers [26]. The focus of this study is a deeper understanding of the BlBDH's enzymatic characteristics, in particular its substrate range, stereoselectivity for non-natural substrates and its use as a biocatalyst for the enantioselective synthesis of α-hydroxy ketones and vicinal diols.

2 Material and methods

2.1 Chemical and reagents

Unless otherwise stated, chemicals were of analytical grade and purchased from Sigma-Aldrich. (*R*)-1-hydroxy-1-phenylpropan-2-one ((*R*)-PAC), (*S*)-1-hydroxy-1-phenylpropan-2-one ((*S*)-PAC), (*R*)-2-hydroxy-1-phenylpropan-1-one ((*R*)-HPP), (*S*)-2-hydroxy-1-phenylpropan-1-one ((*S*)-HPP) were synthesized as described elsewhere [27,28]. 1-Hydroxy-1-[2-(trifluoromethyl)phenyl]-2-propanon and 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-butanon were synthesized in the group of K. Zeitler (University Leipzig; Germany).

Reagents for molecular biology were purchased from Thermo Scientific. DNA oligonucleotide synthesis and DNA sequencing were performed by Eurofins Genomics (Germany). Stargate® cloning vectors and streptactin columns were from IBA GmbH (Germany).

2.2 Bacterial strains and plasmids

Cloning was done in *Escherichia coli* DH5α by using the Stargate®-pENTRY vector (kanamycin resistance). *Escherichia coli* BL21(DE3) was used for protein production. The Stargate® pASG.5 vector (pASG-Bl02066-5; ampicillin resistance) and pASG.3 (pASG-Bl02066-3; ampicillin resistance) vector were used for heterologous gene expression (IBA, Germany).

2.3 Cloning of the *meso*-2,3-butanediol dehydrogenase gene from *Bacillus licheniformis* DSM 13^T

The primers (Bl02066-fw: AGCGGCTCTTC**AATG**AGTAAAGTATCTGGAAAAATTGC and Bl02066-rev: AGCGGCTCTTC**TCCC**ATTAAATACCATTCCGCCATCA) were deduced using the known sequence of the *B. licheniformis* DSM 13^T gene (budC). The gene was amplified from genomic DNA obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) by using these primers for PCR amplification. Stargate® cloning of the PCR product was performed as described in the user manual. The budC gene in the expression vectors was sequenced using the primers 5'-GAGTTATTTTACCACTCCCT-3' (forward) and 5'-CGCAGTAGCGGTAAACG-3' (reverse).

2.4 Determination of protein concentration and purity

Protein concentrations were determined using the Bradford method and bovine serum albumin (BSA) as a standard [29].

SDS-PAGE was carried out according to Laemmli [30] using 12-% polyacrylamide gels and Roti®-Mark PRESTAINED ladder as standard.

2.5 Production of recombinant *meso*-2,3-butanediol dehydrogenase in *E. coli* and purification

E. coli BL21(DE3) was transformed with either the pASG-budC.3 or pASG-budC.5 plasmid to produce enzyme variants with an N- or C-terminal-fused StrepII-tag. Cells were grown at 30 °C, 180 rpm, 200 ml in lysogenic broth (LB-medium) supplemented with carbenicillin (100 µg/ml). Cells were cultivated and gene expression was induced with anhydrotetracycline (200 ng/ml) during the exponential growth phase. The cells were harvested 3 h post induction and resuspended in TRIS-HCl buffer (10 mM, pH 7.4) supplemented with NaCl (150 mM). After disruption of the cells by sonication, cell debris was removed by centrifugation (15,000 × g, 4 °C, 40 min). Recombinantly produced BDHs were purified using Strep-Tactin® macroprep columns according to the manufacturer's instructions in TRIS-HCl buffer (10 mM, pH 7.4) supplemented with NaCl (150 mM). The fractions containing BDH activity

were pooled and concentrated in Vivaspin 6 concentrators (10.000 MWCO; Sartorius). In this process, the buffer was exchanged to TRIS-HCl buffer (10 mM, pH 7.4).

2.6 Butanediol dehydrogenase activity standard assay

Enzyme reactions were followed by substrate-dependent oxidation of NADH at 340 nm over a period of 90 s using a temperature-controlled photometer (Bioscience Ultrospec 2100 Pro, Amersham). All reactions were performed at 30 °C. The reduction reactions were determined in potassium phosphate buffer (50 mM, pH 6.8) with substrate (10 mM) and NADH (0.3 mM). Activities for the oxidation reaction was determined in glycine-NaOH buffer (50 m, pH 10.0) with substrate (10 mM) and NAD^+ (0.3 mM). For highly hydrophobic molecules 5% DMSO as solubilizer was added. The reaction was initiated by addition of an appropriately diluted sample of purified BlBDH. A correction was made by measuring a control without enzyme. Variability is expressed as standard deviation (in triplicates).

One unit of BlBDH is defined as the amount of enzyme that oxidizes one µmol of NADH per minute at 30 °C under the given conditions. k_{cat} was calculated per enzyme subunit.

2.7 Substrate spectrum of BlBDH

Unless otherwise stated, activities towards various potential substrates were tested using standard reduction assay conditions (chapter 2.6) with 10 mM of potential substrates (see results). For hydrophobic compounds, a final concentration of 5% (v/v) dimethyl sulfoxide (DMSO) was used as a solubilizer.

2.8 Effect of organic solvents on the activity of BlBDH

The stability of the purified enzyme towards organic co-solvents was tested in the presence or absence of various water miscible organic co-solvents in different concentrations (see results), respectively in potassium phosphate buffer (50 mM, pH 6.8). Residual activities were measured after 1 h incubation at 22 °C using the standard assay (chapter 2.6).

2.9 Kinetic parameters of BlBDH for various substrates

Kinetic parameters were determined under standard conditions (chapter 2.6.) with substrate concentrations in the range of 0 - 50 mM for acetoin, 0 - 250 mM for diacetyl, 0 - 175 mM for 2,3-hexanedione, 0 - 50 mM for 2,3-heptanedione, and 0 - 175 mM for 5-methyl-2,3-hexanedione. To

improve solubility of hydrophobic compounds 5% (v/v) DMSO was added for all substrates. Kinetic data were fitted to the Michaelis-Menten equation using the GraphPad Prism 7 software.

2.10 pH optimum

pH-optima were determined by using the following buffers (50 mM each, adjusted to the desired pH with its corresponding acid/base at 30 °C) with the standard assay (2.6.) consisting of acetoin or diacetyl (10 mM each), NADH (0.3 mM) for reduction reaction and *meso*-2,3-butanediol (10 mM), NAD^+ (0.3 mM) for oxidation reaction without DMSO: sodium acetate (pH 4.0 - 6.0), potassium phosphate (pH 6.0 - 8.0), glycine-NaOH buffer (pH 8.0 - 11.0).

2.11 Biocatalytic characterization of BlBDH

Stereoselectivity and conversion were analyzed by carrying out the reduction reaction of selected diketones and α-hydroxy ketones. Formate dehydrogenase (FDH) from *Candida boidinii* (Megazyme) was used for cofactor regeneration. Biotransformations were carried out in 1.5 ml Eppendorf vials at 30 °C for 60 min without agitation in a total volume of 1 ml. The standard reaction mixtures for the reduction reaction consisted of: substrate (10 mM), purified BlBDH (1 U/ml of the corresponding substrate), FDH (5 U/ml), sodium formate (30 mM) and NADH (0.3 mM) in potassium phosphate buffer (50 mM; pH 6.8). For high hydrophobic molecules like 1-phenyl-1,2-propanedione or 2,3-heptanedione 5% methanol (v/v) was added as solubilizer. Samples (100 µl) were taken at different points in time during the reaction, extracted with diethyl ether (300 µl) and applied to GC analysis (GC-2010 Plus (Shimadzu) with a flame ionization detector) equipped with a Hydrodex γ - DIMON (25 m x 0,25 mm ID Macherey & Nagel) column. The following temperature profile was used: 45 °C (2 min), 45 - 70 °C (2 °C/min); 70 - 180 °C (10 °C/min); 180 °C (10 min).

Retention times of educts (as standards purchased from Sigma-Aldrich or synthesized) were: diacetyl (2,3-butanedione) 4.1 min; (*R*)-acetoin 11.7 min; (*S*)-acetoin 14.4 min; racemic 4-hydroxy-3-hexanone 18.6 and 19.7 min; 2,3-pentanedione 9.8 min; 3-hydroxy-3-methyl-2-butanone 10.5 min; 2,3-hexanedione 12.9 min; 3,4-hexanedione 15.9 min; 5-methyl-2,3-hexanedione 13.3 min; 2,3-heptanedione 15.3 min; ethylpyruvate (ethyl-1,2-oxopropanoate) 17.7 min; (*S*)-2-hydroxy-1-phenylpropan-1-one (HPP) 24.9 min; (*R*)-2-hydroxy-1-phenylpropan-1-one 24.7 min; (*S*)-1-hydroxy-1-phenylpropan-2-one (PAC) 24.8 min; (*R*)-1-hydroxy-1-phenylpropan-2-one 25.0 min; 1-phenyl-1,2-propanedione 22,7 min; racemic 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-propanone 23.7 and 23.8 min; racemic 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-butanone 24.7 and 24.8 min.

Retention times of the products were: (*S*,*S*)-butane-2,3-diol 17.6 min; (*R*,*R*)-butane-2,3-diol 17.8 min; *meso*-butane-2,3-diol 18.1 min; 2-hydroxy-3-pentanone 18.3 min; 3-hydroxy-2-pentanone 17.7 min; 2-hydroxy-3-hexanone 19.5 min; 3-hydroxy-2-hexanone 19.9 min; 4-hydroxy-3-hexanone 19.4 min; 3,4-hexanediol 20.9 min; 3,4-hexanediol 21.1 min; 5-methyl-2-hydroxy-3-hexanone 19.7 min; 5-methyl-3-hydroxy-2-hexanone 20.2 min; 2-hydroxy-3-heptanone 20.2 min; 3-hydroxy-2-heptanone (enantiomer 1) 20.3 min; 3-hydroxy-2-heptanone (enantiomer 2) 20.1 min; 2,3-heptanediol 22.3 min; 3-methyl-2,3-butanediol 17.8 min; (*R*,*S*)-1-phenyl-1,2-propanediol 27.1 min; (*R*,*S*)-1-phenyl-1,2-propanediol 26.9 min; (*S*)-2-hydroxy-1-phenylpropane-1-on 24.7 min; (*R*)-1-hydroxy-1-phenylpropane-2-on 24.8 min; products of the reduction of racemic 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-butanone 27.7 and 27.8 min; products of the reduction of racemic 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-propanone 26.6 and 26.7 min.

2.12 Chemical synthesis of vicinal diols

Standards of 2,3-pentanediol, 2,3-hexanediol, 3,4-hexanediol, and 5-methyl-2,3-hexanediol were obtained by reduction of the corresponding diketones (2 mmol) with sodium borohydride ($NaBH_4$, 2 mmol) in 20 ml methanol. The reaction mixture was incubated for 3 hours 20 °C under stirring. After the slowly addition of 1 ml 10% HCl and 30 ml H_2O the reaction was stirred for another 10 min. The reaction products were extracted with diethyl ether, neutralized, dried with $MgSO_4$ (anhydrous), filtrated and the solvent was removed under vacuum [21].

2.13 GC-MS analysis

Samples were analyzed by GC-MS (GC-2010 Plus, Shimadzu) with a flame ionization detector coupled with a quadrupol-mass spectrometer (GC-MS-QP2010S, Shimadzu). Molecule fragmentation was achieved by electron ionization (70 eV).

3 Results and discussion

3.1 Cloning, heterologous expression of the *meso*-butane-2,3-diol dehydrogenase encoding gene bud C from *Bacillus licheniformis* DSM 13^T and purification of the StrepII-tagged enzyme

The BlBDH gene encoding the *meso*-2,3-BDH from *Bacillus licheniformis* DSM 13 was amplified from genomic DNA, cloned into the Stargate® ENTRY vector by using *Lgu*I restriction sites yielding the pE-Bl02066 vector. Sequencing verified a 100% identity with the previously published *meso*-2,3-BDH [31] from *Bacillus licheniformis* DSM 13 on nucleotide level.

In order to perform further biochemical studies, the enzyme here was sub-cloned from pE-Bl02066 into the vectors pASG.3 and pASG.5, respectively. By using *Esp3*I restriction sites the gene was fused in frame with an either C-terminal or N-terminal vector-encoded StrepII-affinity tag having a glycine, serine linker between tag and enzyme. The vectors pASG.3 and pASG.5 were used to transform *E. coli* BL21(DE3) cells for heterologous gene expression. By inducing gene expression with anhydrotetracycline, both enzyme variants were produced. Only the variant with an N-terminal StrepII-affinity tag proofed to be active (45 U/mg in crude cell extract, estimated overexpression of 50%), was purified to homogeneity and used for further studies. SDS-PAGE gels (Figure 1) revealed a prominent band at approximately 30 kDa. This is in agreement with the calculated molecular mass of 28.2 kDa according to the amino acid sequence. As mentioned in the study of Xu *et al*. this enzyme forms a homotetramer in its active form [31]. The specific activity of the purified enzyme varied between 106 - 120 U/mg with acetoin as substrate (standard reaction assay; chapter 2.6). With diacetyl as a substrate an activity of 76 U/mg was measured. Differences to studies earlier published by Xu et al. using the His-tagged enzymes found 120 U/mg with diacetyl as a substrate. These variants can be explained by differences in the concentrations of diacetyl, NADH, potassium phosphate and pH [31]. However, our conditions ensured that V_{max} was reached.

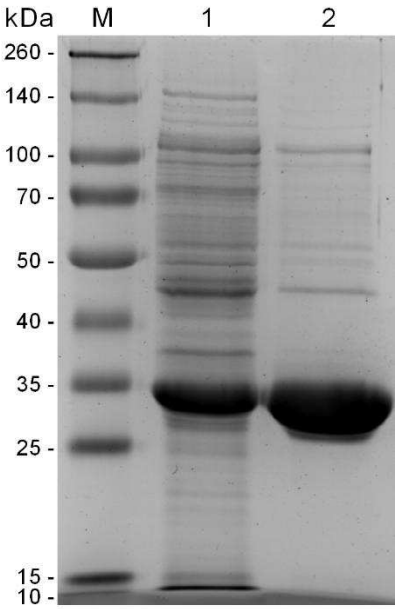


Figure 1: Purification of recombinant BlBDH. Cell-free extract (10 µg protein, lane 1) and purified enzyme (10 µg, lane 2) were analyzed by SDS-PAGE and stained with Coomassie blue.

Sequence alignment with the biochemically characterized *meso*-BDH from *Klebsiella pneumoniae* [26] and *Serratia marcescens* H30 [32] revealed a nucleotide identity of 65.3% and 51.0%, respectively. Regarding the protein sequence the sequence identity is 67.1% and 32.8%, respectively (Figure 2).

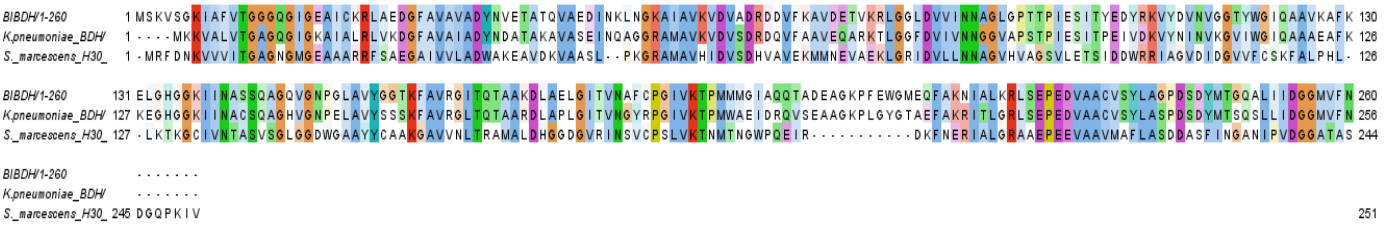


Figure 2: Alignment of the amino acid sequence from BlBDH and the *meso*-BDHs from *Klebsiella pneumoniae* (accession number: JN865245.1) [26] and *Serratia marcescens* H30 (accession number: AFH00999.1) [32].

3.2 Substrate scope

Even for biochemically characterized *meso*-BDHs the substrate range besides the physiological substrates diacetyl, acetoin and 2,3-butanediol is hardly known. The relative activity towards acetoin and diacetyl varies between different BDHs. BlBDH shows higher reductase activities for acetoin over diacetyl like the *meso*-BDH from *Serratia marcescens* H30 [32]. Instead, the acetoin reductase from *Rhodococcus erythropolis* shows a two times higher activity for diacetyl over acetoin, under the given conditions [33]. To further elucidate the biocatalytical properties of BlBDH, we extended the studies on several aliphatic and alkylaryl α-diketones such as α-keto acids and α-keto ester. Enzyme activity was measured spectrophotometrically as shown in Table 1. For the reduction reaction this enzyme exhibits the highest activity towards vicinal diketones and α-hydroxy ketones.

Important to mention is that this enzyme shows some remarkably good activity towards vicinal diketones with longer aliphatic chains like 2,3-heptanedione and 2,3-hexanedione and even branched molecules like 5-methyl-2,3-hexanedione. Furthermore, not negligible activities with α-keto aldehydes such as methylglyoxal or α-hydroxy aldehydes such as (*R*)-lactaldehyde were measured. Additionally, moderate to low activities were observed with bulky vicinal diketones and α-hydroxy ketones, like 1-phenyl-1,2-propanedione. While most BDHs struggle with molecules with longer alkyl chains, BlBDH shows good activity towards such molecules. In fact, we observed even higher activities for 2,3-hexanedione (150%) and 2,3-heptanedione (119%) as for acetoin under the assay conditions. While for 2,3-pentanedione the (*R*)-2,3-butanediol dehydrogenase from *Saccharomyces cerevisiae* and the YAL060W gene product (*R*,*R*)-2,3-butanediol dehydrogenase from *Saccharomyces cerevisiae* shows only low activity for this compound [34,35]. Instead, the acetoin reductase from *Enterobacter aerogenes* exhibits a higher activity for this molecule with 85% (in comparison to diacetyl) [36]. Exceptionally high activities for molecules with larger alkyl chains like 2,3-pentanedione, 2,3-hexanedione and 2,3-heptanedione were found with the 2,3-butanediol dehydrogenase from *Serratia marcescens* CECT 977 [22]. For the reduction of 1-phenyl-1,2-propanedione both enzymes exhibit a similar activity under the given reaction conditions [22]. Instead, with bulky substrates like benzoin or benzil no activity of BlBDH was detected. Low activity for lactaldehyde and methylglyoxal could be measured.

Furthermore, when using enantiomerically pure substrates the enzyme always, to the best of our knowledge, prefer the (*R)*-enantiomer over the (*S)*-enantiomer. For the reduction of (*R*)-1-hydroxy-1-phenylpropan-2-one ((*R)*-PAC) a relative activity of 8% and for the reduction of (*S*)-1-hydroxy-1-phenylpropan-2-one ((*S)*-PAC) a relative activity of 5% compared to acetoin was found. A low activity for the reduction of (*R*)-2-hydroxy-1-phenylpropan-1-one ((*R)*-HPP) was observed (2.5%) but no activity for the reduction of (*S*)-HPP. The same is true for (*R*)- and (*S*)-lactaldehyde (Table 1).

In the oxidative reaction BlBDH displays only activity towards *meso*-2,3-butanediol (chapter 2.6). No activity with (2*S*,3*S*)-2,3-butanediol and (2*R*,3*R*)-2,3-butanediol was detected. Likewise, no activity with primary, secondary alcohols or other diols was observed. Several already characterized BDHs show the same pattern like the *R*-selective BDH from *Clostridium beijerinckii* or the *R*-selective BDH from *Bacillus clausii* DSM 8716^{T} with no activity detected for primary and secondary alcohols like ethanol, 2-propanol and ethyleneglycol [37,38]. On the contrary, the *meso*-BDH from *Serratia marcescens* H30, for example, exhibits some low activity with (*S*,*S*)-2,3-butanediol, 1,2-propanediol, glycerol, and 1,2-pentanediol under respective assay conditions [32].

Table 1: Relative activities of *meso*-BDHs towards NADH-oxidizing substrates (50 mM potassium phosphate buffer, pH 6.8, 30 °C, 0.3 mM NADH, 10 mM substrate (100% = 120 U/mg)) and NAD^+-reducing substrates (50 mM glycine-NaOH buffer, pH 10.0, 30 °C, 0.3 mM NAD^+ and 10 mM substrate (100% = 73.4 U/mg)). Data for enzymes from this study, *S.* marcescens [32] (assay conditions: potassium phosphate buffer (50 mM, pH 8.0); NAD^+ (4 mM); 40°C; substrate (100 mM) for oxidation and sodium acetate-buffer (50 mM, pH 5.0); NADH (0.2 mM); 40 °C; substrate (100 mM) for reduction reaction) and *K. pneumoniae* [26] (assay conditions: sodium pyrophosphate (33 mM, pH 8.0); NAD^+ (5 mM); 40 °C; substrate (100 mM) for oxidation and sodium pyrophosphate (33 mM, pH 7.0); NADH (5 mM); 40 °C; substrate (50 mM) for reduction reaction) refer to the activity with racemic acetoin. Data for *meso*-BDH from *B. licheniformis* [31] (assay conditions: glycine-NaOH buffer (100 mM, pH 10.0); NAD^+ (1 mM); 30°C; substrate (5 mM) for oxidation and potassium phosphate (100 mM, pH 6.0); NADH (1 mM); 30 °C; substrate (5 mM) for reduction reaction) refer to diacetyl for the reductive reaction and all data for the oxidative reaction refer to the activity with *meso*-2,3-butanediol except data taken from Xu *et al.*[31] n.d., no data.

	meso-butanediol dehydrogenase from	*B. licheniformis*	*B. licheniformis*[31]	*S. marcescens* H30[32]	*K. pneumoniae*[26]
	Substrate	activity [%]	activity [%]	activity [%]	activity [%]
Entry No.	**Reductive reaction**				
1		100.0 ± 0.0	97 ± 2	100 ± 0.0	100 ± 0.0
2		67.1 ± 0.0	100 ± 3	75 ± 3	n.d.
3		55.7 ± 0.0	n.d.	n.d.	0.1 ± 0.0
4		92.1 ± 0.1	69 ± 4	n.d.	n.d.
5		150.3 ± 0.0	66 ± 2	n.d.	n.d.
6		36.1 ± 0.0	10 ± 1	n.d.	n.d.
7		38.0 ± 0.0	n.d.	n.d.	n.d.
8		115.7 ± 0.0	n.d.	n.d.	n.d.
9		119.3 ± 6.4	n.d.	n.d.	n.d.
10		14.4 ± 0.0	n.d.	n.d.	n.d.
11		0.1 ± 0.0	n.d.	n.d.	n.d.
12		7.6 ± 0.6	n.d.	n.d.	0
13		5.9 ± 0.7	n.d.	n.d.	n.d.
14		0.0	n.d.	n.d.	n.d.
15		2.5 ± 0.0	n.d.	n.d.	n.d.
16		2.0 ± 0.0	n.d.	n.d.	n.d.
17		18.2 ±0.0	n.d.	n.d.	n.d.
18		7.9 ± 0.0	n.d.	n.d.	n.d.
19		5.2 ± 0.1	n.d.	n.d.	n.d.
20		4.1 ±0.0	n.d.	n.d.	n.d.
21		2.9 ±0.0	n.d.	n.d.	n.d.
22		2.5 ± 0.1	n.d.	n.d.	n.d.
23		0.0	n.d.	n.d.	n.d.
	Oxidative reaction				
24		100.0 ± 0.0	100.0 ± 2 (all stereoisomers)	100.0 ± 0.0	100.0 ± 0.0
25		0.2 ± 0.0	n.d.	11 ± 3	0
26		0.1 ± 0.0	n.d.	0	0
27		0.8 ± 0.0	n.d.	n.d.	n.d.
28		0.5 ± 0.0	n.d.	n.d.	n.d.
29		0.4 ± 0.0	0.57 ± 0.15	24 ± 3	n.d.
30		0.0	n.d.	n.d.	n.d.
31		0.0	n.d.	n.d.	n.d.
32		0.0	n.d.	n.d.	n.d.
33		0.0	n.d.	n.d.	n.d.
34		0.0	n.d.	n.d.	n.d.

3.3 Solvent stability of BlBDH

The effect of organic solvents on the enzyme stability was determined by incubating the enzyme with a solvent concentration of 0 – 60% (v/v) over 1 hour at 25 °C. DMSO, ethanol, acetone, methanol and acetonitrile were used as water miscible solvents (Figure 3). BlBDH shows the highest tolerance towards DMSO. The enzyme is stable up to 20% DMSO (v/v) under these conditions. A complete loss of activity was observed after incubation with 60% (v/v) DMSO. In contrast, this enzyme inactivates rapidly in the presence of acetonitrile, ethanol or acetone, respectively, whereas methanol this slightly better tolerable. For further biocatalytic studies of hydrophobic substrates we choose an organic solvent concentration of 5% (v/v) DMSO. This was the lowest amount of organic solvent to achieve solubility of the given substrates.

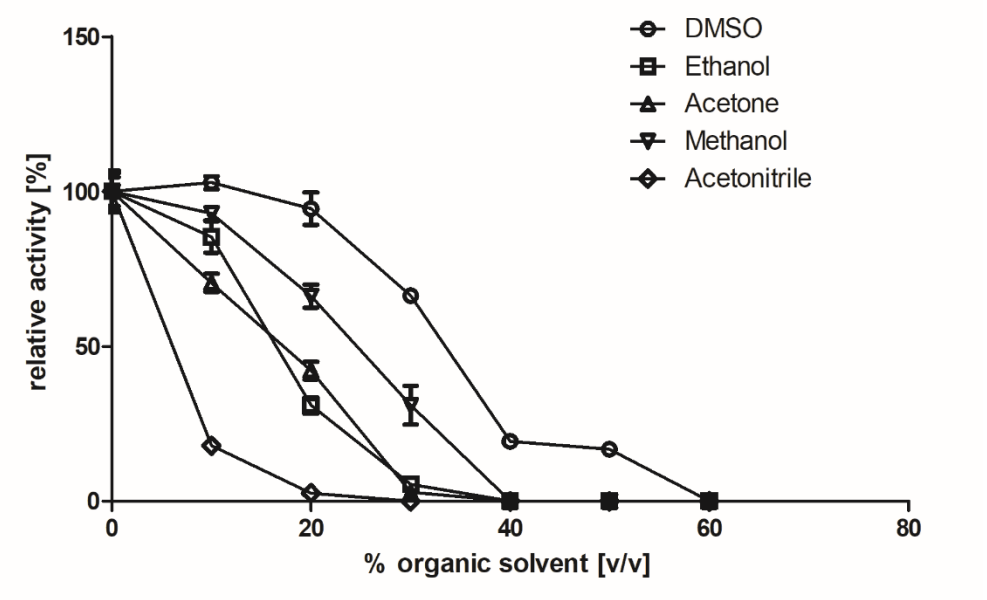


Figure 3: Effect of water-miscible organic solvents on the stability of BlBDH. BlBDH was incubated for 1 hour (25°C) with 0 to 60% (v/v) solvent in potassium phosphate buffer (50 mM, pH 6.8). Activity was determined with the standard photometric assay.

3.4 Biochemical properties of BlBDH

BlBDH exhibits different pH optima for the reduction and oxidation reaction consisting of: pH 5.0 for the reduction of diacetyl, pH 6.0 – 8.0 for acetoin and pH 9.0 for the oxidation of *meso*-2,3-butanediol. In the study of Xu *et al.*[31] a pH optimum of 10.0 was measured for the oxidation of 2,3-butanediol (all stereoisomers; see suplements).

Similar pH optima for the oxidation and reduction reaction were observed with the *meso*-BDH from *Serratia marcescens* H30, the BDH from *B. stearothermophilus* and the BDH from *Leuconostoc pseudomesenteroides* [32,39,40]. Generally the pH-optimum of *meso*-BDHs for the reduction of acetoin and diacetyl lies in within an acidic to low basic, whereas the pH-optimum for the oxidation of *meso*-2,3-butanediol lies more in the basic pH range. In summary, BlBDH exhibits the same pH optima

range like other reported butanediol dehydrogenases. The highest enzyme activity was observed at 37 °C (data not shown and Xu *et al*.[31]) and the K_m and k_{cat} values for acetoin and diacetyl are 2.76 ± 0.63 mM; 81.78 s^{-1} and 77.5 ± 6.2; 872.3 s^{-1}, respectively. Comparison between the measured kinetics with and without 5% (v/v) DMSO with acetoin as substrate shows that DMSO has a slight impact on the enzyme kinetics. Adding DMSO leads to a slightly higher K_m value and slightly lower turnover number. All kinetics were fitted with the Michaelis-Menten equation, although acetoin shows a slight substrate inhibition. Considering that diacetyl is one of the physiological substrates of butanediol-dehydrogenases, BlBDH exhibits a high K_m-value for this molecule compared to acetoin. On the other hand, this enzyme reveals its highest maximum activity for diacetyl.

We further investigated the kinetic parameters for the non-physiological substrates 2,3-pentanedione, 2,3-hexanedione, 2,3-heptanedione and 5-methyl-2,3-hexanedione, because of the high activities this enzyme displays for these substrates (Figure 4). The measured K_m and k_{cat} values are 29.0 mM ± 6.5 mM and 535.8 s^{-1} for 2,3-hexanedione, 42 mM ± 15.4 and 423.8 s^{-1} for 5-methyl-2,3-hexanedione, 11.0 mM ± 1.6 and 306 s^{-1} for 2,3-heptanedione, respectively (Table 2). Although DMSO (5% v/v) was added to counter solubility issues, V_{max} was not reached in the here tested substrate concentrations for 5-methyl-2,3-hexanedione because of the solubility limitations. For better substrate solubility, higher solvent concentrations are needed. But this would have an impact on the enzyme activity and stability. Therefore, these measurements can only be used as an estimation of the kinetic parameters. However, the data indicate that all diketones are converted with very high activity (80-870 s-1). Among these tested diketones, the largest (2,3-heptanedione) resulted in the lowest K_m-value correlated with the lowest activity compared to the hexanediones. Between the two tested hexanediones the lowest K_m-value compared with the highest activity was measured for 2,3-hexanedione.

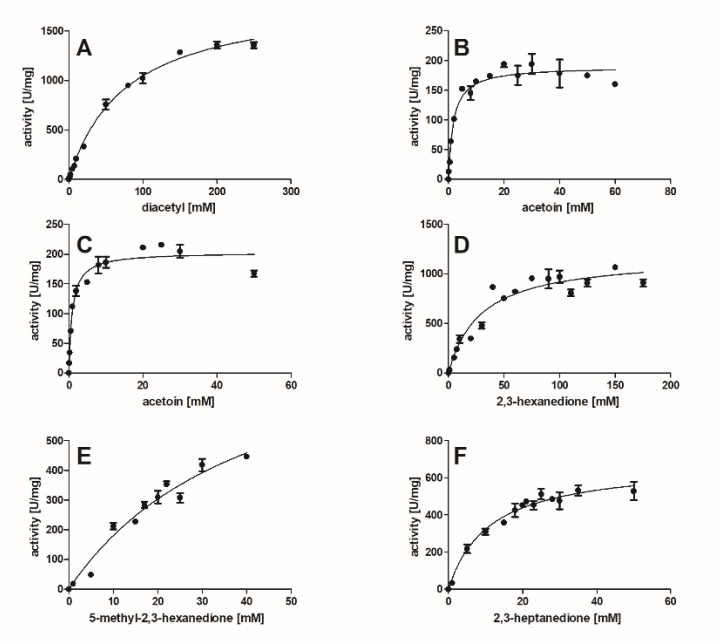


Figure 4: Michaelis-Menten plot for the reduction of diacetyl (A), acetoin with 5% (v/v) DMSO (B), acetoin without 5% (v/v) DMSO (C), 2,3-hexanedione (D), 5-methyl-2,3-hexanedione (E), 2,3-heptanedione (F). Activity was determined in potassium-phosphate buffer (50 mM; pH 6.8), with 5% (v/v) DMSO as solubilizer and NADH (300 µM) at 30°C. The kinetic for acetoin was also measured.without 5% (v/v) DMSO for comparison. Substrate concentrations were varied as indicated in the plot. Measurements were done in triplicates. The curve was fitted with the software GraphPad Prism 7 with the Michaelis-Menten equation.

Table 2: Kinetic parameter of BlBDH for different substrates.

Substrate	K_m [mM]	*kcat* [s^{-1}]	V_{max} [U/mg]
Acetoin without 5% (v/v) DMSO	0.9 ± 0.2	92.28	203.3 ± 6.7
Acetoin with 5% (v/v) DMSO	1.8 ± 0.3	81.78	190.0 ± 5.4
Diacetyl	77.5 ± 6.2	872.3	1856.0 ± 57.5
2,3-Hexanedione	29.0 ± 6.5	535.8	1183.0 ± 79.0
5-Methyl-2,3-hexanedione	42.0 ± 15.4	423.8	941.7 ± 218.1
2,3-Heptanedione	11.0 ± 1.6	306.0	680.5 ± 32.71

3.5 Biocatalytic characterization of BlBDH

To gain a deeper insight into the biocatalytical potential of BlBDH, biotransformations with its physiological substrates as well as non-physiological vicinal diketones and α-hydroxyketones as substrates (Figure 5) and BlBDH over 1 hour were done (chapter 2.11). Samples were taken and analyzed by GC and GC-MS every 20 min. NADH was regenerated by using formate dehydrogenase and formate (Figure 6).

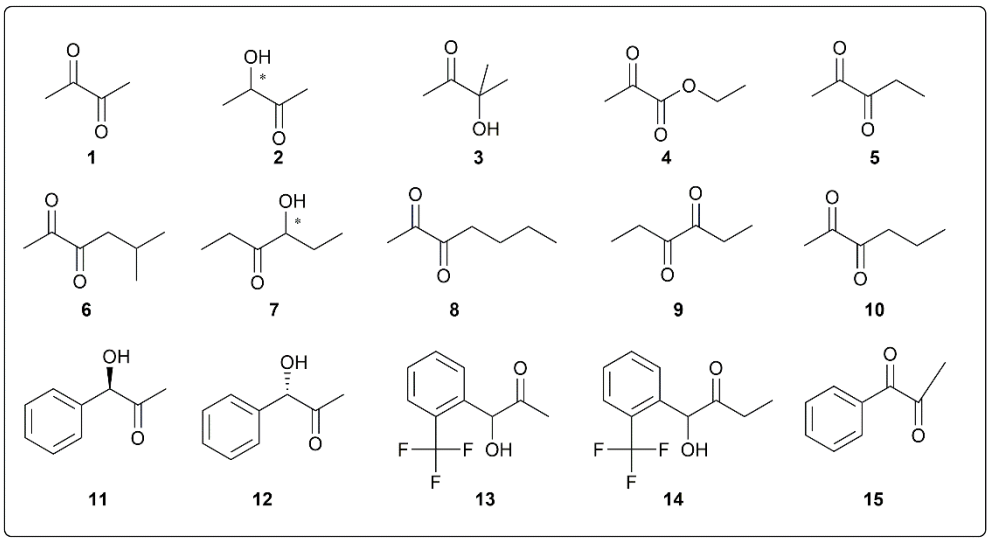
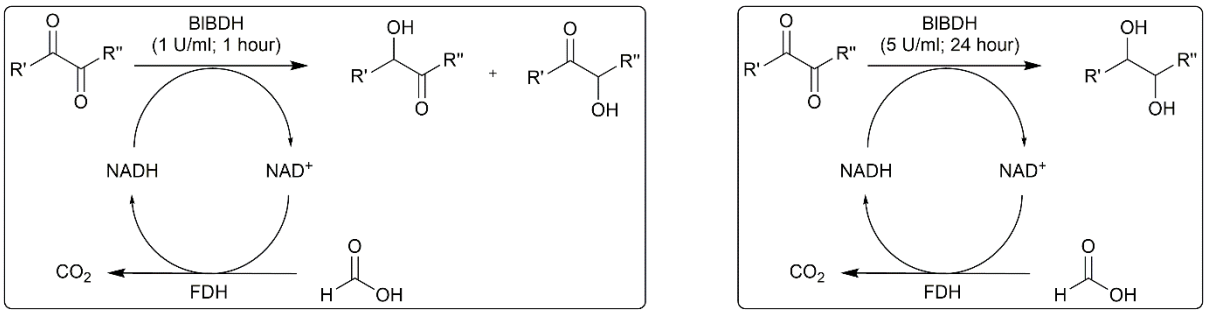


Figure 5: Vicinal diketones and α-hydroxy ketones (10 mM) reduced by BlBDH (1 U/ml for the corresponding substrate). A cofactor regeneration system was applied by using formate dehydrogenase (5 U/ml) and formate (30 mM).



1: R'=R"=CH_3 **2**:R'=R"=CH_3 **3**:R'=CH_3, R"=OH-CH_3-CH_3 **4**:R'=CH_3, R"=O-CH_2-CH_3 **5**:R'=CH_3, R"=CH_2-CH_3 **6**:R'=CH_3, R"=CH_2-$(CH_3)_2$ **7**:R'=R"=CH_2-CH_3 **8**:R'=CH_3, R"=$(CH_2)_3$-CH_3 **9**:R'=R"=CH_2-CH_3 **10**:R'=CH_3, R"=$(CH_2)_2$-CH_3 **11**:R'=Ph, R"=CH_3 **12**:R'=Ph, R"=CH_3 **13**:R'=Ph-$(F)_3$, R"=CH_3 **14**:R'=Ph-$(F)_3$, R"=CH_2-CH_3 **15**:R'=PH, R"=CH_3

Figure 6: Approach for the BlBDH catalyzed reduction of diketones. Cofactor regeneration is maintained by using formate dehydrogenase.

As there are no commercial sources of enantiomerically pure α-hydroxy ketones and diols as reference products available, we synthesized the corresponding racemic diols by reduction of the diketones (2,3-pentanedione, 2,3-hexanedione, 3,4-hexanedione, 5-methyl-2,3-hexanedione) with sodium borohydride ($NaBH_4$) [41]. Thus, the reaction products of BlBDH and the constitutional isomer of the α-hydroxy ketones were identified. By comparing the reaction products of BlBDH with our previous published (*R*,*R*)-2,3-butanediol dehydrogenase from *Bacillus clausii* DSM 8716^T the identification of some enantiomers stereoisomers was possible [38,41] .

Under the chosen conditions (1 U/ml, 30°C, 1 h) the reduction of diacetyl leads to the production of (*S*)-acetoin with a conversion of 60% after 1 hour. In some batches a very slow reduction of (*S*)-acetoin to (*S*,*S*)-2,3-butanediol (traces) was detected. The latter is in line with results from Xu *et al.* who showed that diacetyl is converted via (*S*)-acetoin to mainly (*S*,*S*)-2,3-butanediol using 100 U/ml enzyme and 12 h reaction time [31]. In comparison to diacetyl, the BlBDH-catalyzed reduction of

racemic acetoin targeted almost exclusively the (*R*)-enantiomer, which was completely reduced after 1 hour, yielding *meso*-2,3-butanediol. Only a minor conversion (1.7%) of the (*S*)-enantiomer to (*S*,*S*)-2,3-butanediol was observed. Obviously BlBDH exhibits a much higher affinity to the (*R*)-enantiomer and introduces a (*S*)-configurated stereo center. This leads to the opportunity to use this enzyme for dynamic kinetic resolutions by stopping the reaction when the (*R*)-acetoin is consumed, to produce *meso*-2,3-butanediol, with (*S*)-acetoin left. The same stereo-preference is also described for the *meso*-butanediol dehydrogenase from *Serratia marcescens* CECT 977, which also belongs to the SDR superfamiliy. It introduces an (*S*)-configured stereo center but favors (*R*)-acetoin [22]. The same holds true for the *meso*-butanediol dehydrogenase from *Serratia marcescens* H30 [32]. Fermentative production of *meso*-2,3-butanediol was demonstrated by using the *Bacillus licheniformis* MW3 (Δgdh encoding for a (*R*,*R*)-2,3-butanediol dehydrogenase gene) strain with concentrations of 90.1 g/l after 32 hours [42]. This is a modified *Bacillus licheniformis* DSM 13 strain harboring the gene of the butanediol dehydrogenase investigated in the present study [42,43].

Under the initially chosen reaction conditions, the reduction of 2,3-pentanedione led to the generation of mainly a 2-hydroxy-3-pentanone (95%) and small amount of a 3-hydroxy-2-pentanone (5%) with a conversion of 100% after 20 min. Note, that as not all isomers were available, the data in this chapter refer to relative peak areas and not to a calibration curve of the products. 2,3-Hexanedione was completely converted after 20 min and no further conversion up to 1 hour was detected yielding two α-hydroxy ketones as products with 2-hydroxy-3-hexanone as the main product (88%) and 3-hydroxy-2-hexanone as side product (12%). The reduction of racemic 4-hydroxy-3-hexanone leads to the complete consumption of only one 4-hydroxy-3-hexanone enantiomer and the synthesis of one diol enantiomer after 20 min. According to the reaction of this enzyme with diacetyl and acetoin, we assume the reduction of the (*R*)-4-hydroxy-3-hexanone enantiomer and the production of the *meso*-3,4-hexanediol (conversion after 1 hour: >99% of (*R*)-4-hydroxy-3-hexanone). Running the reaction over 24 hours led also to the reduction of the second enantiomer, presumably (*S*)-4-hydroxy-3-hexanone, to probably the (*S*,*S*)-3,4-hexanediol (conversion after 24 hours: 54%). 3,4-Hexanedione was reduced to one enantiomer of 4-hydroxy-3-hexanone as the main product. This peak has the same retention time as the peak of the enantiomer of 4-hydroxy-3-hexanone, which was not reduced and therefore it is presumably the (*S*)-configured enantiomer, with traces (which does not increase even with a belonged reaction time, data not shown) of a 3,4-hexanediol as a side product (conversion: 100% after 20 min, no further conversion after 1 hour to the diol). Full conversion of the 4-hydroxy-3-hexanone to this diol was possible by prolonged reaction time (24 h) and a higher concentration of BlBDH (5 U/ml). Performing the reduction of 3,4-hexanediol over 24 hours leads to a product with the same retention time like in the 24 hour reduction of the racemic 4-hydroxy-3-hexanone and therefore be annotated as the (*S*,*S*)-3,4-hexanediol. The reduction of 3,4-

hexanedione and the racemic 4-hydroxy-3-hexanone mimics the behavior of BlBDH with diacetyl and racemic acetoin. It shows again that this enzyme introduces an *S*-configured stereo center, while preferring the *R*-enantiomer of the hydroxy ketone intermediate. The (*S*)-enantiomer of the hydroxy ketone intermediate can be reduced but only by applying a higher enzyme concentration and/or a prolonged reaction time.

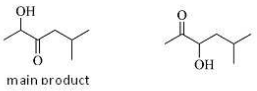
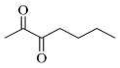
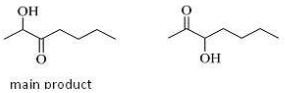
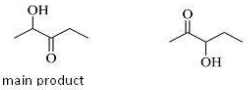
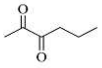
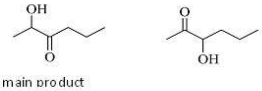
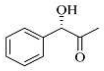
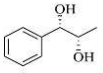
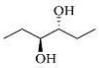
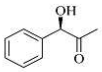
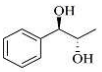
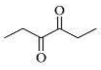
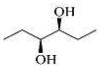
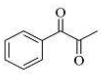
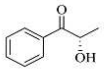
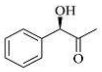
By reducing 5-methyl-2,3-hexanedione with BlBDH the generation of two products was detected with 2-hydroxy-5-methyl-3-hexanone (86%) as the main product and a small amount of 3-hydroxy-5-methyl-2-hexanone as a side product (14%; conversion after 20 min >99%, no further products after 1 hour). In some batches traces of 5-methyl-2,3-hexandiol were detected.

Besides, the reduction of 2,3-heptanedione was very unspecific under this conditions, yielding one 2-hydroxy-3-heptanone enantiomer as the major product (50%) with two 3-hydroxy-2-heptanone enantiomers (37% and 3%, respectively) and one 2,3-heptanediol (7.8%) enantiomer as side products (conversion after 1 hour: > 99%). Reduction of 3-hydroxy-3-methyl-2-butanone yielded one 2-methyl-2,3-butandiol enantiomer (conversion after 20 min: > 99%). Ethyl pyruvate was reduced to one product (conversion after 20 min, > 99%).

Surprisingly, BlBDH is also capable to catalyze the reduction of molecules, which contain a phenyl moiety. The reduction of (*S*)-1-hydroxy-1-phenyl-2-propanone ((*S*)-PAC) led to (*S*,*S*)-1-phenyl-1,2-propandiol with a conversion of 85% after 1 hour. Accordingly, the transformation of (*R*)-1-hydroxy-1-phenyl-2-propanone ((*R*)-PAC) led to (*R*,*S*)-1-phenyl-1,2-propandiol with a conversion of 96.8% and >99% *ee* after 1 hour. This identification was possible by comparing the retention times to the reference compounds (*R*,*R*)-1-phenyl-1,2-propandiol and (*S*,*S*)-1-phenyl-1,2-propandiol. The retention time of the reduction product of (*R*)-PAC did not match with both references and therefore can be assumed as the (*R*,*S*)-stereoisomer, whereas the reduction of 1-phenyl-1,2-propanedione leads to (*S*)-2-hydroxy-1-phenyl-1-propanone ((*S*)-HPP) (86.5%) as the major product and (*R*)-1-hydroxy-1-phenyl-2-propanone ((*R*)-PAC) as a side product (11.3%) after one hour reaction time. Thus, from four theoretical products only two are formed. No diol formation of the theoretical accessible diols was observed under the tested conditions. Transformation of racemic 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-propanone and 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-propanone over 24 hours led to the production of two diol enantiomers, respectively (conversion >99%). In general, the reduction of a vicinal diketone, either symmetric or asymmetric, leads mainly to the synthesis of the α-hydroxy ketone intermediate under these conditions. But this enzyme catalyzes this reduction in most cases not absolute stereo selectively (Table 3). It should be noted that the same results with these substrates were obtained also in biotransformations with crude cell extracts of recombinant *E.coli* instead of purified enzyme (data not shown). The reaction pattern of BlBDH is

similar to the *meso*-butanediol dehydrogenase from *Serratia marcescens* CECT 977 [22]. Whereas *Serratia marcescens* CECT 977 BDH primary catalyzes the reduction of 2,3-pentanedione and 3,4-hexanedione primarily to the corresponding (*S*)-diols. Both enzymes, BlBDH as well as *Serratia marcescens* CECT 977 BDH, show the potential to control the reaction outcome by adjusting the reaction parameters, e.g. enzyme concentration and reaction time, to obtain defined target products.

Table 3: Overview of the product formation in biotransformation of several vicinal diketones and α-hydroxyketones with BlBDH. Substrate concentration 10 mM;potassium phosphate buffer (50mM, pH 6.8), reaction time 1 hour; 1 U/ml BlBDH (measured for each substrate), NADH (0.3 mM). A cofactor regeneration system was applied by using formate dehydrogenase (5 U) and formate (30 mM).

Substrate	Product formation	Substrate	Product formation
			 main product
 racemic			 main product
	 main product		
	 main product		
 racemic			
	  traces		 main product 

4 Conclusion

We investigated the biocatalytic properties of the *meso*-2,3-butanediol dehydrogenase from B*acillus licheniformis* DSM 13^{T}. For its physiological substrates this enzyme exhibited the highest activity for acetoin in the reductive reaction and for *meso*-2,3-butanediol in the oxidative reaction. Surprisingly, even more pronounced activities could be measured with the non-physiological substrates 2,3-hexanedione, 2,3-heptanedione and 5-methyl-2,3-hexanedione under this assay conditions (Table 1). Therefore, the kinetic data of BlBDH for this molecules were fitted to the Michaelis-Menten

equation, revealing that this enzyme converts these molecules with high activity. Although, because of solubility issues it was not possible to reach V_{max} for 5-methyl-2,3-hexanedione (Figure 4). Deeper investigation of the stereoselectivity of this enzyme for its physiological substrates revealed that BlBDH introduces an (*S*)-configured stereo center preferably into (*R*)-configured α-hydroxy ketones, which leads to the synthesis of *meso*-2,3-butanediol starting from racemic acetoin. With prolonged reaction time the reduction of this (*S*)-hydroxy ketone is also observed leading to the (*S*,*S*)-diol in traces. Furthermore, this enzyme catalyzes the reduction of several non-physiological substrates with varying aliphatic chains and even bulky ones containing a phenyl moiety, which can additionally be modified with demanding groups like trifluoro-residues. This open the opportunity for a wider substrate usage in biocatalysis. Although, in many cases the reduction is not strictly stereoselective leading to one main product and some side products. Concerning this reaction pattern, the reaction outcome can be controlled by varying the reaction time or the amount of enzyme given into the reaction to either yield the (*S*)-α-hydroxy ketone intermediate or the (*S*,*S*)-diol starting from a diketone. The generation of the *meso*-product is possible by starting from a racemic or a solely (*R*)-configured substrate.

Acknowledgments

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Table 1

Entry No.
1
2
3
4
5
6
7
8
9
10
11
12
13

Table 2

Substrate	K_m [mM]	*kcat* [s^{-1}]
Acetoin without 5% (v/v) DMSO	0.9 ± 0.2	92.28
Acetoin with 5% (v/v) DMSO	1.8 ± 0.3	81.78
Diacetyl	77.5 ± 6.2	872.3
2,3-Hexanedione	29.0 ± 6.5	535.8
5-Methyl-2,3-hexanedione	42.0 ± 15.4	423.8
2,3-Heptanedione	11.0 ± 1.6	306.0

Caption: Kinetic parameter of BlBDH for different substrates.

Table 3

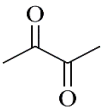
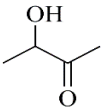
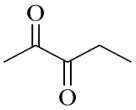
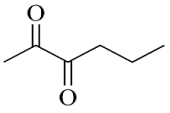
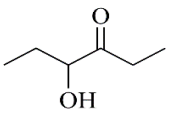
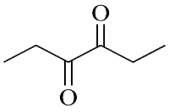
Substrate

 racemic


 racemic


Figure 1

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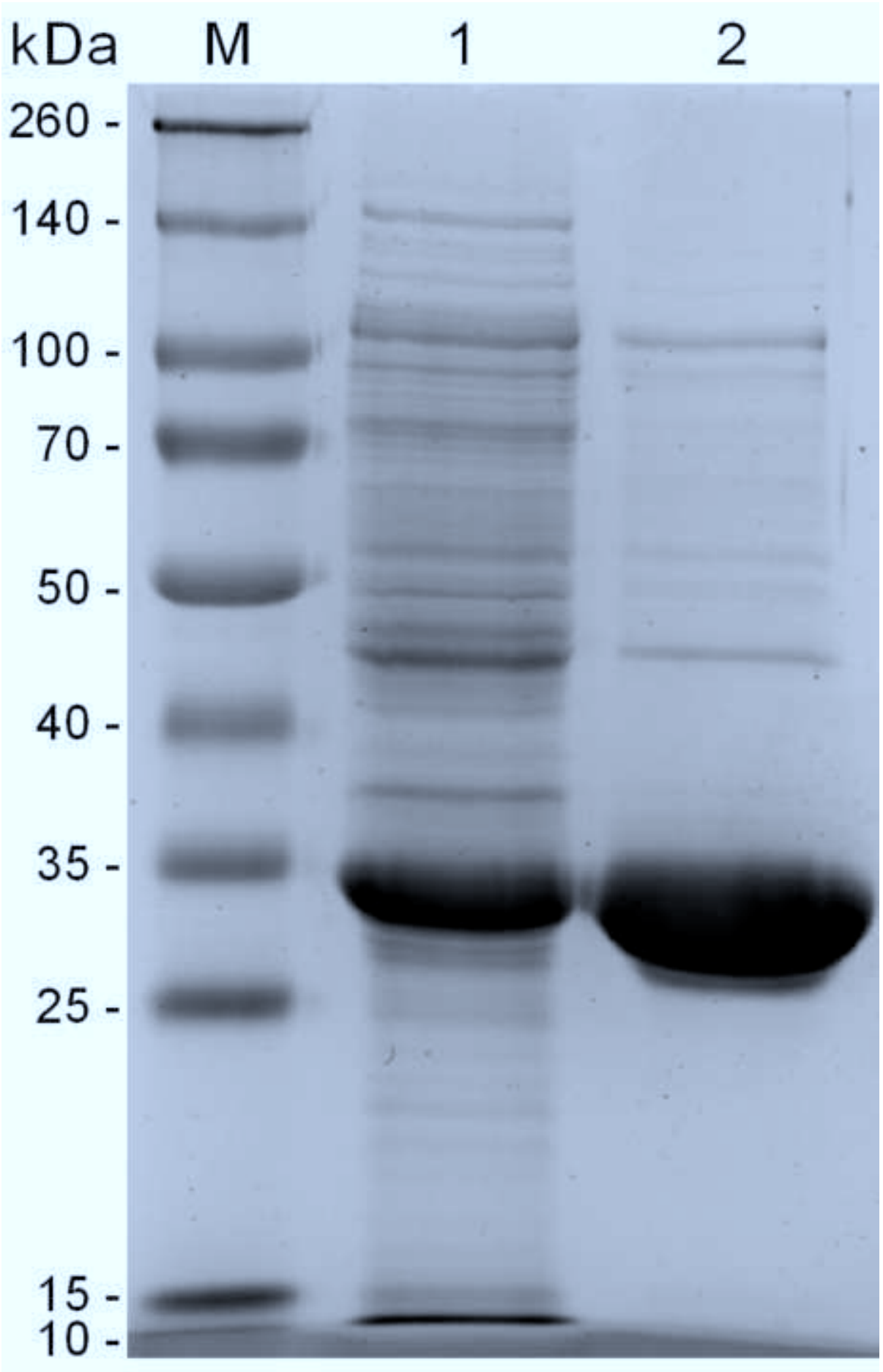

Figure 2

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Figure 3

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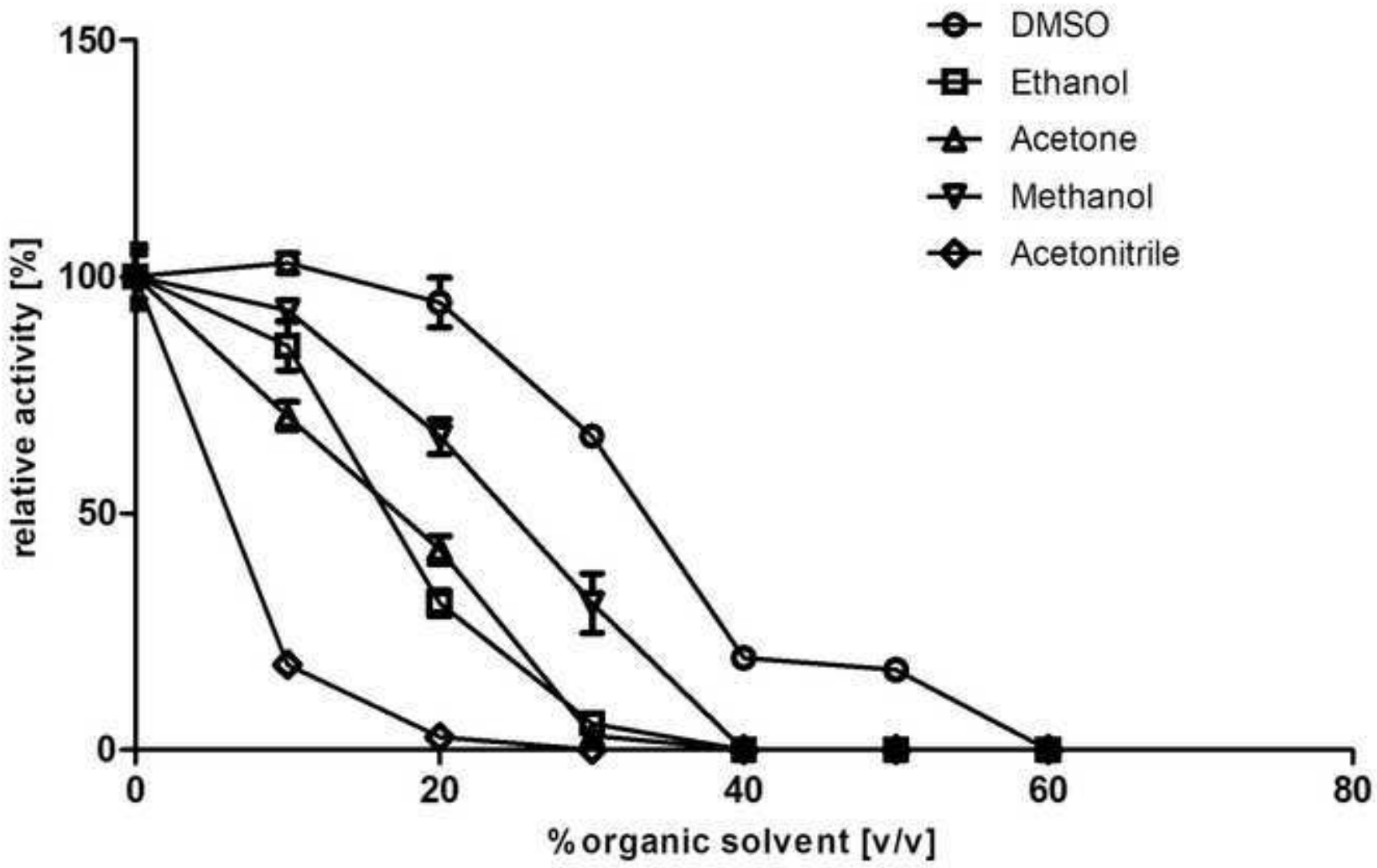

Figure 4

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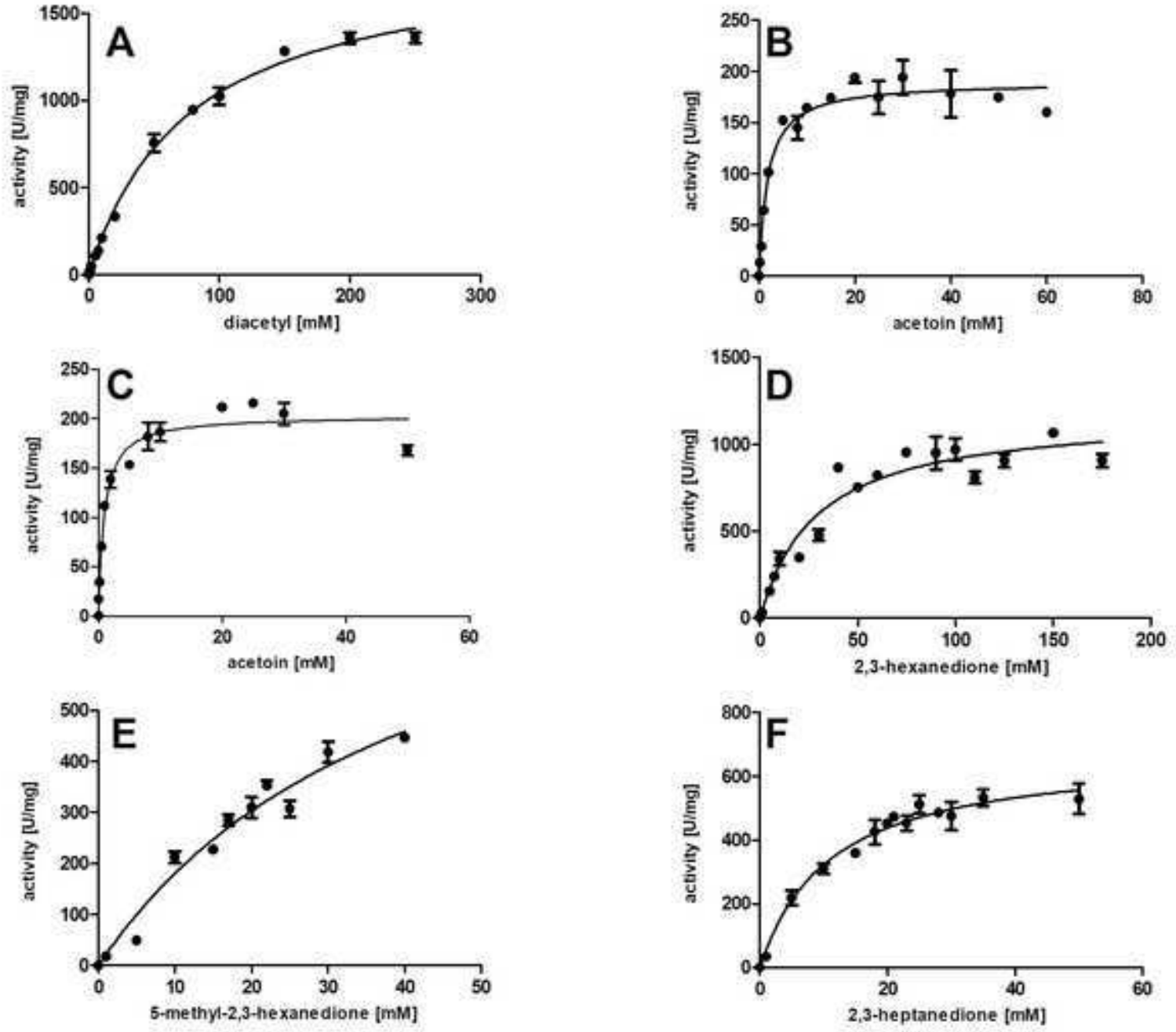

Figure 5
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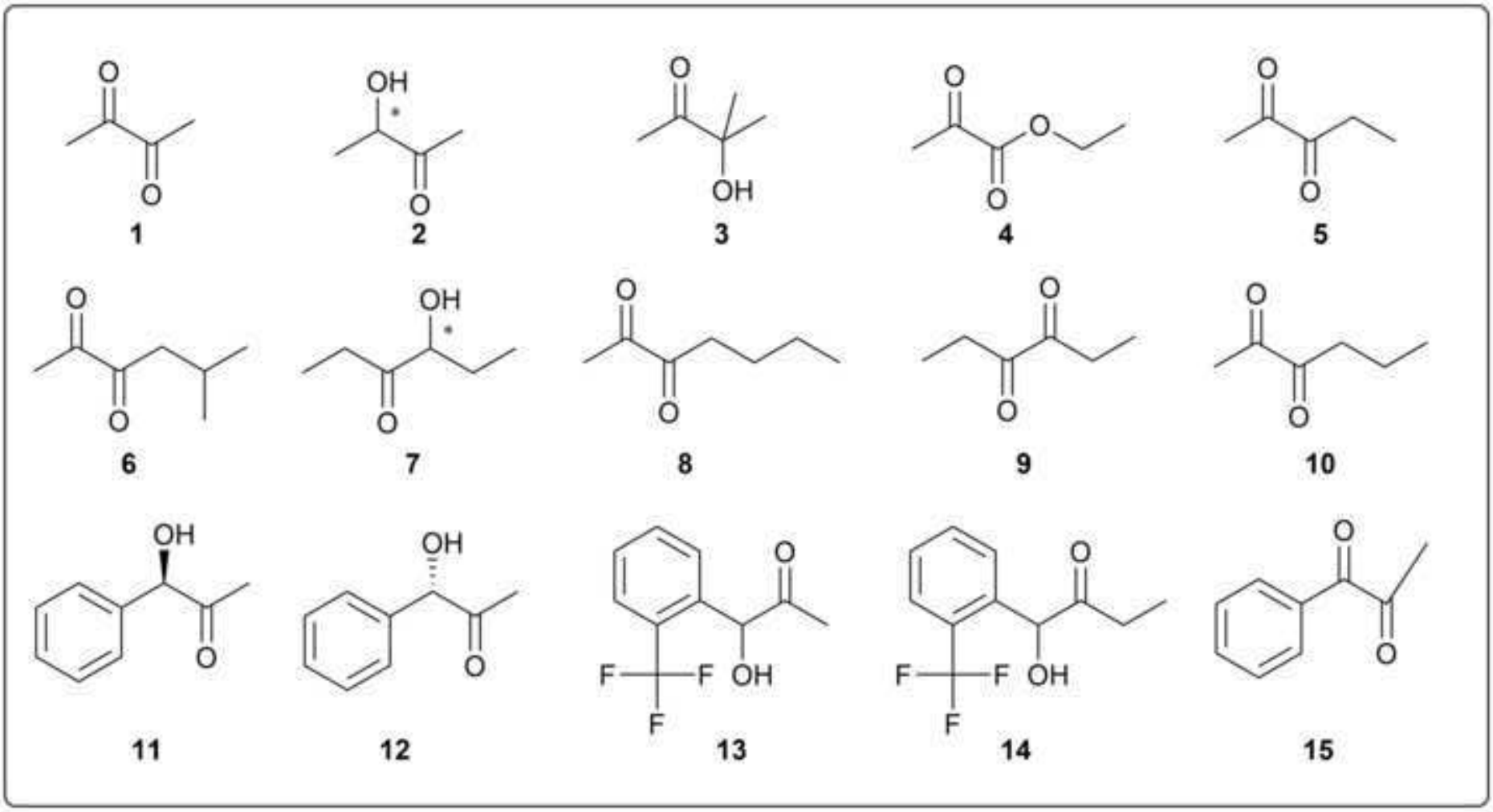

Figure 6

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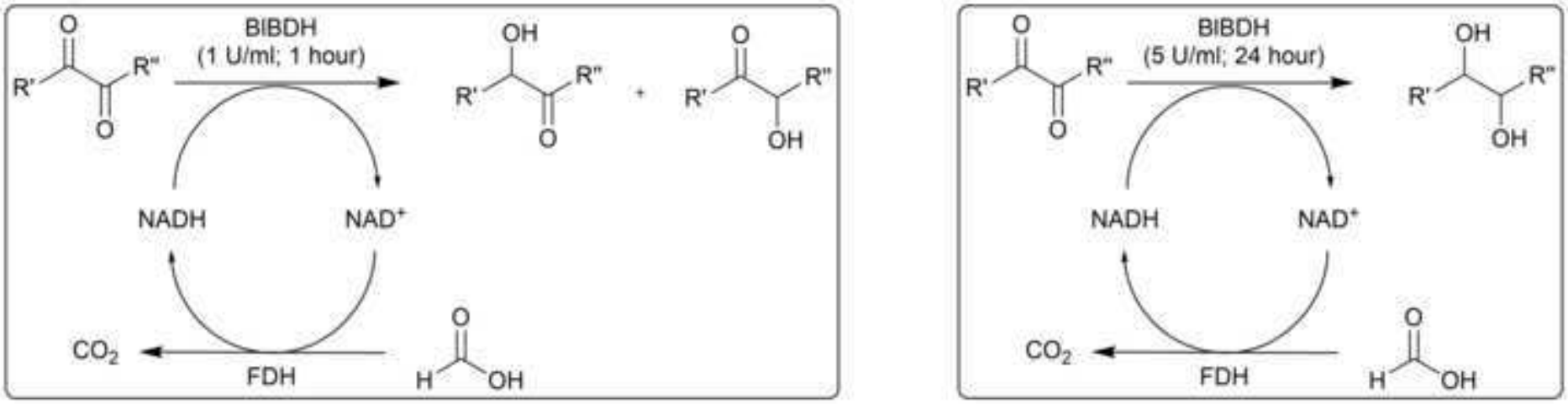

1: R'=R"=CH_3 **2**:R'=R"=CH_3 **3**:R'=CH_3, R"=OH-CH_3-CH_3 **4**:R'=CH_3, R"=O-CH_2-CH_3 **5**:R'=CH_3, R"=CH_2-CH_3 **6**:R'=CH_3, R"=CH_2-$(CH_3)_2$ **7**:R'=R"=CH_2-CH_3 **8**:R'=CH_3, R"=$(CH_2)_3$-CH_3 **9**:R'=R"=CH_2-CH_3 **10**:R'=CH_3, R"=$(CH_2)_2$-CH_3 **11**:R'=Ph, R"=CH_3 **12**:R'=Ph, R"=CH_3 **13**:R'=Ph-$(F)_3$, R"=CH_3 **14**:R'=Ph-$(F)_3$, R"=CH_2-CH_3 **15**:R'=PH, R"=CH_3

Supplementary File

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***Declaration of Interest Statement**

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: