

**Biochemische und biokatalytische Charakterisierung  
der Butandioldehydrogenasen aus *Bacillus clausii*  
DSM 8716<sup>T</sup> und *Bacillus licheniformis* DSM 13<sup>T</sup>**

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## **Eidesstattliche Erklärung**

Ich, Lukas Muschallik, versichere an Eides statt, dass die vorliegende Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Ich habe bisher keine erfolglosen Promotionsversuche unternommen und diese Dissertation nicht an einer anderen Fakultät vorgelegt.

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## Publikationen im Rahmen dieser Arbeit

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- **Muschallik L.**, Molinnus D., Bongaerts J., Pohl M., Wagner T., Schöning M.J., Siegert P., Selmer T. (*R,R*)-butane-2,3-diol dehydrogenase from *Bacillus clausii* DSM 8716<sup>T</sup>: cloning and expression of the bdhA -gene, and initial characterization of enzyme. *J Biotechnol.* 2017;258:41-50. doi:10.1016/j.jbiotec.2017.07.020
- Molinnus D., **Muschallik L.**, Gonzalez L.O., Bongaerts J., Wagner T., Selmer T., Siegert P., Keusgen M., Schöning M.J. Development and characterization of a field-effect biosensor for the detection of acetoin. *Biosens Bioelectron.* 2018;115(March):1-6. doi:10.1016/j.bios.2018.05.023
- **Muschallik L.**, Molinnus D., Jablonski M., Kipp C.R., Bongaerts J., Pohl M., Wagner T., Schöning M.J., Selmer T., Siegert P. Synthesis of  $\alpha$ -hydroxy ketones and vicinal (*R,R*)-diols by *Bacillus clausii* DSM 8716<sup>T</sup> butanediol dehydrogenase. *RSC Adv.* 2020;10:12206-12216. doi: 10.1039/D0RA02066D
- **Muschallik L.**, Recker I., Jablonski M., Gellissen M., Kipp C.R., Bongaerts J., Pohl M., Schöning M.J., Selmer T., Siegert P., Synthesis of  $\alpha$ -hydroxy ketones and vicinal diols with *Bacillus licheniformis* DSM 13<sup>T</sup> butane diol dehydrogenase.

in Vorbereitung

### Poster-Präsentationen:

- Development of an enzyme-based diacetyl/acetoin biosensor for monitoring crucial parameters in fermentation processes. **Muschallik L.**, Molinnus D., Müschen M., Bongaerts J., Wagner T., Selmer T., Schöning M.J. and Siegert P.; Aachen Protein Engineering Symposium, Aachen (Germany), 21.09.-23.09.2016.
- Development of a biosensor for the detection of acetoin during wine fermentation. Molinnus D., **Muschallik L.**, Bongaerts J., Selmer T., Wagner T., Siegert P., Keusgen M., Schöning M.J., 5th International Symposium on Sensor Science 2017, Barcelona (Spain), 27.09.-29.09.2017.
- (*R,R*)-Butane-2,3-diol dehydrogenase from *Bacillus clausii* DSM 8716<sup>T</sup> – a versatile biocatalyst. **Muschallik L.**, Molinnus D., Jablonski M., Kipp C., Bongaerts J., Wagner T., Pohl M., Selmer T., Schöning M.J., Siegert P., Poster, Symposium zum 10-jährigen des INB, FH Aachen intern, 2018, Jülich (Germany), 23.02.2018.
- Monitoring of biotechnological processes Using a diacetyl/acetoin biosensor and evaluation of acetoin reductases for use in biotransformations. **Muschallik, L.**, Molinnus, D.; Bongaerts, J.; Wagner, T.; Siegert, P.; Schöning, M.J.; Selmer, T., Tag der Forschung der FH Aachen, Aachen (Germany) 15.03.2018.

- Development and characterization of a field-effect biosensor for the detection of acetoin during fermentation processes. Molinnus D., **Muschallik L.**, Jablonski M., Bongaerts J., Wagner T., Selmer T., Siegert P., Keusgen M., Schöning M.J., 11th International Workshop on Engineering of Functional Interfaces (EnFI 2018), Lutherstadt Wittenberg (Germany), 01.07.-03.07.2018.
- Field-effect biosensor for acetoin detection during fermentation process of alcoholic beverages. Molinnus D., **Muschallik L.**, Jablonski M., Bongaerts J., Wagner T., Selmer T., Siegert P., Keusgen M., Schöning M.J., IMCS 2018, Vienna (Austria), 15.07.-19.07.2018.
- (*R,R*)-Butane-2,3-diol dehydrogenase from *Bacillus clausii* DSM 8716<sup>T</sup> - a versatile biocatalyst for synthesis of  $\alpha$ -hydroxy ketones / diols and biosensor development. **Muschallik L.**, Molinnus D., Jablonski M., Kipp C., Bongaerts J., Wagner T., Pohl M., Selmer T., Schöning M.J., Siegert P., 9<sup>th</sup> International Congress on Biocatalysis: BIOCAT2018, Hamburg (Germany), 26.08.-30.08.2018.
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- Acetoin Biosensor based on Acetoin reductase-Modified Field-Effect Sensor Applied for Acetoin Detection in Beer Samples. Jablonski M., Münstermann F., Molinnus D., **Muschallik L.**, Bongaerts J., Wagner T., Keusgen M., Siegert P., Schöning M.J.. 237th ECS Meeting and the 18th International Meeting on Chemical Sensors (IMCS 2020)

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## Abkürzungsverzeichnis

ADH	Alkoholdehydrogenase
ADH-A	Alkoholdehydrogenase aus <i>Rhodococcus ruber</i>
ADH-T	Alkoholdehydrogenase aus <i>Thermoanaerobacter sp.</i>
AgOTf	Silbertrifluormethansulfonat
ALDC	Acetolactat-Dehydrogenase
ATP	Adenosintriphosphat
BcBDH	Butandioldehydrogenase aus <i>Bacillus clausii</i> DSM 8716T
BDH	Butandiol-Dehydrogenase
BINAP	(1,1-Binaphthalene-2,2-diyl)bis(diphenylphosphine)
BIBDH	Butandioldehydrogenase aus <i>Bacillus licheniformis</i> DSM13T
bpQOR	bakterielle Quinone-Oxidoreduktasen
CAD	Zimtalkohol-Dehydrogenasen
CAL-B	Lipase aus <i>Candida antarctica</i>
CD	Circulardichroismus
DADH	Alkoholdehydrogenase aus <i>Drosophila melanogaster</i>
<i>de</i>	Diastereomerenüberschuss
DMSO	Dimethylsulfoxid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylendiamintetraessigsäure
<i>ee</i>	Enantiomerenüberschuss
E-Wert	Enantioselektivität
FAD	Flavinadenindinukleotid
FAS	Fettsäure-Synthasen
FDH	Glutathion abhängigen Formaldehyd-Dehydrogenasen, Formiatdehydrogenase
FMN	Falvinmononukleotid
G-6-P-DH	Glucose-6-Phosphat-Dehydrogenase
GDH	Glukosedehydrogenase
HLADH	Alkoholdehydrogenase aus der Pferdeleber
HPP	2-Hydroxy-1-phenylpropan-1-on
INB	Institut für Nano- und Biotechnologien der FH Aachen
$k_{cat}$	katalytische Konstante
$K_m$	Michaelis-Menten Konstante
K <sub>pi</sub>	Kaliumphosphat
LBADH	Alkoholdehydrogenase aus <i>Lactobacillus brevis</i>
LogP-Wert	Oktanol-Wasser-Verteilungskoeffizient
MAE	Malatenzym
MDR	„medium-chain“ Dehydrogenasen
MES	2-(N-Morpholino)ethansulfonsäure
NAD <sup>+</sup> / NADH	Nicotinamadenindinukleotid (oxidiert / reduziert)
NADP <sup>+</sup> / NADPH	Nicotinamadenindinukleotidphosphat (oxidiert / reduziert)
PAC	1-Hydroxy-1-phenylpropan-1-on
PAGE	Polyacrylamidgelelektrophorese
PDH	Sorbitol/Xylitol-Dehydrogenasen
pK <sub>s</sub>	negativer dekadischer logarithmus der Säurekonstanten

PQQ	Pyrrlochinolinchinon
PTGR	Prostaglandin-Reduktase
RDH	Ribitoldehydrogenase aus <i>Klebsellia aereogenes</i>
SDH	Sorbitoldehydrogenase aus der Schafsleber
SDR	„short-chain“ Dehydrogenasen
SDS	Natriumdodecylsulfat
TDH	Threonin-Dehydrogenasen
ThDP	Thiamindiphosphat
U	Unit (Enzymeinheit)
$V_{\max}$	maximale Reaktionsgeschwindigkeit
YADH	Alkoholdehydrogenase aus der Hefe

## Kurzfassung

Obwohl Dehydrogenasen und besonders Alkoholdehydrogenasen biokatalytisch bestens untersucht sind, ist der Einsatz von Butandioldehydrogenasen in der Biokatalyse bisher kaum beschrieben. Physiologisch katalysieren diese Enzyme die stereoselektive Reduktion von Diacetyl und Acetoin final zum 2,3-Butandiol. Auch die Rückreaktion, die Oxidation von 2,3-Butandiol, wird von diesen Enzymen katalysiert, jedoch nur bis zum Acetoin Intermediat. Vor allem finden diese Enzyme daher oft Erwähnung in der biologischen Produktion von 2,3-Butandiol.

Doch gerade durch ihre natürliche Affinität gegenüber  $\alpha$ -Hydroxyketonen und vicinalen Diolen bieten diese Enzyme großes Potential als Biokatalysatoren für die enantioselektive Synthese dieser Molekülgruppen, die als Aromastoffe, Pheromone und Synthons für Pharmazeutika Anwendung finden können. Auch ist die rein chemische Synthese dieser oft schwierig und verbunden mit der Verwendung schwermetallhaltiger Katalysatoren. Daher wurden in dieser Arbeit die Butandioldehydrogenasen aus *Bacillus licheniformis* DSM 13<sup>T</sup> (BIBDH) und *Bacillus clausii* DSM 8716<sup>T</sup> (BcBDH) biochemisch und biokatalytisch charakterisiert und für der Einsatz in Biotransformationen evaluiert.

BcBDH und BIBDH unterscheiden sich auf struktureller Ebene grundlegend und gehören zu den Superfamilien der „medium-chain“ bzw. „short-chain“-Dehydrogenasen“. Beide Enzyme konnten mit sehr guten Ausbeuten heterolog in *E. coli* B121 (DE3) produziert werden. Sie akzeptieren eine große Auswahl an nicht natürlichen vicinalen Diketonen und  $\alpha$ -Hydroxyketonen als Substrate, auch sterisch anspruchsvolle Moleküle, die z.B. einen Phenylrest beinhalten. Aus biokatalytischer Sicht unterscheiden sich beide Enzyme deutlich. So diskriminiert die BcBDH zwischen symmetrischen und asymmetrischen Molekülen wodurch die Reaktion entweder am  $\alpha$ -Hydroxyketon oder Diol ausgehend von einem Diketon stoppt. Auch wird als erstes immer die Hydroxygruppe am größeren Molekülrest reduziert. Hier konnte die *R*-Selektivität für die natürlichen und einige nicht natürliche Substrate demonstriert werden. Die BIBDH hingegen fügt ein *S*-konfiguriertes Stereozentrum ein, weißt aber unterschiedliche kinetische Parameter für *R*- und *S*-Enantiomere dieser Substrate auf. Ausgehend von einem Diketon stagniert die Reaktion bei der Bildung des  $\alpha$ -Hydroxyketons bei kurzer Reaktionsdauer. Eine Weiterreduktion zum vicinalen Diol erfolgt oft erst bei längerer Reaktionsdauer oder größerem Enzymeinsatz. Im Gegensatz zur BcBDH reduziert die BIBDH als erstes immer die Hydroxygruppe am kleineren Molekülrest des Substrats. Eine besonders hohe Stereoselektivität konnte in Biotransformationen mit der BcBDH beobachtet werden. Bei der Umsetzung von 5-Methyl-2,3-hexandion, 2,3-Hexandion und 2,3-Heptandion mit diesem Enzym wurden so Enantioselektivitäten von 97%, 89% und 99% *ee.* erreicht.

Als erstes direktes Anwendungsbeispiel konnte der Einsatz der BcBDH bei der Entwicklung eines potentiometrischen Biosensors zur Messung der Acetoinkonzentration in Bier und Wein im Rahmen eines Kooperationsprojekts (AG Prof. Schöning, INB FH Aachen) gezeigt werden.

# 1 Einleitung

## 1.1 Enzyme in der Biokatalyse

Bereits die alten Ägypter nutzten Mikroorganismen zur Herstellung von Nahrungsmitteln und Getränken, ohne die dabei zugrunde liegenden biochemischen Prozesse zu kennen. Doch erst im Laufe des 19. Jahrhunderts gelang es, durch Erkenntnisse im Bereich der Mikrobiologie diese Prozesse besser zu verstehen. So konnten Wissenschaftler wie Louis Pasteur zeigen, dass Mikroorganismen für die Gärungsprozesse bei der Herstellung diverser Lebensmittel verantwortlich sind. Es gelang ihm auch zu zeigen, dass die optische Aktivität einer Substanz auf eine molekulare Asymmetrie zurück zu führen ist und dass gewisse Mikroorganismen unter bestimmten Bedingungen nur ein Enantiomer eines Racemats verstoffwechseln können <sup>1</sup>. Weitere Arbeiten durch Eduard Buchner zeigten die Möglichkeit einer zellfreien alkoholischen Gärung durch Enzyme <sup>2</sup>, ein Begriff der 1878 durch Willhelm Friedrich Kühne eingeführt wurde <sup>3</sup>. Enzyme sind Proteine und haben die Funktion von Biokatalysatoren, wodurch sie maßgeblich an den biochemischen Prozessen in Organismen beteiligt sind. Als Katalysatoren sind sie dabei in der Lage, Reaktionen um das bis zu  $10^{17}$  fache zu beschleunigen <sup>4</sup>. Ausgehend von ihrer katalysierten Reaktion, können Enzyme in die Klassen der Oxidoreduktasen, Transferasen, Hydrolasen, Lyasen, Isomerasen, Ligasen und Translocasen eingeteilt werden (Tabelle 1) <sup>5</sup>.

Tabelle 1: Übersicht der Enzymklassen. Nach „Biotransformations in organic chemistry“ <sup>5</sup>.

Enzyme class	Number		Reaction Type	Utility <sup>a</sup>
	Classified	Available		
1. Oxidoreductases	~700	~100	Oxidation-reduction: oxygenation of C-H, C-C, C=C bonds, or overall removal or addition of hydrogen atom equivalents	+++
2. Transferases	~750	~100	Transfer of groups: aldehydic, ketonic, acyl, sugar, phosphoryl, methyl, NH <sub>3</sub>	++
3. Hydrolases	~650	~180	Hydrolysis formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydrides, glycosides, organohalides	+++
4. Lyases	~300	~40	Addition-elimination of small molecules on C=C, C=N, C=O bonds	++
5. Isomerases	~150	6	Isomerizations such as racemization, epimerization, rearrangement	+
6. Ligases	~80	5	Formation-cleavage of C-O, C-S, C-N, C-C bonds with concomitant triphosphate cleavage	±
7. Translocases			Transport a molecule from side 1 to side 2	

<sup>a</sup>The estimated 'utility' of an enzyme class for the transformation of nonnatural substrates ranges from +++ (very useful) to ± (little use)

Im Gegensatz zur klassischen organischen Synthese, bei der oft harsche Prozessbedingungen angewendet werden wie eine hohe Temperatur, hoher Druck, extreme pH-Werte oder die Verwendung von giftigen Katalysatoren, sind Enzyme in der Lage, viele Reaktionen unter milden Synthesebedingungen zu katalysieren<sup>6</sup>. Zusätzlich weisen Enzyme oft eine hohe Stereo- und Regioselektivität auf. Damit können sie zwischen funktionellen Gruppen gleicher Reaktivität unterscheiden und die Reaktion an einer definierten funktionellen Gruppe im Molekül bewirken. Lipasen z.B. können genutzt werden für die regioselektive Veresterung von Zuckern, die mit der klassischen organischen Synthese den Einbau von Schutzgruppen erfordern würde. Dies jedoch ist für industrielle Prozesse kaum praktikabel<sup>7,8</sup>. Besonders in der pharmazeutischen Industrie ist die Synthese stereoisomerenreiner Produkten erforderlich. Enantiomere eines Moleküls verhalten sich wie Bild und Spiegelbild zueinander und weisen, bis auf die optische Aktivität, gleiche physikalische Eigenschaften auf. Ist die Verbindung biologisch aktiv, kann das eine Enantiomer die gewünschte Wirkung entfalten, hingegen kann das andere Enantiomer gar toxisch sein. Die unterschiedliche biologische Wirkung zweier Enantiomere einer Verbindung lässt sich zum Beispiel an dem Molekül Carvon veranschaulichen. Während das (*R*)-konfigurierte Carvon nach Minze riecht, so zeigt das (*S*)-konfigurierte Carvon ein Geruch von Kümmel<sup>9</sup>. Enzyme als Biokatalysatoren sind in der Lage, viele Synthesen hoch enantioselektiv durchzuführen. So ist eine kostspielige und aufwendige Racemattrennung, wie es in der chemischen Synthese oft der Fall ist, nicht notwendig<sup>10</sup>. Auch im Hinblick auf die Kostenreduzierung und Nachhaltigkeit ist die Nutzung von Enzymen oft vorteilhaft<sup>11</sup>. So kann die Nutzung diverser schwermetallhaltiger Katalysatoren umgangen werden. Auch ist es möglich, bestehende Prozesse zu verkürzen und kosteneffektiver zu gestalten. Beispiel dafür ist die Synthese von Atorvastatin als aktive Komponente des Cholesterol-senkenden Medikaments Lipitor®. Auf chemischen Wegen müssen vier Reaktionsschritte durchgeführt werden, darunter eine Claisen-Kondensation und eine Boran-Reduktion bei -70°C. Durch die Verwendung einer Ketoreduktase und einer Halohydrin-dehalogenase ließ sich die Synthese auf zwei Reaktionsschritte verkürzen. Zudem war es möglich, zwei energieintensive Schritte zu umgehen<sup>12</sup>. Durch diese vorteilhaften Eigenschaften finden Enzyme in diversen industriellen Prozessen ihren Einsatz. So werden Proteasen und Lipasen in Wasch- und Spülmitteln zur Entfernung von Protein- oder Fettflecken, wie auch in der Bearbeitung von Leder oder in der Lebensmittelindustrie eingesetzt. Oxidoreduktasen können zum Bleichen eingesetzt werden<sup>13</sup>.

## 1.2 Dehydrogenasen

Dehydrogenasen gehören zu der Klasse der Oxidoreduktasen. Enzyme aus dieser Klasse katalysieren in prokaryotischen und eukaryotischen Mikroorganismen, sowie in Pflanzen und tierischen Zellen, Redoxreaktionen. In biologischen Systemen erfolgt dies z.B. durch Übertragung von Hydridionen. Der Transfer, zumeist stereospezifisch und stereo- sowie regioselektiv, findet dabei zwischen dem Substrat und einem Cofaktor statt. Als Cofaktoren benötigen Oxidoreduktasen für gewöhnlich Nicotinamidadenindinukleotid (NAD), Nicotinamidadenindinukleotidphosphat (NADP), Flavinadenindinukleotid (FAD), Flavinmononukleotid (FMN) oder auch (selten) Pyrrolochinolinchinon (PQQ). So sind diese Enzyme in der Lage, die Reduktion von Ketogruppen in Hydroxygruppen, wie auch die Rückreaktion, die Oxidation von Hydroxygruppen, zu katalysieren (Abbildung 1). Einige Reduktasen ermöglichen zudem die Reduktion aktivierter C=C Doppelbindungen. Ein Beispiel hierfür sind die „Old Yellow Enzymes“<sup>14,15</sup>.

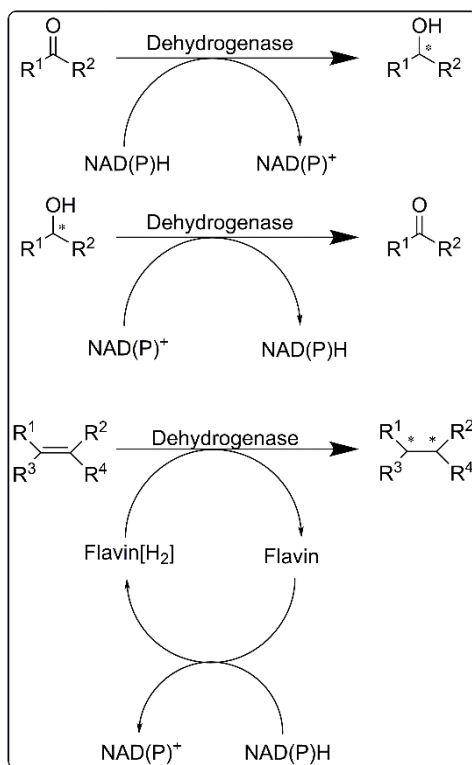


Abbildung 1: Übersicht der Reaktionsschemata für Dehydrogenasen.

Dehydrogenasen können dabei in die Superfamilien der „medium-chain“ Dehydrogenasen (MDR) oder „short-chain“ Dehydrogenasen (SDR) eingeordnet werden. So bestehen Dehydrogenasen aus der „medium-chain“ Dehydrogenase Superfamilie zumeist aus zwei Domänen. Eine für die Cofaktorbindung mit der enthaltenen Rossmann-Faltung. Diese besteht aus abwechselnd sechs  $\alpha$ -Helices und sechs  $\beta$ -Faltblättern mit einem zentralen  $\beta$ -Faltblatt, welches von zwei  $\alpha$ -Helices flankiert wird<sup>16</sup>.

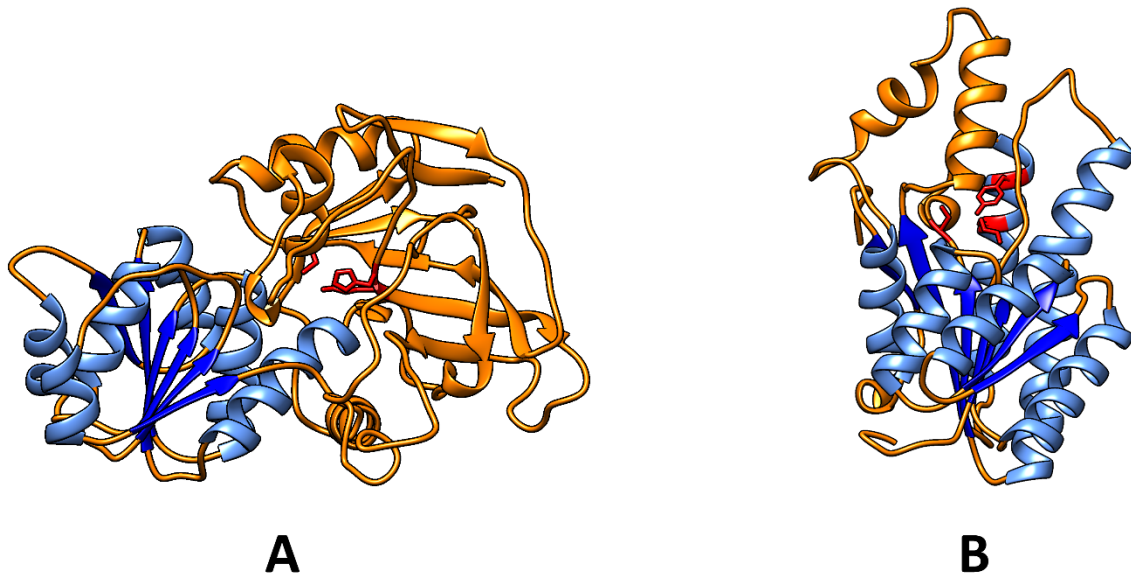


Abbildung 2: Homologiemodelle eines Monomers der Butandioldehydrogenase aus *Bacillus clausii* DSM 8716<sup>T</sup> (A; gehört zu den „medium-chain“ Dehydrogenasen; Modell basierend auf der Kristallstruktur der (2*R*, 3*R*)-Butandiol-Dehydrogenase aus *Bacillus subtilis* (PDB ID: 6IE0) und eines Monomers der Butandioldehydrogenase aus *Bacillus licheniformis* DSM 13<sup>T</sup> (B; gehört zu den „short-chain“ Dehydrogenasen; Modell basierend auf der Kristallstruktur der Oxidoreduktase Gox2036 aus *Gluconobacter oxidans* 621H (PDB ID: 3WTC)). Der Rossmannfold ist in blau markiert. Die katalytischen Aminosäuren sind rot eingefärbt. Die Tertiärstruktur von BcBDH weist zwei Domänen auf, während die von BIBDH nur aus einer Domäne besteht.

Die andere Domäne dient der Bindung des Substrats. Beide Domänen werden durch eine tiefe Tasche getrennt, die das aktive Zentrum beherbergt und der Bindung des Cofaktors dient. Dehydrogenasen aus der Gruppe der „short-chain“ Dehydrogenasen weisen hingegen oft nur eine Domäne auf<sup>17</sup> (Abbildung 2).

### 1.3 „Medium-chain“ und „short-chain“ Dehydrogenasen

Die Einteilung von Dehydrogenasen in die Superfamilie der „short-chain“ Dehydrogenasen und der „medium-chain“ Dehydrogenasen resultierte aus der Untersuchung verschiedener Alkoholdehydrogenase- (ADH) Strukturen. So konnte festgestellt werden, dass die ADH aus *Drosophila melanogaster* (DADH) große strukturelle Unterschiede im aktiven Zentrum zu den ADHs aus Pferdeleber (HLADH) und aus Hefe (YADH) aufweist<sup>18</sup>. Hingegen wurden strukturelle Gemeinsamkeiten bei den ADHs aus Pferdeleber und Hefe festgestellt, die unter anderem beide größere Untereinheiten haben als DADH<sup>19,20</sup>. Zusätzlich wurde gezeigt, dass diese Einteilung auch bei den Polyoldehydrogenasen auftritt. So zeigt die Sorbitoldehydrogenase aus Schafsleber (SDH) strukturelle Ähnlichkeiten zu HLADH und YADH, die beide größere Untereinheiten besitzen. Umgekehrt besitzt die Ribitoldehydrogenase aus *Klebsellia aereogenes* (RDH) eine kleine Untereinheit und zeigt

strukturelle Gemeinsamkeiten zur DADH, nicht jedoch zur HLADH oder YADH <sup>21</sup>. Die Einteilung erfolgte daher zunächst nach der Größe der Untereinheit, wobei „short-chain“-Dehydrogenasen ca. 250 Aminosäuren pro Untereinheit und „medium-chain“-Dehydrogenasen ca. 350 Aminosäuren pro Untereinheit aufweisen. Zusätzlich zeigen beide Untergruppen Unterschiede in der Struktur und im Reaktionsmechanismus. Dehydrogenasen aus der Gruppe der „medium-chain“-Dehydrogenasen (MDR) haben entweder einen Zink-abhängigen Reaktionsmechanismus oder nutzen ein konserviertes Tyrosin zur Katalyse. Dabei bestehen die Enzyme aus dieser Klasse für gewöhnlich aus zwei Domänen. Die C-terminale Domäne ist dabei für die Cofaktorbindung und die N-terminale Domäne für die Substratbindung verantwortlich <sup>22</sup>. Bei den zehn größten Unterfamilien der MDR Superfamilie handelt es sich um die Alkohol-Dehydrogenasen (ADH), Prostaglandin-Reduktasen (PTGR), Fettsäure-Synthasen (FAS), Zimtalkohol-Dehydrogenasen (CAD), bakterielle Quinone-Oxidoreduktasen (bpQOR), mutmaßliche Quinone-Oxidoreduktasen (QORX), einer bakteriellen Familie mit dem Namen YHDH, die Familie der Glutathion abhängigen Formaldehyd-Dehydrogenasen (FDH) zu der auch die (S)-(Hydroxymethyl)glutathion-Dehydrogenase aus *Methylobacter marinus* gehört, die Threonin-Dehydrogenasen (TDH) und Sorbitol/Xylitol-Dehydrogenasen (PDH) <sup>23</sup>. Enzyme aus der Superfamilie der SDR bestehen in ihrer klassischen Form nur aus einer Domäne mit der Substratbindestelle in der hochvariablen C-terminalen Region <sup>17,24</sup>. Im Gegensatz zu den Enzymen der MDR Superfamilie benötigen diese auch keine metallischen Cofaktoren. Wie auch die MDR Superfamilie lässt sich die SDR Superfamilie in Subkategorien unterteilen. Hierbei wird unterschieden zwischen den „classical“ und „intermediate“ SDRs, „divergent“ SDRs, „complex“ SDRs und den „extended“ SDRs <sup>25</sup>. Die „classical“ SDRs besitzen dabei ein konserviertes Motiv zur Cofaktorbindung TGXXX[AG]XG und eins für die Substratbindung YXXXX <sup>25</sup>. Diese Motive variieren jedoch zu einem gewissen Grad in den verschiedenen SDR Subgruppen (Tabelle 2).

Tabelle 2: Cofaktor- und Substratbindemotive für fünf SDR Untergruppen. Entnommen aus Kavanagh *et al.* <sup>25</sup>

Subfamily	Cofactor binding	Active site
classical'	TGxxx[AG]xG	YxxxK
extended'	[ST]GxxGxxG	YxxxK
intermediate'	[GA]xxGxx[GA]	YxxxK
divergent'	GxxxxxSxA	YxxMxxxK
complex'	GGxGxxG	YxxxN

x, any amino acid residue. Brackets denote alternatives that can be present or absent



## 1.4 Butandiol-Dehydrogenasen

Butandiol-Dehydrogenasen (auch Acetoin-Reduktasen, EC 1.1.1.4) gehören in die Gruppe der Oxidoreduktasen und können, wie auch Alkoholdehydrogenasen, in die Superfamilie der „medium-chain“ Dehydrogenasen und „short-chain“ Dehydrogenasen unterteilt werden. Wie Alkoholdehydrogenasen sind Butandiol-Dehydrogenasen cofaktorabhängig und benötigen entweder NAD(P)H bzw. NAD(P)<sup>+</sup>. Physiologisch gesehen katalysieren diese Enzyme die Reduktion von Diacetyl und Acetoin, die final stereoselektiv zu 2,3-Butandiol reduziert werden können. Auch wird die Rückreaktion, die Oxidation von 2,3-Butandiol zu Acetoin, von diesen Enzymen katalysiert. Eine weitere Oxidation des Acetoin zum Diacetyl konnte bisher nicht gezeigt werden<sup>26-28</sup>. Diacetyl, Acetoin und 2,3-Butandiol stellen Überflussmetabolite dar, die zum Beispiel bei der Hefefermentation (Wein- und Bierherstellung) gebildet werden. Diese Moleküle sind dabei Nebenprodukte des Kohlenhydratmetabolismus<sup>29</sup>. Neben Hefen bilden auch Mikroorganismen, vor allem aus der Ordnungen der Enterobakterien und Bacilli, besonders häufig Acetoin und 2,3-Butandiole als Überflussmetabolite<sup>30,31</sup>. Die Bildung von Acetoin und 2,3-Butandiol wird unter anderem durch die Kondensation von zwei Pyruvatmolekülen durch die  $\alpha$ -Acetolactat-Synthase zu (*S*)- $\alpha$ -Acetolactat und die darauffolgende Decarboxylierung durch die  $\alpha$ -Acetolactat-Decarboxylase zu (*R*)-Acetoin initiiert. (*S*)- $\alpha$ -Acetolactat kann auch durch nicht enzymatische Decarboxylierung zu Diacetyl überführt werden. In Hefen kann in einem Reaktionsweg über die Verknüpfung von zwei Acetaldehydmolekülen mittels der Pyruvatdecarboxylase Acetoin synthetisiert werden<sup>29</sup>. Diacetyl wie auch Acetoin können im Anschluss durch eine Acetoin-Reduktase/Butandiol-Dehydrogenase zu (*R,R*)-, (*S,S*)- bzw. *meso*-2,3-Butandiol reduziert werden (Abbildung 3)<sup>29,32</sup>.

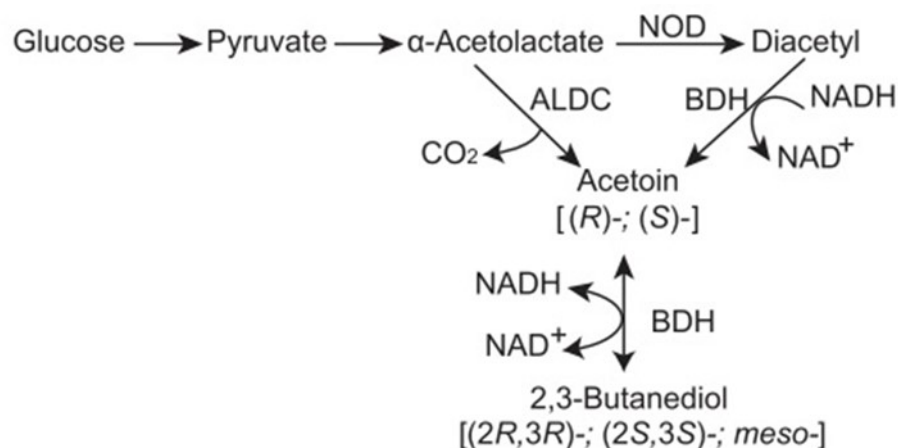


Abbildung 3: Beispiel eines Stoffwechselwegs für die Bildung von 2,3-Butandiol in Bakterien. Entnommen aus Hao *et al.*<sup>32</sup>. Zwei Moleküle Pyruvat können von der  $\alpha$ -Acetolactat-Synthase zu (*S*)- $\alpha$ -Acetolactat kondensiert werden. Darauf folgt die decarboxylierung zu (*R*)-Acetoin durch  $\alpha$ -Acetolactat-Decarboxylase (ALDC). Ebenfalls kann  $\alpha$ -Acetolactat nicht enzymatisch

zu Diacetyl decarboxyliert werden (NOD). Sowohl Diacetyl wie auch Acetoin können durch die Acetoin-Reduktase/Butandiol-Dehydrogenase (BDH) zu 2,3-Butandiol reduziert werden.

Butandiol-Dehydrogenasen aus der jeweiligen Superfamilie (SDR oder MDR) weisen dabei für diese Superfamilie typische Charakteristika auf. Butandiol-Dehydrogenasen aus der MDR Superfamilie bilden zumeist Homodimere, sind oft *R*-selektiv und benötigen ein Zinkion, welches von den konservierten katalytischen Aminosäuren Cys<sub>37</sub>, His<sub>70</sub>, Glu<sub>71</sub> gebunden wird. Einige Butandiol-Dehydrogenasen aus dieser Superfamilie komplexieren noch ein zweites strukturelles Zinkion mit den konservierten Aminosäuren Cys<sub>100</sub>, Cys<sub>103</sub>, Cys<sub>106</sub> und Cys<sub>114</sub><sup>33</sup>. Hingegen bilden Butandiol-Dehydrogenasen aus der SDR Superfamilie vorwiegend Homotetramere und erzeugen meist ein (*S*)-Stereozentrum. Diese Enzyme sind nicht metallionenabhängig<sup>27,34,35</sup>.

Der Reaktionsmechanismus von Butandiol-Dehydrogenasen (BDH) entspricht dem der Alkoholdehydrogenasen. Ausgehend von kristallographischen Strukturen der *S*-selektiven BDH aus *Brevibacterium saccharolyticum* C-1012 und der *meso*-BDH aus *Klebsiella pneumoniae* IAM 1063 können beide Enzyme der Superfamilie der SDR zugeordnet werden und besitzen dasselbe konservierte Motiv für die katalytischen Aminosäuren bestehend aus Asn, Ser, Tyr und Lys (Abbildung 4). Tyrosin dient dabei dem Protonentransfer. Die Lysin-Seitenkette ist für die Cofaktorbindung und für die Verringerung des pK<sub>s</sub> Wertes der Hydroxygruppe der Tyrosin-Seitenkette verantwortlich. Die Serin-Seitenkette spielt eine Rolle beim Binden des Substrats, des Intermediats und des Produkts, während die Asparagin-Seitenkette eine wichtige Rolle beim Erhalt der Konfiguration des aktiven Zentrums einnimmt, wodurch ein Proton-Relay-System gebildet werden kann, bestehend aus der Tyrosin-Seitenkette, Lysin-Seitenkette, der Nicotinamid-Ribose-Gruppe des Cofaktors und dem Substrat<sup>36</sup>.

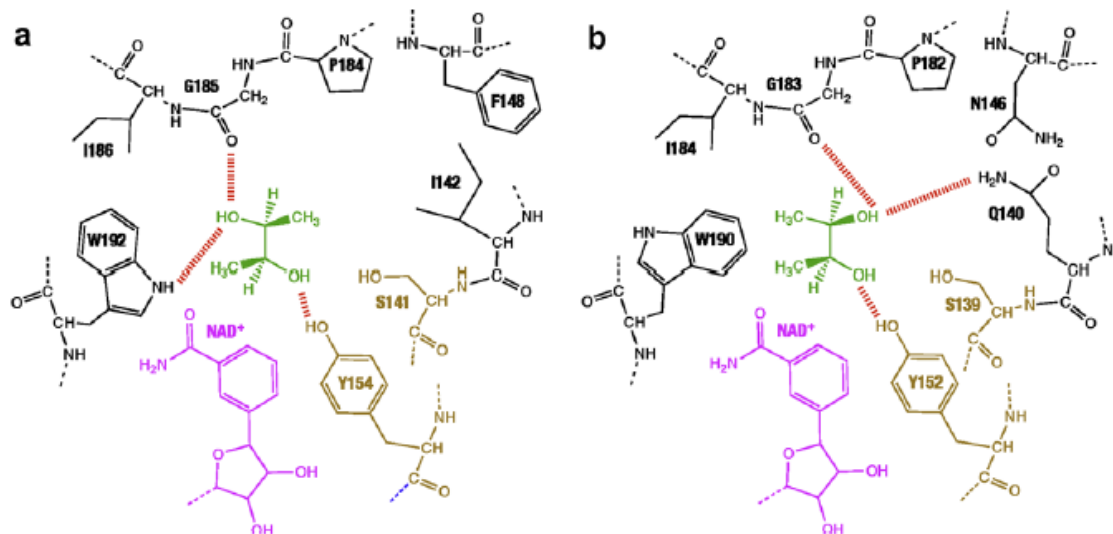


Abbildung 4: Schematische Darstellung der Substratbindungsmodelle für BDHs aus der Superfamilie der „short-chain“-Dehydrogenasen auf der Basis der *S*-BDH aus *Brevibacterium saccharolyticum* C-1012 (a) und der *meso*-BDH aus *Klebsiella pneumoniae* IAM 106 (b). Wasserstoffbrückenbindungen sind mit gestrichelten Linien dargestellt. Die katalytischen Aminosäuren Tyr und Ser sind braun markiert. Entnommen aus Otigiri *et al.* <sup>36</sup>

BDHs aus der Superfamilie der MDR sind bisher kristallographisch kaum untersucht. Im Wesentlichen kann der Reaktionsmechanismus der Butandiol-Dehydrogenasen aus der MDR Superfamilie mit denen der ADHs aus der MDR Superfamilie verglichen werden. Als Modellenzym dient hier oft die HLADH. Die Bindung des Cofaktors erfolgt an der Rossmann-Faltung: das Substrat wird in einer hydrophoben Tasche zwischen den beiden Domänen des Enzyms gebunden mit der Ausrichtung der Hydroxy- und Ketogruppe zum Zinkion. Es erfolgt ein Austausch eines Wassermoleküls am vierfach koordinierten Zinkion gegen die Sauerstoffgruppe des Substrats <sup>37</sup>. Der polarisierende Effekt bewirkt dabei den stereoselektiven Transfer des Hydrids vom Cofaktor auf das Substrat <sup>38</sup>.

## 1.5 Synthesen mittels Dehydrogenasen

Die Verwendung von Enzymen für die Synthese bietet einige Vorteile im Vergleich zur klassischen chemischen Synthese. Bei dem Einsatz von Dehydrogenasen in der biokatalytischen Synthese von Feinchemikalien sind Alkoholdehydrogenasen bereits außerordentlich gut etabliert. Ein Beispiel ist der Einsatz von Alkoholdehydrogenasen in einer Enzymkaskade zur Synthese von allen Stereoisomeren von 4-Methoxyphenyl-1,2-propandiol. Dieses Molekül kann als Synthone für die Pharmazeutika (*R*)-Tamsulosin oder Silibinin genutzt werden. Im Rahmen dieses Prozesses wird eine stereoselektive C-C Knüpfung von 4-Methoxy-Benzaldehyd und Acetaldehyd durch die Benzaldehydlyase aus *Pseudomonas fluorescens* oder

Benzoylformiatdecarboxylase (Variante L461A) aus *Pseudomonas putida* katalysiert. Die Ketogruppe des resultierenden 2-Hydroxyketones wird anschließend von der (*R*)-selektiven Alkoholdehydrogenase aus *Ralstonia sp.* oder der (*S*)-selektiven Alkoholdehydrogenase aus *Lactobacillus brevis* reduziert, wodurch das Endprodukt gebildet wird mit 99% *ee* (Abbildung 5)<sup>39</sup>.

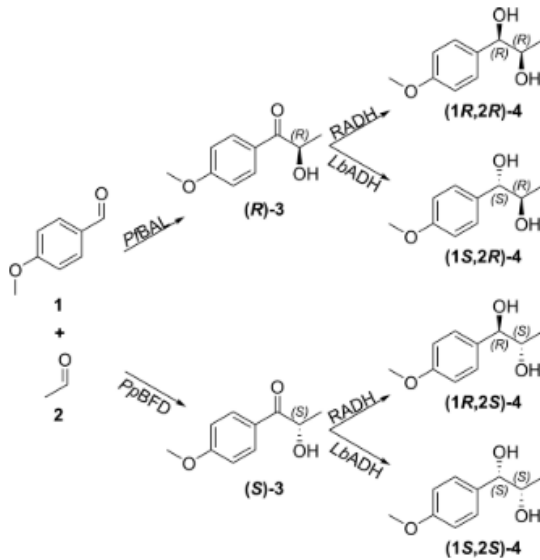


Abbildung 5: Kombination von Enzymen mit unterschiedlicher Stereoselektivität zur Synthese aller Isomeren von 4-Methoxyphenyl-1,2-propandiol. Entnommen aus Oeggel *et al.*<sup>39</sup>

Ausgehend von 2-Acetylthiophenen kann in einem chemo-enzymatischen Prozess das Pharmazeutikum (*S*)-Duloxetine ((*S*)-*N*-methyl-3-(1-naphthalenyloxy)-3-(2-thienyl)propanamine) gebildet werden. Der Schlüsselschritt ist dabei die stereoselektive Reduktion des Intermediats 3-(Dimethylamino)-1-(2-thienyl)-1-propanon durch die Keto-Reduktase (Alkoholdehydrogenase) aus *Rhodospiridium toruloides* in einem *E. coli* Ganzzell-Biokatalysator mit einer Enantioselektivität von 99,9% *ee* (Abbildung 6)<sup>40</sup>.

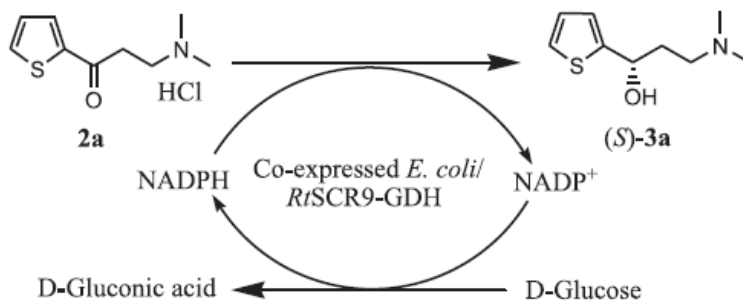


Abbildung 6: Asymmetrische Reduktion von 3-(Dimethylamino)-1-(2-thienyl)-1-propanon als stereoselektiver enzymatischer Zwischenschritt zur Synthese von (*S*)-Duloxetine. Entnommen aus Chen *et al.*<sup>40</sup>

Weiterführende nukleophile aromatische Substitution und N-Demethylierung führt zur Bildung von (*S*)-Duloxetine<sup>40</sup>.

Auch das Pharmazeutikum Travoprost wird chemo-enzymatisch hergestellt. Unter Verwendung der Alkoholdehydrogenase aus *Lactobacillus brevis* und *Ralstonia sp.*<sup>41</sup> werden (1*R*,3*S*)-Cyclopent-4-ene-1,3-diol und 1-(3-(trifluoromethyl)phenoxy)but-3-yn-2-on stereoselektiv oxidiert bzw. reduziert mit einem Enantiomerenüberschuss von 96% *ee* bzw. 99% *ee* (Abbildung 7). Das Einfügen einer Schutzgruppe für die Alkoholfunktion in Gegenwart von Imidazol führt zu der Bildung der chiralen Intermediate A und B für die weitere Synthese des Endprodukts.

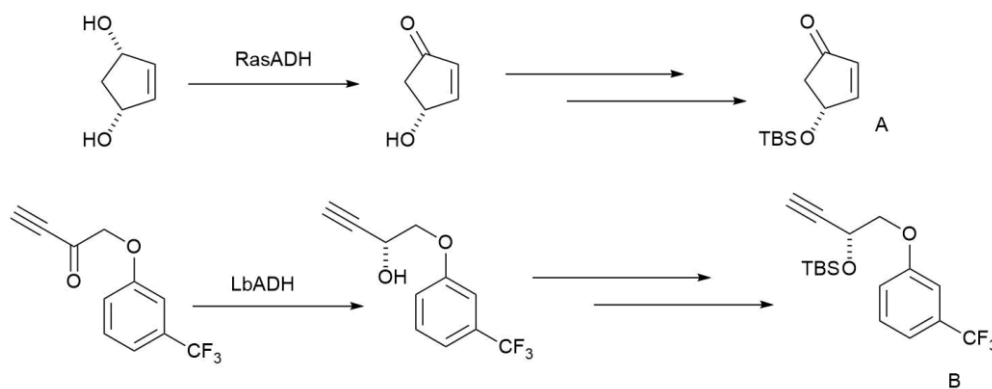


Abbildung 7: Enzymatische stereoselektive Oxidation von (1*R*,3*S*)-Cyclopent-4-ene-1,3-diol und Reduktion 1-(3-(trifluoromethyl)phenoxy)but-3-yn-2-on als Zwischenschritt zur Synthese der Intermediate A und B für die Synthese des Pharmazeutikums Travoprost. Entnommen aus Holec *et al.*<sup>41</sup>.

Generell können für biokatalytische Synthesen Ganzzellkatalysatoren oder isolierte Enzyme eingesetzt werden. Die Verwendung ganzer Zellen, in denen für die Synthese essenzielle Enzyme (über-)produziert werden, haben den Vorteil, dass aufwendige und kostspielige Reinigungsverfahren entfallen. Die für die Reaktion benötigten Cofaktoren, wie NAD(P)H oder NAD(P)<sup>+</sup>, können dabei vom Zellmilieu bereitgestellt werden. Problematisch bei der Verwendung von Ganzzellkatalysatoren können jedoch wirtseigene Enzyme sein, die unter Umständen die gleiche Reaktion, jedoch mit unterschiedlichen Selektivitäten, katalysieren oder Substrate bzw. Endprodukte abbauen. So besitzt zum Beispiel der für heterologe Genexpression gut etablierte Organismus *Escherichia coli* sechs eigene Gene (*adhE*, *adhP*, *eutG*, *viaY*, *yqhD* und *yjgB*), die für Alkoholdehydrogenasen kodieren<sup>42</sup>. Alternativ können Enzyme isoliert für Synthesen eingesetzt werden. So ist es möglich, die Stereoselektivität der Reaktion genau zu bestimmen. Dies bedingt jedoch, neben dem unter Umständen kostspieligen Reinigungsverfahren für das Enzym, die externe Zugabe der Cofaktoren. Dehydrogenasen katalysieren den stereoselektiven Transfer eines Hydridions vom Cofaktor auf eine

Carbonylverbindung, wie auch die Rückreaktion. Abhängig von der Reaktionsrichtung wird dabei der Cofaktor reduziert oder oxidiert. Aufgrund des hohen Preises für die Cofaktoren NAD(P)H und NAD(P)<sup>+</sup> ist eine stöchiometrische Zugabe sehr kostenintensiv. Daher ist die Verwendung eines Cofaktorregenerierungssystems erforderlich, um die Synthesen kosteneffizient gestalten zu können. Die am häufigsten realisierte Methode ist dabei die enzymatische Regenerierung des Cofaktors. Bei Alkoholdehydrogenasen besteht die Möglichkeit, das gleiche Enzym zur Regenerierung nutzen zu können, indem ein günstiger Alkohol oder Keton als Cosubstrat angeboten wird<sup>43,44</sup>. Für die Regeneration von NADH kann unter anderem die Glucosedehydrogenase (GDH)<sup>45</sup>, eine Hydrogenase<sup>46</sup>, das Malatenzym (MAE)<sup>47</sup> und die Posphitdehydrogenase<sup>48</sup> eingesetzt werden. Ein weiteres bereits verwendetes Enzym zur Regeneration von NADH ist die Formiatdehydrogenase (FDH). Ein besonderer Vorteil ist dabei die Irreversibilität der Reaktion, da Formiat zu CO<sub>2</sub> oxidiert wird unter Reduktion von NAD<sup>+</sup> zu NADH. Zudem ist Formiat äußerst kostengünstig<sup>49</sup>. Für die Regeneration von NADPH können die Enzyme GDH<sup>50</sup>, Glucose-6-Phosphat-Dehydrogenase (G-6-P-DH)<sup>51</sup> und MAE<sup>52</sup> verwendet werden.

## **1.6 Enantioselektive Synthese von $\alpha$ -Hydroxyketonen und vicinalen Diolen auf chemischem und biokatalytischem Weg**

Die Synthese von chiralen  $\alpha$ -Hydroxyketonen und vicinalen Diolen ist besonders interessant, da es für diese Moleküle breite Anwendungsmöglichkeiten als Synthesebausteine für Feinchemikalien und Pharmazeutika, als Geschmacksstoffe in Nahrungsmitteln oder als Pheromone für bestimmte Insektenarten gibt. So konnte gezeigt werden, dass gerade längere lineare aliphatische  $\alpha$ -Hydroxyketone und vicinale Diole, wie das 2,3-Hexandiol, 3-Hydroxy-2-hexanon, 2,3-Oktandiol und 3-Hydroxy-2-oktanon als Pheromone für Insekten der Familie Cerambycidae (Bockkäfer) fungieren<sup>53-56</sup>. Auch sind in vielen natürlich vorkommenden Pharmazeutika  $\alpha$ -Hydroxyketone als Strukturbausteine vorhanden. So zum Beispiel in den Farnesyltransferase Inhibitoren Kurasoin A und B<sup>57</sup> (Abbildung 8), in den Antitumor Pharmazeutika Olivomycin A und Chromomycin A3<sup>58</sup> oder in den Inhibitoren für die Produktion von Amyloid- $\beta$  (A $\beta$ ) Protein zur Behandlung von Alzheimer<sup>59</sup>.



Abbildung 8: Kurasoin A und B. Inhibitoren für die Farnesyltransferase. Nach Iwasaki *et al.*<sup>57</sup>

### 1.6.1 Enantioselektive chemische Synthese von $\alpha$ -Hydroxyketonen und vicinalen Diolen

Aufgrund der breiten Anwendungsmöglichkeit von  $\alpha$ -Hydroxyketonen und vicinalen Diolen als Synthesebausteine, wurden zahlreiche chemische Verfahren zur Herstellung dieser Moleküle entwickelt. Über eine Zweistufensynthese können enantioselektiv Acyloine aus Alkenen synthetisiert werden. Im ersten Schritt wird das Alken über asymmetrische Sharpless-Dehydroxylierung enantioselektiv zum Diol überführt. Im zweiten Schritt mittels eines Ruthenium-Katalysators und Oxone erfolgt die regioselektive Oxidation einer Hydroxygruppe unter Bildung des  $\alpha$ -Hydroxyketons<sup>60</sup> (Abbildung 9).

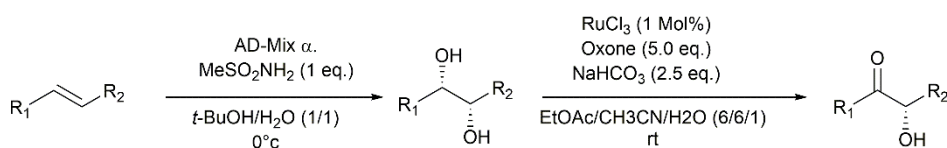


Abbildung 9: Enantioselektive Synthese von  $\alpha$ -Hydroxyketonen ausgehend von Alkenen. Nach Plietker *et al.*<sup>60</sup>

Eine weitere Möglichkeit zur chemischen Synthese von cyclischen  $\alpha$ -Hydroxyketonen ist die enantioselektive Reaktion von Zinn-Enolaten mit dem Diphosphinliganden BINAP ((1,1-Binaphthalene-2,2-diyl)bis(diphenylphosphine)), AgOTf (Silbertrifluormethansulfonat) und Nitrosobenzol. Die Stickstoff-Sauerstoffbindung in dem resultierenden  $\alpha$ -Aminooxyketon kann durch katalytische Mengen Kupfersulfat abgespalten werden. Das Endprodukt bildet das  $\alpha$ -Hydroxyketon mit 94% Ausbeute und bis zu 97% *ee* (Abbildung 10)<sup>61</sup>.

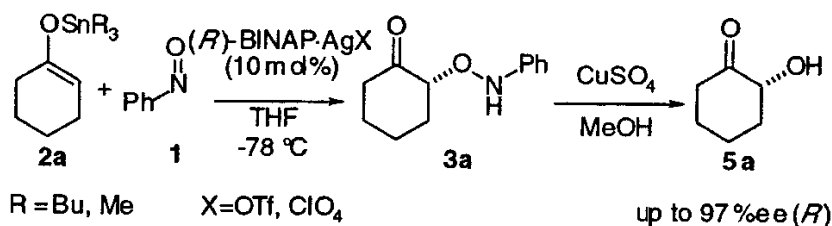


Abbildung 10: Synthese von cyclischen  $\alpha$ -Hydroxyketonen durch BINAP und AgOTf. Entnommen aus Momiyama *et al.*<sup>61</sup>

Die Synthese von enantiomerenreinen vicinalen Diolen kann durch eine hydrolytische kinetische Racematspaltung von terminalen Epoxide realisiert werden. Verwendet wird hier ein chiraler (Salen)-Co<sup>III</sup>-Komplex. Die Geschwindigkeit der ringöffnenden Reaktion kann durch die Menge eingesetzten Wassers kontrolliert werden. So konnte die Synthese von 1-Chlor-2,3-Pentandiol aus racemischem 2-(Chloromethyl)oxiran mit >99% *ee* gezeigt werden (Abbildung 11)<sup>62</sup>.

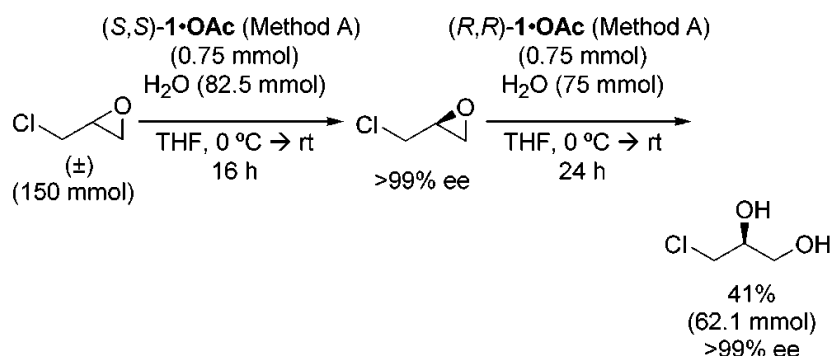


Abbildung 11: Synthese von 1-Chlor-2,3-Pentandiol aus racemischem 2-(Chloromethyl)oxiran. Entnommen aus Schaus *et al.*<sup>62</sup>

Auch ist die Synthese von vicinalen Diolen über die  $\alpha$ -Aminooxilation von Aldehyden mit enantiomerenreinen Prolin als Katalysator und Nitrobenzen als Sauerstoffquelle möglich. Gefolgt von einer *in situ* Reduktion mit Natriumborhydrid. Die N-O Bindung des Aminoxyalkohols kann durch den Einsatz von Platindioxid in Tetrahydrofuran abgetrennt werden. Die Bildung des vicinalen Diols kann so mit bis zu einem *ee* von bis zu 99% realisiert werden (Abbildung 12)<sup>63</sup>.

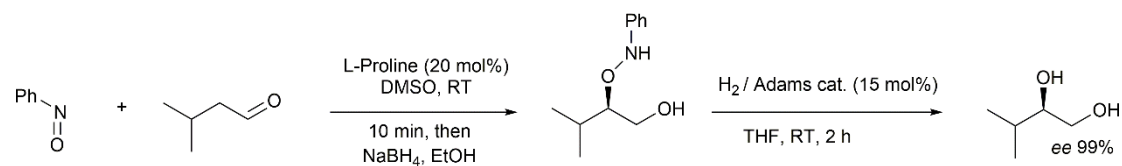


Abbildung 12: Synthese von vicinalen Diolen ausgehend von Aldehyden. Entnommen aus Zhong *et al.*<sup>63</sup>

### 1.6.2 Biokatalytische Darstellung von $\alpha$ -Hydroxyketonen und vicinalen Diolen

Diverse Prozesse für die enzymatische Synthese von  $\alpha$ -Hydroxyketonen und vicinalen Diolen wurden bereits etabliert. Neben dem Einsatz von Oxidoreduktasen sind auch weitere enzymatische Prozesse zur Generierung von  $\alpha$ -Hydroxyketonen und vicinalen Diolen bekannt. Zum einen ist der Einsatz von Thiamindiphosphat (ThDP)-abhängigen Enzymen zur Verknüpfung von Aldehyden möglich, die auch ausgehend von  $\alpha$ -Ketocarbonsäuren, wie Pyruvat oder Benzoylformiat vom Enzym *in situ* gebildet werden können. Fortschritte im



Proteindesign haben zu Enzymvarianten geführt, die die Synthese von  $\alpha$ -Hydroxyketonen mit hoher Enantioselektivität katalysieren<sup>64</sup>. So konnte durch Protein- und Reaktionsdesign mit der Pyruvatdecarboxylase aus *Acetobacter pasteurianus* die Bildung von (*S*)-Phenylacetylcarbinol mit über 97% *ee* erreicht werden (Abbildung 13)<sup>65</sup>.

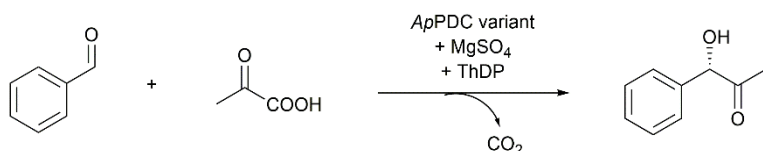


Abbildung 13: Synthese von (*S*)-Phenylacetylcarbinol aus Benzaldehyd und Pyruvat mit Varianten der Pyruvatdecarboxylase aus *Acetobacter pasteurianus*. Nach Sehl *et al.*<sup>65</sup>

Durch die kinetische Racematspaltung von racemischen Estern durch hydrolytische Spaltung mittels Lipasen oder Esterasen lassen sich ebenfalls enantiomerenreine  $\alpha$ -Hydroxyketone gewinnen (Abbildung 14). Die hydrolytische Spaltung von Acyloinestern mit der Lipase aus *Candida antarctica* B (CAL-B) zeigte zum Beispiel ausgesprochen hohe Enantioselektivitäten (*E*-Werte) für die Bildung von 2-Hydroxy-3-heptanon (*E* = 165) und 2-Hydroxy-3-nonanon (*E* = 152)<sup>66</sup>.

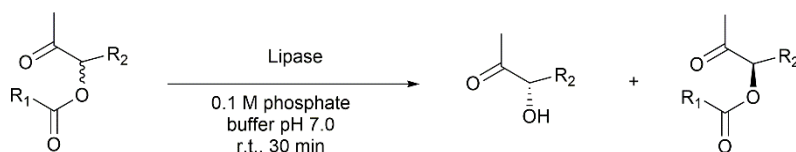


Abbildung 14: Kinetische Auflösung von racemischen Acyloinestern durch Lipasen. Nach Scheid *et al.*<sup>66</sup>

### 1.6.3 Synthese von $\alpha$ -Hydroxyketonen und vicinalen Diolen mittels Butandioldehydrogenasen

Aufgrund der natürlichen Affinität für vicinale Diektone,  $\alpha$ -Hydroxyketone und vicinale Dirole wie Diacetyl, Acetoin und 2,3-Butandiol sind Butandioldehydrogenasen (BDH) besonders interessant für die biokatalytische Anwendung. Im besonderen Forschungsfokus steht diese Enzymgruppe jedoch für die mikrobielle Produktion von 2,3-Butandiol. Besonders etabliert hierfür hat sich der Einsatz von *Klebsiella sp.*, *Paenibacillus polymyxa* oder *Serratia marcescens* in fermentativen Verfahren. So konnte die mikrobielle Produktion von *meso*-2,3-Butandiol und (*S,S*)-2,3-Butandiol bei Einsatz des Mikroorganismus *Klebsiella pneumoniae* in einer Fed-Batch Fermentation ausgehend von Glucose mit Konzentration von 150 g/L dargestellt werden. Die Verwendung des Mikroorganismus *Serratia marcescens* führte in einer Fed-Batch Fermentation mit Sucrose als Substrat zu 152 g/L *meso*-2,3-Butandiol<sup>67</sup>. Neben der fermentativen Produktion, konnte auch der Einsatz von gereinigter BDH in einem zellfreien

System ausgehend von Diacetyl mit racemischen Phenylethanol zur Cofaktorregenerierung oder die Anwendung von Ganzzellkatalysatoren für die Produktion von 2,3-Butandiol gezeigt werden (Abbildung 15) <sup>68,69</sup>.

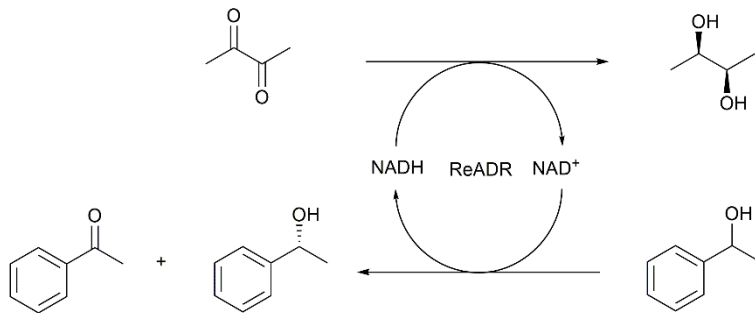


Abbildung 15: Bildung von (*S,S*)-2,3-Butandiol ausgehend von Diacetyl. Phenylethanol wird zur Cofaktorregenerierung genutzt. Nach Wang *et al.* <sup>68</sup>

Hingegen ist die Synthese von nicht physiologischen Acyloinen und vicinalen Diolen mittels BDH bisher nur wenig untersucht worden. Mit der BDH aus *Saccharomyces cerevisiae* ist die stereoselektive Reduktion von 2,3-Pentandion, 2,3-Hexandion und 3,4-Hexandion zu den jeweiligen Diolen mit (*R*)-konfigurierten Stereozentrum möglich <sup>70</sup>. Mit der BDH aus *Serratia marcescens* CECT 977 konnte die enantioselective Synthese von  $\alpha$ -Hydroxyketonen, wie auch vicinalen Diolen ausgehend von prochiralen Diketonen demonstriert werden. So reduziert das Enzym 2,3-Pentandion, 3,4-Hexandion und 1,2-Cyclohexandion zu den korrespondierenden (*S*)-konfigurierten Diolen. Jedoch stoppt die Reduktion von 2,3-Hexandion sowie bei Substraten mit einem Phenylrest, wie 1-Phenyl-1,2-propandion, am (*S*)- $\alpha$ -Hydroxyketonen Intermediat <sup>27</sup>.

## 1.7 Motivation und Zielsetzung

Die Synthese von enantiomerenreinen  $\alpha$ -Hydroxyketonen und vicinalen Diolen ist aufgrund des breiten Einsatzspektrums dieser Moleküle besonders interessant. Obwohl Butandioldehydrogenasen bekannt sind für die natürliche Umsetzung des Diketons Diacetyl zu Acetoin als  $\alpha$ -Hydroxyketon und dieses weiter reduziert werden kann zum 2,3-Butandiol, so sind diese Enzyme im Hinblick auf die stereoselektive biokatalytische Synthese von nicht natürlichen Acyloinen oder vicinal Diolen kaum untersucht. Ziel der vorliegenden Arbeit war die biochemische und biokatalytische Charakterisierung der Butandioldehydrogenasen aus *Bacillus licheniformis* DSM 13<sup>T</sup> und *Bacillus clausii* DSM 8716<sup>T</sup>, welche unterschiedlichen

Superfamilien zugeordnet werden können. So gehört die BDH aus *Bacillus clausii* DSM 8716<sup>T</sup> zu den meist metallionenabhängig „medium-chain“-Dehydrogenasen. Enzyme aus dieser Superfamilie, bilden Homodimere und sind oft *R*-selektiv. Im Rahmen der grundlegenden Charakterisierung des Enzyms galt es zu überprüfen ob diese Eigenschaften auch auf die BDH aus *Bacillus clausii* DSM 8716<sup>T</sup> zutreffen.

Hingegen gehört die BDH aus *Bacillus licheniformis* DSM 13<sup>T</sup> zu den „short-chain“-Dehydrogenasen <sup>71</sup>. Für die biokatalytische Charakterisierung sollten beide Enzyme hinsichtlich der Reduktion diverser vicinaler Diketone untersucht werden. Neben der Untersuchung des zugänglichen Produktspektrums, stand dabei auch die Bestimmung der Stereoselektivität dieser Enzyme für die Reduktion nicht natürlicher Diketone und Hydroxyketone im Vordergrund.

In Kooperation mit der Arbeitsgruppe für Chemo- und Biosensorik (FH Aachen, Prof. Schöning, Promotion Dr. Molinnus) sollte darüberhinaus mit den in dieser Arbeit rekombinant bereitgestellten und biochemisch charakterisierten Enzymen ein potentiometrischer Biosensor zur Quantifizierung von Acetoin und Diacetyl in Fermentationsprozessen entwickelt werden.

## 2 Ergebnisse

### 2.1 Publikation 1

#### **(*R,R*)-butane-2,3-diol dehydrogenase from *Bacillus clausii* DSM 8716 T: cloning and expression of the *bdhA*-gene, and initial characterization**

Lukas Muschallik, Denise Molinnus, Johannes Bongaerts, Martina Pohl, Torsten Wagner, Michael J. Schöning, Petra Siegert, Thorsten Selmer

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#### Zusammenfassung:

In dieser Publikation wurde das Gen codierend für die (*R,R*)-2,3-Butandioldehydrogenase aus *Bacillus clausii* DSM13<sup>T</sup> zum ersten Mal im Expressionsstamm *E. coli* B121 (DE3) heterolog exprimiert. Es erfolgte eine initiale Charakterisierung des Enzyms. Dies beinhaltete die Aufnahme von Substratspektren, Temperaturoptima, pH-Optima und die Ermittlung der kinetischen Parameter für die natürlichen Substrate Diacetyl, Acetoin und 2,3-Butandiol. Durch den Einsatz dieser Butandioldehydrogenase in Biokatalysen mit den physiologischen Substraten konnte nachgewiesen werden, dass es sich bei dem Enzym um eine *R*-selektive Butandioldehydrogenase handelt.

#### Anteile:

L. Muschallik plante und führte alle Experimente zur Expression, Reinigung und Charakterisierung dieses Enzyms durch. D. Molinnus arbeitete an der Entwicklung eines Biosensors als Anwendungsbeispiel, in dem dieses Enzym als Schlüsselpart eingesetzt werden kann und trug mit diesen Arbeiten zur biochemischen Charakterisierung bei. Das Gen wurde initial von T. Selmer aus dem Stamm *B. clausii* DSM13<sup>T</sup> kloniert. P. Siegert und T. Selmer führten die konzeptionelle Planung und Betreuung des Projekts durch. Alle Autoren beteiligten sich an der Erstellung des Manuskripts.



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## (*R,R*)-Butane-2,3-diol dehydrogenase from *Bacillus clausii* DSM 8716<sup>T</sup>: Cloning and expression of the *bdhA*-gene, and initial characterization of enzyme



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### ABSTRACT

The gene encoding a putative (*R,R*)-butane-2,3-diol dehydrogenase (*bdhA*) from *Bacillus clausii* DSM 8716<sup>T</sup> was isolated, sequenced and expressed in *Escherichia coli*. The amino acid sequence of the encoded protein is only distantly related to previously studied enzymes (identity 33–43%) and exhibited some uncharted peculiarities. An N-terminally StrepII-tagged enzyme variant was purified and initially characterized. The isolated enzyme catalyzed the (*R*)-specific oxidation of (*R,R*)- and *meso*-butane-2,3-diol to (*R*)- and (*S*)-acetoin with specific activities of 12 U/mg and 23 U/mg, respectively. Likewise, racemic acetoin was reduced with a specific activity of up to 115 U/mg yielding a mixture of (*R,R*)- and *meso*-butane-2,3-diol, while the enzyme reduced butane-2,3-dione ( $V_{\max}$  74 U/mg) solely to (*R,R*)-butane-2,3-diol via (*R*)-acetoin. For these reactions only activity with the co-substrates NADH/NAD<sup>+</sup> was observed. The enzyme accepted a selection of vicinal diketones,  $\alpha$ -hydroxy ketones and vicinal diols as alternative substrates. Although the physiological function of the enzyme in *B. clausii* remains elusive, the data presented herein clearly demonstrates that the encoded enzyme is a genuine (*R,R*)-butane-2,3-diol dehydrogenase with potential for applications in biocatalysis and sensor development.

### 1. Introduction

Acetoin is a naturally formed  $\alpha$ -hydroxy ketone and a major fermentation product of bacilli and *Enterobacteriaceae*. Some of these organisms produce significant amounts of diacetyl (butane-2,3-dione), acetoin and butane-2,3-diol, and the detection of acetoin is an important biochemical marker for the identification of pathogens (Levine, 1916; Voges and Proskauer, 1898). Diacetyl and acetoin exhibit a butter-like flavor in alcoholic beverages like beer and wine. These compounds are undesired in lighter beers to give a clean, crisp taste, whereas higher concentrations of these compounds are desired in wines for a smoother taste and feel.

Vicinal diketones like butane-2,3-dione are not only naturally produced in fermentation processes (Ji et al., 2011) but also chemically accessible e.g. by ruthenium-catalyzed oxidation of alkenes (Chen et al., 2011), catalytic oxidation of silyl enol ethers (Hayashi et al., 2012) or alkynes (Santoro et al., 2010), and mild oxidation of vicinal diols (Li and Zhang, 2009). Upon stepwise reduction, these compounds can yield a large selection of chiral  $\alpha$ -hydroxy ketones and vicinal diols, which

are interesting synthons for complex chiral structures or monomers for mixed polymers with unique properties, respectively. The chemical synthesis of enantiomerically pure  $\alpha$ -hydroxy ketones or vicinal diols is difficult to achieve (Enders and Lotter, 1981) and the direct, biocatalytic conversion of vicinal diketones with enzymes is advisable.

Butane-2,3-diol dehydrogenases (BDHs) – also referred to as acetoin reductases – are capable of catalyzing the enantioselective reduction of vicinal diketones with NAD(P)H to the corresponding  $\alpha$ -hydroxy ketones but also the further reduction of the latter to yield either (*R,R*)- or (*S,S*)-butane diols (Taylor and Juni, 1960), respectively (Fig. 1). Using racemic acetoin or other racemic  $\alpha$ -hydroxy ketones as substrates for the oxidation of NAD(P)H, a mixture of either (*R,R*)- or (*S,S*)- and *meso*-(*R,S*) vicinal diols is formed by enantioselective enzymes. Unlike the reduction of vicinal diketones via the corresponding  $\alpha$ -hydroxy ketones to the vicinal diols, which proceeds smoothly, the enzymatic oxidation of vicinal diols with NAD(P)<sup>+</sup> remains stuck at the level of the intermediately formed  $\alpha$ -hydroxy ketones, while an oxidation of the latter by oxygen occurs spontaneously (Pound, 1947).

The unique properties of BDHs as outlined in Fig. 1 permit the use of

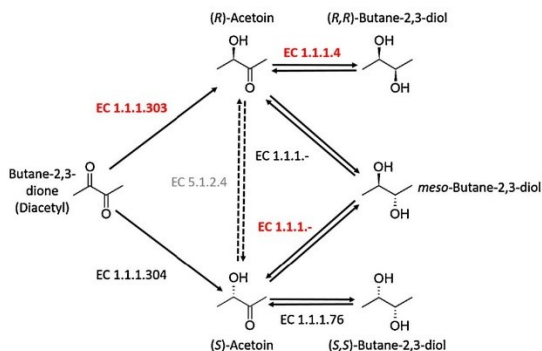
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**Fig. 1.** Butane-2,3-diol dehydrogenase-mediated interconversions of C<sub>4</sub>-compounds. The EC numbers of the reactions catalyzed by (R,R)-BDH (EC 1.1.1.4) are shown bold, those mediated by (S,S)-BDH (EC 1.1.1.76) and meso-BDH (EC 1.1.1.-) are given in plain text. The interconversion of the acetoin enantiomers by acetoin racemase (gray, EC 5.1.1.2.4, dotted arrows) is given to complete the picture. Note that BDHs catalyze the reduction of diacetyl (EC 1.1.1.304 or EC 1.1.1.304, respectively) to acetoin but not the oxidation of the latter.

these enzymes in the preparation of enantiomeric pure acetoin and butane-2,3-diols: The two step reduction of diacetyl with (R,R)-BDH will solely yield (R,R)-butane-2,3-diol via (R)-acetoin while in the oxidation of meso-butane-2,3-diol mediated by this enzyme exclusive (S)-acetoin is formed (Gao et al., 2013; Gonzalez et al., 2000; Yu et al., 2011). Likewise, oxidation of meso-butane-2,3-diol by a (S,S)-BDH or meso-BDH will yield (R)-acetoin.

Our interest in butane-2,3-diol dehydrogenases is motivated by an interdisciplinary approach aiming at increasing the number of characterized enzymes available for biocatalysis, synthetic biology, and sensor chip development. The genome of *B. clausii* KSM-K16 (Kageyama et al., 2007) harbors a gene (ABC0235) which apparently encodes an, yet not characterized, zinc-dependent (R,R)-butanediol dehydrogenase/diacetyl reductase (EC 1.1.1.4/1.1.1.303). Since the encoded amino acid sequence is only distantly related to previously studied (R,R)-BDHs from other organisms, we decided to clone and to express the orthologous gene from *B. clausii* DSM 8716<sup>T</sup> in *Escherichia coli*. In this communication, we demonstrate that this novel BDH is indeed a genuine (R,R)-butane-2,3-diol and solely NADH/NAD<sup>+</sup> dependent dehydrogenase and summarize the initial biochemical characterization of the recombinant enzyme.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Unless stated otherwise, chemicals were analytical grade and purchased from Sigma-Aldrich. Reagents for molecular biology were from Thermo Scientific. DNA oligonucleotide synthesis and DNA sequencing were performed by Eurofins Genomics (Germany). Stargate<sup>®</sup> cloning vectors and StrepTactin<sup>®</sup>-columns were from IBA GmbH (Germany). Genomic DNA of *Bacillus clausii* DSM 8716<sup>T</sup> was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany).

### 2.2. Bacterial strains and plasmids

For initial cloning of the *bdhA* gene, the Stargate<sup>®</sup>-pENTRY vector (kanamycin resistance) was used. Stargate<sup>®</sup>-pASG.3 and pASG.5 vectors (ampicillin resistance) were used for gene expression (IBA, Germany). Cloning was done in *Escherichia coli* DH5α, and *E. coli* BL21(DE3) was used for heterologous expression of the *bdhA* gene.

### 2.3. Cloning of the (R,R)-butane-2,3-diol dehydrogenase encoding gene (*bdhA*) from *Bacillus clausii* DSM 8716<sup>T</sup>

The primers (ABC0235c-s TTGCTCTTCAATGAGAGCAGCCGTATG-GTAC and ABC0235c-as TTGCTCTTACCCTTTCTCGCCGTAAGT-TTCAG, respectively) were deduced using the known sequence of the *B. clausii* KSM-K16 gene (ABC0235). Using these primers and genomic DNA of *B. clausii* DSM 8716<sup>T</sup> for PCR-amplification the *bdhA* gene was obtained. Stargate<sup>®</sup> cloning of the PCR product was performed essentially as described before (Aboulnaga et al., 2013). The *bdhA* gene in the expression vectors was sequenced using the primers GAGTTATTT-TACCCTCCCT (forward) and CGCAGTAGCGGTAAACG3 (reverse). The nucleotide sequence of the *bdhA* gene from *B. clausii* DSM 8716<sup>T</sup> was deposited under the accession number KY380091 in GenBank.

### 2.4. Database searches and alignments

BLAST searches were performed using the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics and sequences of related proteins were aligned using the ClustalW program (Swiss EMBnet node server).

### 2.5. Production of recombinant (R,R)-BDH in *E. coli* and purification

*E. coli* BL21(DE3) was transformed with the plasmid pASG-*bdhA*.3 or pASG-*bdhA*.5 to express gene variants genetically fused 3' or 5' to the vector derived StrepII-encoding sequence of the vector. Cells were grown at 30 °C at 180 rpm 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 exponential growth phase, as previously described (Aboulnaga et al., 2013). 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<sup>®</sup> 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).

### 2.6. Determination of protein concentration, purity, and molecular mass

Protein concentrations throughout enzyme purification were determined using the Bradford method and bovine serum albumin (BSA) as a standard.

SDS-PAGE was carried out according to Laemmli (1970) using 12-% polyacrylamide gels and Roti<sup>®</sup>-Mark PRESTAINED ladder as standard. Native electrophoresis was performed using the Laemmli buffer system without SDS and 4–20% gradient gels with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (250 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (66 kDa) as standards. Proteins were stained with Coomassie Brilliant Blue G250 (Roti<sup>®</sup>-Blue Quick, Roth).

Mass spectrometry was performed using sinapinic acid as the matrix in an Axima Confidence MALDI-TOF-MS essentially as described before (Selmer and Buckel, 1999).

### 2.7. Butanediol dehydrogenase activity assays

Enzyme reactions were followed by substrate-dependent reduction of NAD<sup>+</sup> or oxidation of NADH at 340 nm (0.3 mM each) over a period of 90s using a temperature-controlled photometer (Bioscience Ultrospec 2100 Pro, Amersham). Unless stated otherwise, all reactions were performed at 30 °C.

The acetoin reduction was measured in sodium-MES (2-(N-morpholino)ethane sulfonic acid) buffer (50 mM, pH 6.8) with acetoin

(10 mM) and NADH or NADPH (300  $\mu$ M) as substrates. The butane-2,3-diol oxidation was measured in TRIS-HCl buffer (50 mM, pH 8.0) with (*R,R*)-butane-2,3-diol (10 mM) and  $\text{NAD}^+$  (300  $\mu$ M) as substrate. The reaction was initiated by addition of an appropriately diluted sample of purified BDH. A correction was made by measuring a sample blank without enzyme. Variability is expressed as standard error of the mean.

One unit of BDH was defined as the amount of enzyme that oxidized or reduced one  $\mu$ mol of NADH or  $\text{NAD}^+$  per minute at 30 °C under the given conditions, respectively.

## 2.8. Enzymatic properties and storage stability

### 2.8.1. pH optimum

For the determination of the pH profiles the reaction was carried out using the following buffers at 50 mM concentration each: acetate buffer (pH 4.0–5.0), MES buffer (pH 5.0–7.0), TRIS-HCl (pH 7.0–9.0) for the reduction and 50 mM MES buffer (pH 6.0–7.0), TRIS-HCl (pH 7.0–9.0) and Glycin-NaOH buffer (pH 9.0–10.0) for the oxidation reaction. The pH optima of BDH towards diacetyl, racemic acetoin, (*R,R*)-butane-2,3-diol (10 mM each) were determined in the same buffers supplemented with  $\text{NAD}^+$  or NADH (0.3 mM). Buffer preparation: a solution of TRIS (50 mM), acetate (50 mM), MES (50 mM), glycine (50 mM) was made and adjusted to the desired pH with its corresponding acid/base at 30 °C.

### 2.8.2. Optimal temperature

Determinations of the temperature optima of initial rate activity for the individual reactions were determined in 50 mM MES buffer (pH 6.8) for the reduction reaction and 50 mM TRIS-HCl (pH 8.0) for the oxidation reaction at temperatures in the range from 10 to 70 °C under otherwise standard reaction conditions. The pH of the buffers was set at the chosen temperatures.

### 2.8.3. Substrate specificity

The activities towards various potential substrates were performed using the standard oxidation and reduction assays (see chapter 2.8.) with 10 mM of potential substrates (individual compounds are given in the result section).

### 2.8.4. Kinetic parameters

Kinetic parameters were determined under standard conditions (see chapter 2.8.) with substrate concentrations in the range of 0–60 mM for acetoin; 0–200 mM for diacetyl, (*R,R*)-butane-2,3-diol and *meso*-butanediol; 0–0.6 mM for NADH; 0–2 mM for  $\text{NAD}^+$ . Kinetic data were fitted to the Michaelis-Menten equation with and without substrate inhibition. Determination of kinetic parameters was made with GraphPad Prism 7 software.

### 2.8.5. Storage stability

The storage stability of the purified enzyme was tested in TRIS-HCl buffer (10 mM, pH 7.4) supplemented with NaCl (150 mM) over a period of 6 days at 0; –20 and –80 °C with or without glycerol (10% v/v) as a cryoprotectant, respectively. Residual activities of the enzyme were measured after 1, 2, 3 and 6 days using the acetoin reduction assay (see chapter 2.8.). The shelf stability of the enzyme was addressed at 22 °C in TRIS-HCl buffer (10 mM, pH 7.4) supplemented with NaCl (150 mM) over 16 h.

## 2.9. Stereoselectivity of butane-2,3-diol dehydrogenase from *Bacillus clausii*

Stereoselectivity of the butane-2,3-diol dehydrogenase reduction reaction from *B. clausii* was analyzed by carrying out the asymmetric reduction of diacetyl and racemic acetoin. The stereoselectivity of the oxidation reaction was analyzed by enzymatic oxidation of (*R,R*)-, (*S,S*)- and *meso*-butane-2,3-diol. Formate dehydrogenase (FDH) from *Candida boidinii* (Megazyme; 2.0 U/mg) was used for cofactor regeneration.

The reaction mixtures (1 mL) for the reduction reaction contained 1 U purified BDH, 5 U FDH, 60 mM formate, 20 mM diacetyl, and 0.3 mM NADH in 50 mM MES buffer (pH 6.8) at 30 °C for 70 min. To determine the selectivity of the oxidative reaction, 1 U of purified BDH with equimolar concentrations (10 mM) of substrates in TRIS-HCl buffer (50 mM, pH 8.0) were used. Samples (100  $\mu$ L) were extracted with diethyl ether (300  $\mu$ L) and applied to GC analysis.

A GC-2010 Plus gas chromatograph (Shimadzu) equipped with a flame ionization detector (carrier gas helium with a linear velocity of 40 cm/s) and a Hydrodex  $\gamma$ -DIMON (25 m  $\times$  0.25 mm ID, Macherey-Nagel, Germany) chiral chromatographic column were used for sample analysis. The injector and detector temperature were set to 215 °C and 245 °C, respectively. The column temperature profile was as follows: 45 °C (2 min), 45 °C–70 °C (at 2 °C/min); 70 °C–180 °C (at 10 °C/min); 180 °C (10 min). The Shimadzu GC System software was used for data acquisition and processing. Retention times of educts and products (as standards purchased from Sigma-Aldrich) were: diacetyl 4.1 min; (*R*)-acetoin 11.7 min; (*S*)-acetoin 14.4 min; (*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.

## 3. Results and discussion

### 3.1. Cloning and sequence analysis of the *bdhA* gene from *B. clausii* DSM 8716<sup>T</sup>

The published genome of the extremophile *B. clausii* KSM-K16 harbors an open reading frame encoding a putative (*R,R*)-butane-2,3-diol dehydrogenase (*ABC0235*). The genome-derived sequence was used to deduce a primer pair which was used to amplify a PCR-product of the expected size (1070 bp) using genomic DNA from *B. clausii* DSM 8716<sup>T</sup> as the template. The PCR product was *LguI*-mediated cloned into the Stargate<sup>®</sup> ENTRY vector and sequenced. The DNA sequence of the *bdhA* gene from *B. clausii* DSM 8716<sup>T</sup> shared 96% identity as compared to the *ABC0235* gene of *B. clausii* KSM-K16.

The amino acid sequence identity of the translated nucleotide sequence and the ORF from *B. clausii* KSM-K16 was 97%. Protein similarity search using NCBI BLAST to screen the UniProt Knowledgebase revealed that the amino acid sequences could probably define a novel subgroup of (*R,R*)-butane-2,3-diol dehydrogenases: The closest relatives of *B. clausii* DSM 8716<sup>T</sup> BDH were found in *Sporosarcina psychrophila* (*Bacillus psychrophilus*, A0A127VY90.SPOPS, 71% identity) and *Oceanobacillus caeni* (A0A0N0M728.9BACI, 66% identity) (Fig. 2a), while sequences of previously characterized (*R,R*)-butane-2,3-diol dehydrogenases, e.g. *Bacillus subtilis* (G4EZD3.BACIU, 43% identity), *Paenibacillus polymyxa* (*Bacillus polymyxa*, G9HPK4.PAEPO, 38% identity), *Rhodococcus erythropolis* (T51236.RHOER, 33% identity) and *Saccharomyces cerevisiae* (BDH1.YEAST, 33% identity) are more distantly related to the *B. clausii* enzymes (Fig. 2b). No significant similarities (< 10% identities) to threonine dehydrogenase (from *Clostridium sticklandii*, E3PS87.CLOSD) or glycerol dehydrogenase (*Schizosaccharomyces pombe*, GLD1.SCHPO) were found.

In spite of the comparable low sequence similarity of the *B. clausii* ORFs to previously studied enzymes, key features indicating (*R,R*)-butane-2,3-diol dehydrogenases were readily identified within the amino acid sequence of BDH (Fig. 2): The protein sequence harbors the zinc-containing alcohol dehydrogenases Prosite signature (PS00059, aa 69–83, (Sun and Plapp, 1992)) as well as the (*R,R*)-butanediol dehydrogenase (EC 1.1.1.4) PRATT patch (P39714, aa 148–193). Likewise, amino acids for the ligation of the catalytic  $\text{Zn}^{2+}$ -ion (Cys37, His70 and Glu151) were identified. Interestingly, the ligands for binding of the structural  $\text{Zn}^{2+}$ -ion in the previously studied enzymes (e.g., Cys103, Cys120, Cys123 and Cys131 in the *S. cerevisiae* sequence) were virtually absent in the amino acid sequences of *B. clausii* BDHs. Unexpectedly, the BDH sequences from *B. clausii* strains harbored a zinc-binding region signature (PS00142, aa 39–48 (Jongeneel et al., 1989)) close to the N-terminus, which is otherwise found in neutral zinc metallopeptidases

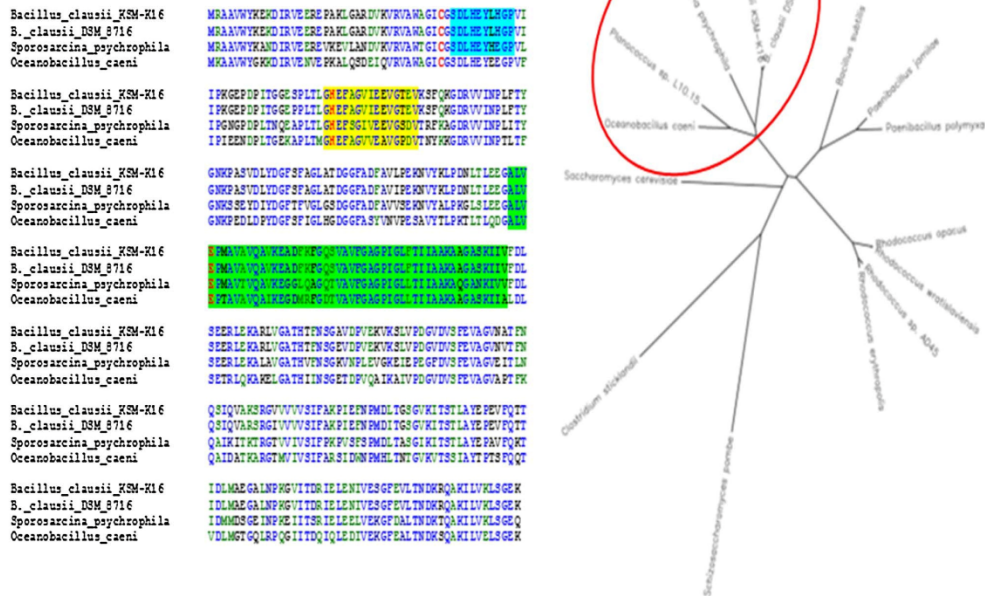


Fig. 2. Sequence alignment of members of characterized (R,R)-BDHs. The left panel shows the amino acid sequence alignment of the BDH from *Bacillus clausii* DSM 8716<sup>T</sup> and the orthologous sequences from *Bacillus subtilis*, *Clostridium beijerinckii*, *Paenibacillus polymyxa*, *Oceanobacillus caeni*, *Saccharomyces cerevisiae* and *Sporosarcina psychrophila*. The putative ligands of the catalytic zinc ion are shown in bold red. The Zn-alcohol dehydrogenase motif (P500059, yellow), the neutral zinc metalloproteases zinc-binding region signature (P500142, blue) and the (R,R)-butanediol dehydrogenase PRATT patch (green) are highlighted. The putative binding region of the structural zinc ion in some BDHs with the conserved cysteinyl ligands (red) is shaded gray. The right panel shows the relationship of the novel subgroup to the previously studied enzymes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

but also present with an only slightly altered sequence in the other (R,R)-BDH sequences.

3.2. Expression of *bdhA* variants in *E. coli* BL21(DE3) and purification of the N-terminally strepII-tagged enzyme

In order to permit biochemical studies of the enzyme, the *bdhA* gene was sub-cloned from pE-*bdhA* into pASG.3 and pASG.5, respectively. In the *Esp3I*-mediated sub-cloning procedure, the gene was fused in frame either 3' or 5' with vector-encoded StrepII-affinity tags. The resulting vectors were used to transform *E. coli* BL21(DE3) cells for recombinant production of C-terminally (pASG-*bdhA*.3) or N-terminally (pASG-*bdhA*.5) StrepII-tagged enzyme variants.

Both enzyme variants were readily produced when gene expression in the recombinant *E. coli* strains was induced by anhydrotetracycline. Maximum expression of the foreign protein was achieved within 3 h post induction, and specific acetoin reductase activities of approximately 1 U/mg and 22 U/mg were reached for the C- and N-terminally tagged enzyme, respectively. Thus, only the latter enzyme variant was purified using Streptactin<sup>®</sup> macroprep columns (IBA Germany) to yield pure enzyme that was used in further studies.

Acetoin reductase activity of BDH was monitored measuring the oxidation of NADH (300 μM) with racemic acetoin (10 mM) in MES buffer (50 mM, pH 6.8). The enzyme was typically enriched 3- to 5-fold and activity was recovered in > 85% yield. The specific activities of individual enzyme preparations varied between 75 U/mg and 105 U/

mg. Although this specific activity is lower than the reported value of the enzyme from yeast (968 U/mg, (Gonzalez et al., 2000)) it is among the highest values observed for enzymes from bacterial origin, which fall between 4,65 U/mg for the enzymes from *Paenibacillus polymyxa* (Yu et al., 2011) and 270 U/mg from *Enterobacter aerogenes* (Carballo et al., 1991), respectively.

As shown in Fig. 3, the recombinant BDH was essentially pure after affinity purification. SDS-PAGE and Coomassie staining revealed a prominent band at approximately 37 kDa. According to MALDI-TOF-MS, the mass of one subunit of recombinant BDH was 39,152 ± 40 Da and in line with the expected molecular mass of 39,190 Da as predicted from the amino acid sequence. Native PAGE and size exclusion chromatography revealed molecular masses of approximately 82 kDa for the functional enzyme. Thus, recombinant BDH from *Bacillus clausii* was shown to be a homodimer as previously described for the BDHs from *P. polymyxa* (Gao et al., 2013), *S. cerevisiae* (Gonzalez et al., 2000) rather than a homotetramer as described for the enzyme of another *S. cerevisiae* strain (Heidlas and Tressl, 1990a) or a monomer as described for BDH from *Rhodococcus erythropolis* (Yu et al., 2015).

3.3. Stability of the recombinant enzyme

The recombinant BDH as purified was unstable at 0 °C (t1/2 < 2,5 d). At 22 °C, the half-life time in TRIS-buffered saline (TBS) was reduced to approximately 12 h. Similar stabilities were observed in MES and phosphate buffers (100 mM each) between pH 5.5 and 9.0. The frozen



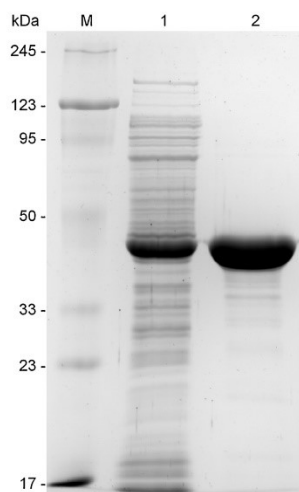
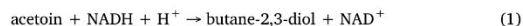


Fig. 3. Purification of recombinant (R,R)-BDH. Cell-free extract (10 µg protein, lane 1) and affinity-purified enzyme (10 µg, lane 2) were analyzed by SDS-PAGE and stained with Coomassie blue.

enzyme was stable in TBS at  $-80^{\circ}\text{C}$  without cryoprotection and activity losses  $< 10\%$  were noticed upon freeze-thawing. Similar instabilities of the BDH from *R. erythropolis* (Yu et al., 2011) and *P. polymyxa* (Hohn-Bentz and Radler, 1978) at elevated temperatures have been reported previously.

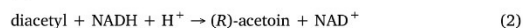
### 3.4. Activity optima of the enzyme

Throughout purification, BDH activity was measured in MES (50 mM, pH 6.8) buffer with racemic acetoin (10 mM) and NADH (300 µM) as substrates. No activity could be observed with NADPH. Here, the acetoin reductase activity of the enzyme was measured according to Eq. (1):



As shown in Fig. 4b the BDH from *B. clausii* showed maximum acetoin reductase activity at pH 7.0. The pH-dependent activity profile of the enzyme was shown to be rather broad, and between pH 5.5 and pH 7.5 more than 80% of the maximum activity was observed.

The enzyme also catalyzes the reduction of diacetyl according to Eq. (2):



As shown in Fig. 4a, the pH-dependence of diacetyl reduction (10 mM) behaved quite differently from the acetoin reductase activity described above. The pH optimum is shifted down to pH 6.0, and the pH-dependence of activity is narrower as compared to the acetoin reductase activity.

$\text{NAD}^+$ -dependent oxidation of (R,R)-butane-2,3-diol to (R)-acetoin, in turn, yields a proton, and a shift of the pH optimum to a higher pH value was expected and found. It turned out that the pH optimum of the diol oxidation reaction was shifted to pH 8.0 (Fig. 4c).

The observed pH dependences of acetoin and diacetyl reduction by the novel BDH from *B. clausii* are in agreement with previously published data for other BDHs. For BDH from *Paenibacillus polymyxa* (formerly *B. polymyxa*) a pH optimum of 8 was found (Yu et al., 2011). It must be stressed, however, the pH optimum for the enzyme from a different *Paenibacillus polymyxa* strain was reported to be at pH 6 (Gao

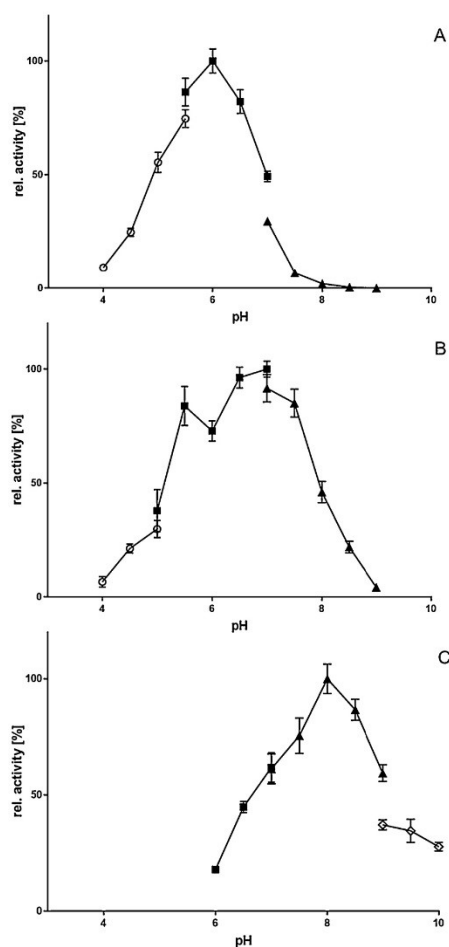


Fig. 4. pH-dependent activity of (R,R)-BDH for (A) the reduction of diacetyl, (B) reduction of acetoin, and (C) oxidation of (R,R)-butane-2,3-diol. The activity was measured with substrate concentrations of 10 mM (300 µM NAD(H)) at  $30^{\circ}\text{C}$  in sodium acetate (pH 4.0–5.0, open circles), sodium MES (pH 5.0–7.0, squares), TRIS-HCl buffer (pH 7.0–9.0, triangles) and sodium glycine (pH 9.0–10.0, diamonds); buffer concentration 50 mM. Experiments were carried out in triplicates.

et al., 2013). The optima for ketone reduction of other BDHs were found between pH 6.4 and 6.7 for the enzymes from *Clostridium beijerinckii* (Raedts et al., 2014), *Enterobacter aerogenes* (Carballo et al., 1991), *Rhodococcus erythropolis* (Yu et al., 2015) and *Saccharomyces cerevisiae* (Heidlas and Tressl, 1990a), while the maxima for diol oxidation were reported between pH 10 and pH 11 for the latter enzymes. Thus, the properties of *B. clausii* BDH with respect to butane-2,3-diol oxidation most closely resemble those reported for the enzyme from *P. polymyxa* (pH 8.0 (Gao et al., 2013)).

As a consequence of these results, the reduction of substrates by NADH was measured in MES buffer (50 mM, pH 6.8) while the oxidation of substrates by  $\text{NAD}^+$  was analyzed in TRIS-HCl buffer (50 mM, pH 8.0) in the ongoing studies. Using these conditions, the temperature dependence of the reactions was analyzed between  $10^{\circ}\text{C}$  and  $70^{\circ}\text{C}$ . As shown in Fig. 5, acetoin reductase, diacetyl reductase and butane-2,3-

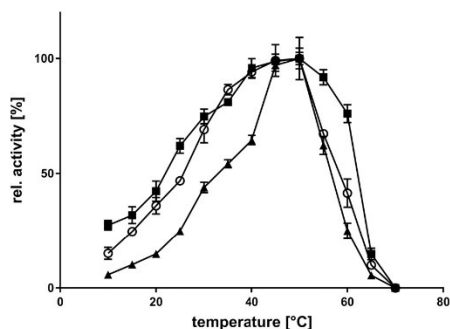


Fig. 5. Effect of the temperature on acetoin (squares) and diacetyl (open circles) reduction and (*R,R*)-butane-2,3-diol oxidation (triangles). The reductase activities were measured with substrate concentrations of 10 mM and 300  $\mu$ M NAD(H) in sodium MES (50 mM, pH 6.8), the oxidase activity was measured in TRIS-HCl buffer (50 mM, pH 8.0).

diol dehydrogenase activities showed temperature optima at 50 °C. Above this value, the enzyme activities rapidly decline. Also, the temperature optimum of BDH from *B. clausii* was in line with previously reported values for orthologous enzymes from other mesophiles, which have been reported between 30 °C and 60 °C. Respective Arrhenius plots permit the calculation of activation energies of 27 kJ/mol, 41 kJ/mol and 60 kJ/mol for the BDH catalyzed acetoin reductase, diacetyl reductase and butane-2,3-diol dehydrogenase activities, respectively. The activation energy of *B. clausii* BDH-catalyzed acetoin reduction was, therefore, significantly lower than the previously reported value of 96 kJ/mol for the NADPH-dependent reduction of acetoin by *C. beijerinckii* BDH (Raedts et al., 2014).

### 3.5. Kinetic properties with natural substrates

The Michaelis-Menten parameters like ( $K_M$ )-values, maximum specific activities ( $V_{max}$ ) and substrate inhibition constants ( $K_i$ ) of BDH have been obtained for its natural substrates diacetyl, racemic acetoin, *meso*-butane-2,3-diol, (*R,R*)-butane-2,3-diol, NADH and  $NAD^+$ . As evident from Fig. 6 and Table 1, BDH from *B. clausii* is a genuine (*S*)-specific acetoin reductase. The enzyme showed highest turnover numbers and specific constants, and the lowest  $K_M$ -values for acetoin and NADH. The turnover numbers and the  $K_M$ -values of these substrates for the novel enzyme are one order of magnitude lower than previously reported for BDH from yeast (Gonzalez et al., 2001). The kinetic parameters of the novel enzyme resemble, therefore, the known properties of other (*R*)-specific BDHs from bacterial origin, which also exhibit low  $K_M$ -values for acetoin and comparable low turnover numbers with this substrate (Carballo et al., 1991; Gonzalez et al., 2000; Raedts et al., 2014; Yu et al., 2015; Zhang et al., 2016). Notably, the yeast enzyme physiologically functions in the degradation of butane-2,3-diol in the absence of glucose (Gonzalez et al., 2001), while the bacterial enzymes have been reported to play an important role in the formation of this fermentation product from glucose.

The BDH from *B. clausii* was significantly substrate inhibited by acetoin at elevated concentrations ( $K_i = 88$  mM). Although a slight substrate inhibition was also observed for diacetyl and (*R,R*)-butane-2,3-diol, the  $K_i$  values of 330 mM and 560 mM suggested, however, that these inhibitions are physiologically irrelevant. Such an inhibition by the natural substrates is without reported precedence when the previously characterized (*R*)-BDHs are concerned, but it has been shown that the enzyme from *E. aerogenes* is substrate inhibited by methyl pyruvate ( $K_i = 81$  mM), ethyl pyruvate ( $K_i = 166$  mM) and methylglyoxal ( $K_i = 204$  mM), respectively (Carballo et al., 1991).

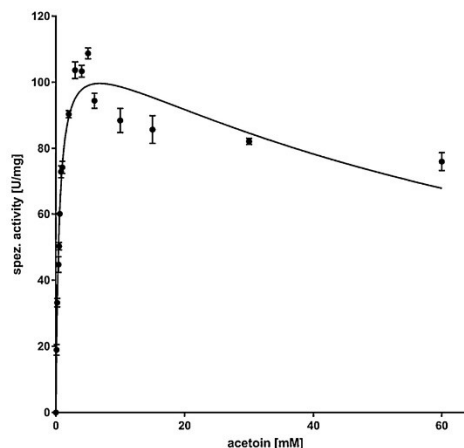


Fig. 6. Michaelis-Menten plot for the reduction of acetoin by (*R,R*)-BDH. The activity was measured in sodium MES buffer (50 mM, pH 6.8) supplemented with 300  $\mu$ M NADH at 30 °C. The concentration of racemic acetoin was varied as indicated in the plot. The curve was fitted with the software GraphPad Prism 7 with the equation for substrate inhibition.

Table 1

Kinetic parameters of (*R,R*)-BDH for its natural substrates acetoin, diacetyl, (*R,R*)-butane-2,3-diol, *meso*-butane-2,3-diol, NADH and  $NAD^+$ . The data were measured in sodium MES buffer (50 mM, pH 6.8) at 30 °C. The data for reduction of acetoin, diacetyl and oxidation of (*R,R*)-butane diol were measured with constant initial NAD(H) concentrations (300  $\mu$ M). The values for reduction of  $NAD^+$  and oxidation of NADH were obtained with acetoin (10 mM). Experiments were carried out in triplicates and the kinetic parameters were calculated with the GraphPad Prism 7 software.

Substrate	$V_{max}$ [U/mg]	$K_M$	$K_i$ [mM]	$k_{cat}$ [ $s^{-1}$ ]	$k_{cat}/K_M$ [ $s^{-1}mM^{-1}$ ]
Acetoin	115.2 $\pm$ 5.2	529 $\pm$ 79 $\mu$ M	88 $\pm$ 22	74	140
Diacetyl	74.0 $\pm$ 3.4	2.5 $\pm$ 0.4 mM	330 $\pm$ 73	48	19
( <i>R,R</i> )-BD	12.4 $\pm$ 0.7	2.2 $\pm$ 0.5 mM	560 $\pm$ 225	8	4
NADH	106.5 $\pm$ 2.8	44 $\pm$ 4 $\mu$ M	–	68	1551
<i>m</i> -BD	22.8 $\pm$ 0.9	23.1 $\pm$ 2.8 mM	–	15	0.7
$NAD^+$	12.7 $\pm$ 0.5	284 $\pm$ 30 $\mu$ M	–	8	29

### 3.6. Stereoselectivity of the BDH catalyzed reactions

To figure out the stereoselective oxidation/reduction of substrates by *B. clausii* BDH, diacetyl, racemic acetoin (20 mM each), *meso*-butane-2,3-diol and (*R,R*)-butane-2,3-diol (10 mM each) were used as substrates. The reductions of racemic acetoin and diacetyl were measured using formate (60 mM) and formate dehydrogenase (5 U/mL) as an NADH-regenerating system. The butane-2,3-diol isomers were extracted with diethyl ether and then analyzed by chiral GC. It turned out that starting with diacetyl as a substrate, exclusively (*R,R*)-butane-2,3-diol was formed. Analyzing the reduction of diacetyl over time revealed the formation of traces of (*R*)-acetoin at early stages of this reaction. While the reduction of racemic acetoin yielded a 1:1 mixture of *meso*-butane-2,3-diol and (*R,R*)-butane-2,3-diol as products where (*R*)-acetoin gets slightly faster reduced over (*S*)-acetoin (Fig. 7).

In turn, the oxidation of *meso*-butane-2,3-diol yielded exclusively (*S*)-acetoin while the substrate (*R,R*)-butane-2,3-diol was oxidized to (*R*)-acetoin, and no further oxidation of the two acetoin enantiomers to

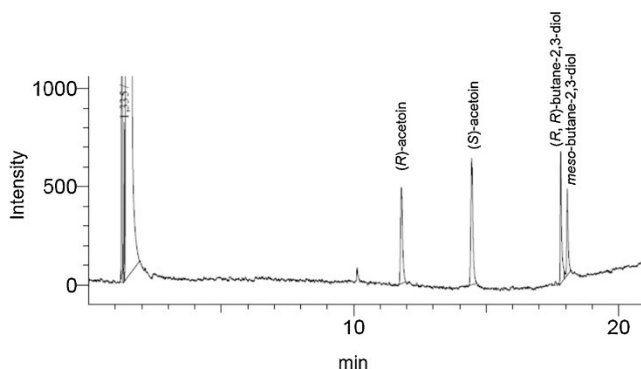


Fig. 7. Formation of (*R*)- and *meso*-butane-2,3-diol from racemic acetoin by (*R,R*)-BDH of *B. clausii*. Racemic acetoin (20 nmol) was reduced by the enzyme (1 U) using formate (60 nmol) and formate dehydrogenase (5 U) to regenerate NADH (300  $\mu$ M) at 30 °C in sodium MES buffer (50 mM, pH 6.8, 1 mL).

diacetyl was observed.

These data suggest that *B. clausii* BDH catalyzes a highly selective oxidation of secondary alcohol groups in (*R*)-configuration of butane-2,3-diols and also reduces the carbonyl function in acetoin and diacetyl in a stereoselective manner to yield the (*R*)-configuration of the resulting chiral center. In contrast, the configuration of the substituents in  $\alpha$ -position to the reaction center appears to be of minor importance for binding to the enzyme as indicated by the fact that the enzyme was capable of producing (*R,R*)- and *meso*-butane-2,3-diol from racemic acetoin. A similar stereoselectivity for these substrates has also been demonstrated for the enzymes from *P. polymyxa* (Gao et al., 2013; Yu et al., 2011) and yeast (Gonzalez et al., 2000). The latter enzyme has also been used for the chiral reduction of pentane-2,3-dione, hexane-2,3-dione and hexane-3,4-dione (Calam et al., 2016). Notably, the pure yeast enzyme yielded exclusively the (*R,R*)-enantiomers of pentane-2,3-diol, hexane-2,3-diol and hexane-3,4-diol, respectively. This suggests that the active center of yeast (*R,R*)-BDH preferentially binds the (*R*)-form of the intermediately formed  $\alpha$ -hydroxy ketones but likewise provides sufficient space to accommodate larger substrates.

### 3.7. Alternative substrates

While (*R,R*)-BDH from *B. clausii* accepted only NAD(H) as co-substrates, a selection of alternative substrates was used by the enzyme as oxidant or reductant, respectively.

In the reductive reaction, the enzyme showed neither activity with primary (acetaldehyde, benzaldehyde) nor with secondary carbonyl compounds (acetone, methyl ethyl ketone, hexan-2-one, acetophenone, 2-furyl methyl ketone),  $\alpha$ -keto acids (pyruvate) and  $\alpha$ -keto esters (ethyl pyruvate). Likewise, no methylene bridged diones (pentane-2,4-dione, hexane-2,4-dione) were reduced. In turn, the enzyme readily reduces substrate analog vicinal diketones, like pentane-2,3-dione, hexane-2,3-dione, hexane-3,4-dione and methylglyoxal (2-oxopropanal), and  $\alpha$ -hydroxy ketones like 3-hydroxy-3-methyl-butanone and 4-hydroxy-hexan-3-one.

The enzyme catalyzed the oxidation of racemic propane-1,2-diol as well as the oxidation of *cis*- and *trans*-1,2-cyclopentane diol, while neither primary (butane-1-ol, ethanol, methanol), nor secondary alcohols (propane-2-ol, ethylene glycol, racemic 1-phenylethanol or di-propylene glycols) or glycerol were oxidized. Thus, BDH from *B. clausii* selectively acts on vicinal diketones,  $\alpha$ -hydroxy ketones and vicinal diols.

As summarized in Tables 2 and 3, the substrate spectra of individual (*R,R*)-BDHs vary considerably. Although only a small selection of substrates was tested for individual enzymes, remarkable differences became obvious. The overview suggests that only the (*R,R*)-BDH from *B. clausii* exhibits a narrow substrate spectrum, which is apparently

restricted to vicinal diketones,  $\alpha$ -hydroxy ketones and vicinal diols. The *C. beijerinckii* enzyme showed a low activity for the reduction of butane-2-one, while no oxidation of the corresponding alcohol was observed and accepts also 3-acetoxy-butane-2-one as a substrate. The *P. polymyxa* (*R,R*)-BDH converts several primary and secondary alcohols and also oxidizes methylene-bridged diols. The enzymes from *S. cerevisiae* and *E. aerogenes* can also act on  $\alpha$ -keto or  $\alpha$ -hydroxy acid esters, while the substrates having proven to be converted by the *R. erythropolis* BDH also included  $\alpha$ -keto- and  $\alpha$ -hydroxy acids, and aromatic compounds (e.g. acetophenone).

## 4. Conclusions and outlook

### 4.1. Properties of (*R,R*)-butanediol dehydrogenase from *B. clausii*

Sequenced genomes harbor many genes that have been tentatively annotated as (*R,R*)-BDH genes or related zinc-dependent secondary alcohol dehydrogenases. Only a few of the encoded enzymes, however, have been characterized in detail and an even smaller number of these enzymes have been shown to be genuine (*R,R*)-butane-2,3-diol dehydrogenases (Carballo et al., 1991; Gao et al., 2013; Gonzalez et al., 2000; Raedts et al., 2014; Yu et al., 2011; Yu et al., 2015). In spite of the low sequence homology of the BDHs from *B. clausii* strains with the previously studied enzymes, the novel enzyme characterized in this work indubitably is a genuine (*R,R*)-BDH, too. This conclusion is justified based on the findings that the recombinant enzyme produced in *Escherichia coli* and purified to apparent homogeneity i) is exclusively active in the reductive direction towards aliphatic vicinal diketones and  $\alpha$ -hydroxy ketones, and ii) oxidizes only vicinal diols, which contain at least one hydroxyl function in (*R*)-configuration.

BDHs vary significantly in their polypeptide chain length as well as in their native molecular masses. While small, monomeric enzymes of approximately 30 kDa are found in archaea (Machielsen et al., 2006), bacterial enzymes are generally composed of subunits ranging from 30 kDa to 45 kDa in mass which form homodimeric structures (Carballo et al., 1991; Gao et al., 2013; Takeda et al., 2011). Eukaryotic BDHs are composed of small subunits (30 kDa) and form di- or tetramers (Gonzalez et al., 2000; Heildas and Tressl, 1990a). The molecular mass of the enzyme from *B. clausii* is comparable to the mass of other (*R,R*)-BDHs from bacterial origin and the enzyme forms a homodimer, too.

### 4.2. Substrate spectrum of (*R,R*)-butanediol dehydrogenase from *B. clausii*

Despite the previously outlined lack of absolute requirement, BDHs need a hydroxy, keto, acetyl or acetoxy group adjacent to the redox-active keto/hydroxy group in the preferred substrates. The electro-negative nature of the neighboring groups suggests that they are

Table 2

Relative activities of (*R,R*)-BDHs towards NADH-oxidizing substrates. All data refer to the activity with diacetyl. Data for enzymes from *P. polymyxa* (Yu et al., 2011), *R. erythropolis* (Yu et al., 2015), *C. beijerinckii* (Raedts et al., 2014), *E. aerogenes* (Carballo et al., 1991) and two *S. cerevisiae* strains (Gonzalez et al., 2000; Heidl and Tressl, 1990b) were taken from literature and adapted.

Substrate	<i>B. clausii</i> DSM 8716	<i>Paenibacillus polymyxa</i> [1] ATCC 12321	<i>Saccharomyces cerevisiae</i> [2] YAL060W Gene Product	<i>Rhodococcus erythropolis</i> [3] WZ010	<i>Clostridium beijerinckii</i> [4] NCIMB 8052	<i>Saccharomyces cerevisiae</i> [5]	<i>Enterobacter aerogenes</i> [6] ATCC 15038
	Activity [%]	Activity [%]	Activity [%]	Activity [%]	Activity [%]	Activity [%]	Activity [%]
Reductive reaction							
Acetoin	180	110	196	144	109	–	73
3-Hydroxy-3-methyl-2-butanone	155	–	–	–	–	–	–
Hexane-2,3-dione	59	–	–	–	–	55	–
Diacetyl	100	100	100	100	100	100	100
Glyoxal	–	–	0	–	–	–	trace
Methylglyoxal	97	–	46	–	–	78	11
4-Hydroxy-3-hexanone	87	–	–	–	–	–	–
Pentane-2,3-dione	70	–	17	–	–	103	86
Hexane-3,4-dione	28	–	–	–	–	–	–
Acetone	0	–	–	–	–	0	0
Hexane-2-one	0	–	–	–	–	0	–
Pentane-2,4-dione	–	–	–	–	–	–	trace
Heptane-3-one	0	–	–	–	–	–	–
2-Hydroxy-acetophenone	–	–	–	13	–	0	–
Acetophenone	0	–	–	4	–	–	–
2-Furyl-methylketone	0	–	–	–	–	–	–
Acetaldehyde	0	–	–	–	–	0	0
Benzaldehyde	0	–	–	–	–	–	–
Ethylpyruvate	0	–	–	–	–	5	58
Pyruvate	0	–	–	–	–	0	–
Glyceraldehyde	–	–	0	–	–	–	0
Glyceraldehyde-3-phosphate	–	13	–	–	–	–	–
1,3-Dihydroxy-acetone	–	–	18	–	–	0	–
1-Hydroxy-2-propanone	–	–	40	–	–	–	–
4-Hydroxy-2-butanone	–	–	–	1	–	–	–
Octane-2-one	–	–	–	13	–	0	–
3-Methyl-2-butenal	–	–	–	24	–	–	–
2,2,2-Trifluoroacetophenone	–	–	–	40	–	–	–
3-Acetoxy-2-butanone	–	–	–	–	72	–	–
Butane-2-one	–	–	–	–	2	–	0
Acetol	–	–	–	–	50	–	–
1,2-Cyclo-hexanedione	–	–	–	–	–	86	–
Phenylglyoxal	–	–	–	–	–	143	–
Ethyl 2-oxobutyrate	–	–	–	–	–	15	–
Ethyl 3-oxobutyrate	–	–	–	–	–	3	–
Methyl pyruvate	–	–	–	–	–	–	49
Acetylthylcarbinol	–	–	–	–	–	–	52

probably involved in hydrogen bonding. Although no crystal structure of an (*R,R*)-BDH is available yet, the crystal structure of (*S,S*)-BDH from *Brevibacterium saccharolyticum* and of *meso*-BDH from *Klebsiella pneumoniae* have been solved and the specific binding of either (*S,S*)- or *meso*-butane-2,3-diol in these enzymes was studied (Otagiri et al., 2010). These investigations revealed that the substituent neighboring the reaction center is also tightly bound by hydrogen bonds and that the hydrogen bonding accounts for the selective binding of the two alternative substrates by the dedicated enzymes. In turn, the selectivity of substrate binding in (*R,R*)-BDH appears to be restricted to the reaction site while the enzymes do not discriminate on the configuration of the ligands at the vicinal carbon. This observation suggests that these enzymes are well capable of distinguishing between  $\alpha$ -hydroxy-,  $\alpha$ -keto-, or  $\alpha$ -acetyl-substituted substrates and compounds lacking these structural features but are unable to resolve (*R*)- and (*S*)-configuration at this site. The structural base of this flexible binding mode is an exciting question for the understanding of the action of (*R,R*)-BDHs and will be addressed by crystallographic studies in the future.

#### 4.3. Potential applications of (*R,R*)-butanediol dehydrogenase from *B. clausii* in biotechnology

The results presented herein allow a preliminary evaluation of the suitability of the (*R*)-BDH from *B. clausii* on using this enzyme for biocatalytic, synthetic biological and analytical purposes. BDH-based biosensors for the detection of acetoin and diacetyl in fermentation processes must provide a high selectivity of the enzyme for these compounds to avoid false positive results caused by other fermentation products, in particular by ethanol. The available data suggest that (*R,R*)-BDH from *B. clausii* is not active towards primary or secondary alcohols,  $\alpha$ -hydroxy-fatty acids or their esters and, therefore, might work well in complex fermentation broth. Likewise, the robustness of the enzyme on the miniaturized, silicon-based biosensor chip is important. Increasing the shelf lifetime of the enzyme and investigating its stability towards the conditions employed for enzyme immobilization on the sensor chip surface are the issues to be solved in the future. The electrochemical sensor chip shall allow the simultaneous detection of acetoin and diacetyl (for e.g., beer and wine fermentation processes) and is designed as differential measurement set-up to additionally

**Table 3**  
Relative activities of (*R,R*)-BDHs towards NAD<sup>+</sup>-reducing substrates. All data refer to the activity with (*R,R*)-butane-2,3-diol. Data for enzymes from *P. polymyxa* (Yu et al., 2011), *R. erythropolis* (Yu et al., 2015), *C. beijerinckii* (Raedts et al., 2014), *E. aerogenes* (Carballo et al., 1991) and two *S. cerevisiae* strains (Gonzalez et al., 2000; Heidlás and Tressl, 1990b) were taken from literature and adapted.

Substrate	<i>B. clausii</i> DSM 8716	<i>Paenibacillus polymyxa</i> [1] ATCC 12321	<i>Saccharomyces cerevisiae</i> [2] YAL060W Gene Product	<i>Rhodococcus erythropolis</i> [3] WZ010	<i>Clostridium beijerinckii</i> [4] NCIMB 8052	<i>Saccharomyces cerevisiae</i> [5]	<i>Enterobacter aerogenes</i> [6] ATCC 15038
	Activity [%]	Activity [%]	Activity [%]	Activity [%]	Activity [%]	Activity [%]	Activity [%]
<b>Oxidative Reaction</b>							
( <i>R,R</i> )-Butane-2,3-diol	100	100	100	100	100	–	–
meso-Butane-2,3-diol	94	72	43	–	99	–	–
rac-Butane-2,3-diol	–	–	–	–	80	–	–
Propane-1,2-diol	41	51	17	–	24	–	–
trans-1,2-cyclopentenediol	12	–	–	–	–	–	–
cis-1,2-cyclopentenediol	10	–	–	–	–	–	–
( <i>S,S</i> )-Butane-2,3-diol	1	0	0	0	0	–	–
Ethanol	0	5	0	–	0	–	–
Butane-1-ol	0	6	–	4	–	–	–
Butane-2-ol	–	–	–	7	0	–	–
Propane-2-ol	0	15	–	–	0	–	–
Propane-1-ol	–	5	–	–	0	–	–
Glycerol	0	25	0	23	1	–	–
Ethylene glycol	0	–	–	–	1	–	–
1-Phenylethanol	0	–	–	–	–	–	–
Methanol	0	–	–	–	–	–	–
Dipropylene glycol	0	–	–	–	–	–	–
Propane-1,3-diol	–	5	–	–	0	–	–
Pentane-1,2-diol	–	65	3	–	–	–	–
Pentane-1,5-diol	–	7	–	–	–	–	–
Butane-1,2-diol	–	–	32	–	–	–	–
Butane-1,3-diol	–	–	0	21	–	–	–
( <i>R</i> )-1-Phenyl-1,2-ethanediol	–	–	–	46	–	–	–
Ethyl 4-chloro-3-hydroxybutyrate	–	–	–	17	–	–	–
Sodium lactate	–	–	–	15	–	–	–
Ethyl lactate	–	–	–	7	–	–	–
Isopropanol	–	–	–	5	–	–	–
Cyclohexanol	–	–	–	2	–	–	–
Pentane-2-ol	–	–	–	2	–	–	–
Octane-2-ol	–	–	–	1	–	–	–

eliminate disturbing factors such as possible influences of temperature and pH variations.

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## 2.2 Publikation 2

### **Synthesis of $\alpha$ -hydroxy ketones and vicinal (*R,R*)-diols by *Bacillus clausii* DSM 8716<sup>T</sup> butanediol dehydrogenase**

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#### Zusammenfassung:

Aufbauend auf der ersten Publikation wird die (*R,R*)-2,3-Butandioldehydrogenase aus *B. clausii* DSM 13<sup>T</sup> biokatalytisch weitergehend untersucht. Es wurde der Einfluss von diversen wassermischbaren Lösungsmitteln sowie von Metallionen auf die Enzymaktivität untersucht. Zudem wurde das Substratspektrum im Hinblick auf Moleküle, die einen Phenylrest beinhalten vertieft. Der Hauptfokus lag in der Untersuchung der Reduktion diverser Diketone und  $\alpha$ -Hydroxyketone mit diesem Enzym und der Bestimmung der Stereoselektivität sowie Produktidentifikation.

#### Anteile:

L. Muschallik plante und führte die Experimente durch. D. Molinnus und M. Jablonski arbeiteten an der Entwicklung eines Biosensors als Anwendungsbeispiel, indem dieses Enzym als Schlüsselpart eingesetzt werden kann und trugen mit diesen Arbeiten zur biochemischen Charakterisierung bei. C. Kipp unterstützte in der Durchführung von Biotransformationen zur biokatalytischen Charakterisierung des Enzyms. J. Bongaerts, M. Pohl, T. Wagner, M. J. Schöning und T. Selmer gaben wertvolle fachspezifische Beratung zur Analytik, Biotransformationen, chemische Synthese und mögliche Anwendung in einem Biosensor. P. Siegert führte die konzeptionelle Planung und Betreuung des Projekts durch. Alle Autoren beteiligten sich am Entwurf des Manuskripts.

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## Synthesis of $\alpha$ -hydroxy ketones and vicinal (*R,R*)-diols by *Bacillus clausii* DSM 8716<sup>T</sup> butanediol dehydrogenase<sup>†</sup>

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$\alpha$ -hydroxy ketones (HK) and 1,2-diols are important building blocks for fine chemical synthesis. Here, we describe the *R*-selective 2,3-butanediol dehydrogenase from *B. clausii* DSM 8716<sup>T</sup> (BcBDH) that belongs to the metal-dependent medium chain dehydrogenases/reductases family (MDR) and catalyzes the selective asymmetric reduction of prochiral 1,2-diketones to the corresponding HK and, in some cases, the reduction of the same to the corresponding 1,2-diols. Aliphatic diketones, like 2,3-pentanedione, 2,3-hexanedione, 5-methyl-2,3-hexanedione, 3,4-hexanedione and 2,3-heptanedione are well transformed. In addition, surprisingly alkyl phenyl dicarbonyls, like 2-hydroxy-1-phenylpropan-1-one and phenylglyoxal are accepted, whereas their derivatives with two phenyl groups are not substrates. Supplementation of Mn<sup>2+</sup> (1 mM) increases BcBDH's activity in biotransformations. Furthermore, the biocatalytic reduction of 5-methyl-2,3-hexanedione to mainly 5-methyl-3-hydroxy-2-hexanone with only small amounts of 5-methyl-2-hydroxy-3-hexanone within an enzyme membrane reactor is demonstrated.

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## 1 Introduction

The biocatalytic synthesis of enantiopure  $\alpha$ -hydroxy ketones and vicinal diols is an intriguing field, due to the broad application of these molecules, e.g. as flavouring compounds, pheromones or as precursors for fine chemicals.<sup>1–4</sup> Additionally, the development of efficient syntheses for enantiomerically enriched  $\alpha$ -hydroxy ketones is an important research focus in the pharmaceutical industry. These compounds can be found in antidepressants and fungicides, in selective inhibitors of amyloid protein production (used in the treatment of Alzheimer's disease), in farnesyl transferase inhibitors (Kurasoin A and B), and in antitumor-antibiotics (Olivomycin A and Chromomycin A3 and Taxol).<sup>5,6</sup> Several chemical approaches, like the  $\alpha$ -hydroxylation of carbonyl compounds such as alkenes and ketone enolates, the hydrolytic kinetic resolution of terminal epoxides, or the asymmetric dihydroxylation of olefins are also reported.<sup>7–10</sup> Besides the chemical synthesis, different biocatalytic routes were reported to efficiently produce  $\alpha$ -hydroxy ketones. For example, the use of thiamine diphosphate-dependent lyases (ThDP lyases) to catalyze the carbonylation of

aldehydes,<sup>11–13</sup> hydrolases and lipases produce  $\alpha$ -hydroxy ketones through dynamic kinetic resolutions (DKRs)<sup>14–16</sup> and redox reactions catalyzed by oxidoreductases, either by means of free enzymes (applying a cofactor regeneration system) or by whole cell biotransformations gave  $\alpha$ -hydroxy ketones and 1,2-diols.<sup>17–21</sup>

Butane-2,3-diol-dehydrogenases, also known as acetoin reductases, belong to the class of oxidoreductases (EC 1.1.1.4) and catalyze the enantioselective reduction of diketones with NAD(P)H to the corresponding vicinal diols via an  $\alpha$ -hydroxy ketone intermediate.<sup>22</sup> Even though they are widely known to play a key role in the biological production of 2,3-butanediols,<sup>2,23</sup> their biocatalytic potential is poorly studied. Nevertheless, the enantioselective synthesis of vicinal diols and  $\alpha$ -hydroxy ketones by the butane-2,3-dehydrogenases from *Saccharomyces cerevisiae* and *Serratia marcescens* CECT 977 (ref. 18 and 19) shows the applicability of this enzyme class as biocatalyst.

Here, we report the stereoselective characteristics of our previously described enzyme, the (*R,R*)-butane-2,3-diol dehydrogenase from *Bacillus clausii* DSM 8716<sup>T</sup>,<sup>24</sup> for various diketones and  $\alpha$ -hydroxy ketones besides its physiological substrates acetoin and diacetyl.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Unless stated otherwise, chemicals were analytical grade and purchased from Sigma-Aldrich. (*R*)-1-Hydroxy-1-phenylpropan-

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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 (M. Pohl and D. Rother, Forschungszentrum Jülich; Germany) as described elsewhere.<sup>25,26</sup> 2-Hydroxy-3-methoxy-1-(4-methoxyphenyl)-1-propanone, 1-(2-chlorophenyl)-1-hydroxy-3-methyl-2-butanone and 1-(2-bromophenyl)-1-hydroxy-2-butanone were synthesized in the group of K. Zeidler (University Leipzig; Germany).

Reagents for molecular biology were 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* DH5 $\alpha$ . *Escherichia coli* BL21(DE3) was used for protein production. The Stargate@ pASG.5 vector (pASG-ABC0235n-5; Ap<sup>R</sup>) and vector pET28a-eforRED (Km<sup>R</sup>, this work) were used for gene expression. Plasmid pET28a-eforRED was derived from pET-28a(+) (Novagen) by introducing the *eforRED* gene encoding a red chromoprotein between the start and stop codons of the pET28a multiple cloning site. Additionally, the *eforRED* gene is flanked by *Bsa*I sites allowing golden gate cloning. The *eforRed* gene serves as stuffer DNA that is entirely replaced by the target gene upon cloning. Concurrently, the color of the colony changes from red to white indicating the cloning success. The resulting pET28a derivatives are identical to those that would be obtained by conventional cloning into the *Nco*I and *Xho*I sites of vector pET-28a(+).

## 2.3 Subcloning of BcBDH with N-terminal StrepII-tag

BcBDH production *via* autoinduction required subcloning of the gene to introduce the genetic information for an N-terminal StrepII-tag into a suitable vector. Using primers (BcBDH-fw: aaaGGTCTCccatgGCTAGCGCATGGAGTCATCCTCAATTC; BcBDH-rv: aaaGGTCTCccctcaGCTCCCTTTCTGCGCGCTAAGTTTC) the gene was amplified from plasmid pASG-ABC0235n-5 as a template. The PCR product was then introduced into vector pET28a-eforRED by golden gate cloning resulting in plasmid pET28a-BcBDH5'. Gene sequencing verified no changes compared to the template plasmid construct.

## 2.4 Determination of protein concentration

Protein concentration throughout enzyme purification were determined using the Bradford method and bovine serum albumin (BSA) as a standard.<sup>27</sup>

## 2.5 Production and purification of recombinant BcBDH in *E. coli*

Heterologous expression of the *bdhA* gene, encoding the (*R,R*)-butane-2,3-diol-dehydrogenase from *B. clausii* DSM 8716<sup>T</sup>, in *E. coli* BL21(DE3) and purification to homogeneity by affinity chromatography *via* strep-tag using Strep-Tactin® macroprep columns was done as described before,<sup>24</sup> this gene was expressed by autoinduction in shake flasks using the pET28a-

based expression vector. Therefore, cells were grown at 30 °C at 180 rpm in autoinduction medium for 24 h.<sup>28,29</sup> Harvesting and cell disruption was done as described before.<sup>24</sup> To achieve a higher biomass, the production of BcBDH in *E. coli* BL21(DE3) was carried out in 2 l-scale cultivation processes by autoinduction. Cells were grown in a defined autoinduction medium<sup>30</sup> at 37 °C and 500 rpm till the diauxic shift was detectable. Then, temperature was reduced to 22 °C continuing the cultivation for further 12 h. Success of purification was controlled by SDS-PAGE and carried out according to Laemmli<sup>31</sup> using 12% polyacrylamide gels and Roti-Mark PRESTAINED ladder as standard.<sup>24</sup>

## 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 Ultraspec 2100 Pro, Amersham). All reactions were performed at 30 °C.<sup>24</sup>

The reduction reactions were determined in MES-NaOH (2-(*N*-morpholino)ethane sulfonic acid) buffer (50 mM, pH 6.8) with acetoin (10 mM) and NADH (0.3 mM) as substrates. The reaction was initiated by addition of an appropriately diluted sample of purified BcBDH. A correction was made by measuring a control without enzyme. Variability is expressed as standard deviation.

One unit of BcBDH is defined as the amount of enzyme that oxidizes one  $\mu$ mol of NADH per minute at 30 °C under the given conditions, respectively.

## 2.7 Substrate spectrum

Unless stated otherwise, the activities toward various potential substrates were tested using standard reduction assays (Section 2.6) with 10 mM of potential substrates (individual compounds are given in the Result section). For hydrophobic compounds a final concentration of 5% dimethyl sulfoxide (DMSO) was used as a solubilizer.

## 2.8 Effect of organic solvents on the activity of BcBDH

The stability of the purified enzyme toward organic co-solvents was tested with or without the addition of organic co-solvents (v/v), respectively (individual compounds are given in the Result section) in Tris-HCl buffer (10 mM; pH 7.4) supplemented with NaCl (150 mM). Residual activities were measured after 1 h incubation at 22 °C using the acetoin reduction assay (see Section 2.6).

## 2.9 Effect of metal ions and EDTA on the activity of BcBDH

The effect of different metal ions on BcBDH activity was determined by adding a final concentration of 1 mM of different metal salts (CoCl<sub>2</sub>·6H<sub>2</sub>O, FeCl<sub>2</sub>, NiSO<sub>4</sub>·6H<sub>2</sub>O, MgCl<sub>2</sub>, MnCl<sub>2</sub>, KCl and ZnSO<sub>4</sub>) to the enzyme preparation without any further pretreatment. The activity without supplement was defined as 100%. In order to remove divalent metal ions from BcBDH 1 mM EDTA (ethylenediaminetetraacetic acid) was added. After incubation for 1 h at 22 °C the residual activity was determined using the standard reduction assay as described above.



To elucidate the effect of metal ion supplementation over a longer period of time, BcBDH was incubated for 25 hours at 22 °C in its storage buffer Tris-HCl (10 mM; pH 7.4) with NaCl (150 mM), supplemented with MnCl<sub>2</sub> and ZnSO<sub>4</sub> (1 mM each), respectively. The reference was prepared in the same way without metal ion addition. Activity was measured after 1, 2, 4, and 25 hours with the standard photometric assay. The activity without supplement was defined as 100%.

### 2.10 Stereoselectivity of BcBDH by GC

Stereoselectivity and conversion was analyzed by carrying out the reduction reaction of selected diketones and  $\alpha$ -hydroxy ketones (see Section 3.5). 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 BcBDH (1 U ml<sup>-1</sup> of the corresponding substrate), FDH (5 U ml<sup>-1</sup>), sodium formate (30 mM) and NADH (0.3 mM) in MES-NaOH buffer (50 mM; pH 6.8). Samples (100  $\mu$ l) were taken at different points in time during the reaction, extracted with diethyl ether (300  $\mu$ l) and applied to GC analysis (GC-2010 Plus (Shimadzu) with a flame ionization detector) equipped with a Hydrodex  $\gamma$ -DIMON (25 m  $\times$  0.25 mm ID Macherey & Nagel) column. The following temperature profile was used: 45 °C (2 min), 45–70 °C (at 2 °C min<sup>-1</sup>); 70–180 °C (at 10 °C min<sup>-1</sup>); 180 °C (10 min). For benzoin analysis the last step was extended to 180 °C (60 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; methylglyoxal (2-oxopropanal) 2.3 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, benzoin 43.0 min.

Retention times of the products were (as standards purchased from Sigma-Aldrich or BcBDH synthesized and identified as shown in Section 3.5): (*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; 3-hydroxy-2-pentanone 16.4 min; 2,3-pentanediol 19.8 min; (*R*)-3-hydroxy-2-hexanone 19.3 min; (*R*)-2-hydroxy-3-hexanone 19.4 min; (*R*)-3,4-hexanediol 21.0 min; (*R*)-5-methyl-2-hydroxy-3-hexanone 19.7 min; (*R*)-5-methyl-3-hydroxy-2-hexanone 19.9 min; 2-methyl-2,3-butanediol 17.8 min; 3,4-hexanediol 21.0 min; (*R,S*)-1-phenyl-1,2-propanediol 27.2 min; (*R,R*)-phenyl-1,2-propanediol 27.0 min; (*S,S*)-phenyl-1,2-propanediol 26.9 min.

### 2.11 Chemical synthesis of 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<sub>4</sub>, 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<sub>2</sub>O the reaction was stirred for another 10 min. The reaction products were extracted with diethyl ether, neutralized, dried with MgSO<sub>4</sub> (anhydrous), filtrated and the solvent was removed in vacuum.<sup>18</sup>

### 2.12 Preparative biocatalytic synthesis

Preparative biotransformations of 2,3-pentanedione, 2,3-hexanedione, 3,4-hexanedione and 5-methyl-2,3-hexanedione were carried out in a volume of 20 ml. The reaction mixture consisted of diketone (200 mM), BcBDH (0.5 U ml<sup>-1</sup>), formate dehydrogenase (FDH) for cofactor regeneration (0.8 U ml<sup>-1</sup>), sodium formate (600 mM), NADH (0.3 mM) and 10% methanol in MES-NaOH buffer (50 mM; pH 6.8). The reaction was incubated at 30 °C for 48 hours under gentle stirring. The products were extracted with diethyl ether, dried with MgSO<sub>4</sub> (anhydrous), filtrated and the solvent removed by distillation and analysed by GC-MS.

The BcBDH reduction product of 5-methyl-2,3-hexanedione was additionally analysed by <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR; NMR spectrometer Spinsolve 60 carbon (60 MHz) (Magritek)). The sample was solved in 1 ml CDCl<sub>3</sub>. <sup>1</sup>H-NMR (CDCl<sub>3</sub>)  $\delta$  0.90 (d, 3H), 1.00 (d, 4H), 1.43 (ddd, 2H), 1.95 (m, 1H), 2.17 (s, 4H), 3.20 (s, 2H) 4.18 (m, 1H).

### 2.13 GC-MS analysis

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

### 2.14 Circular dichroism

Circular dichroism (CD) spectroscopy of different products was performed using a Jasco J-1100 spectrometer with a 1 mm optical path length cell. The extracted biocatalytic products were appropriately diluted in ethanol. The ellipticity readout were converted to molar ellipticity units with the following eqn (1):<sup>32</sup>

$$[\theta] = \frac{100 \times \theta}{c \times l} \quad (1)$$

Here,  $[\theta]$  = molar ellipticity in deg  $\times$  cm<sup>2</sup>  $\times$  dmol<sup>-1</sup>;  $c$  = concentration of an optical active compound in M;  $l$  = cuvette optical path length in cm.

### 2.15 Synthesis of (*R*)-5-methyl-3-hydroxy-2-hexanone with an enzyme membrane reactor

Biotransformation with the enzyme membrane reactor were carried out using a stainless steel reactor with a volume of 10 ml with an internal magnetic stirrer, coupled with a thermostat for temperature control, a bubble trap, a sterile filter, a 10 kDa ultrafiltration membrane (Ultrasel® Merck; regenerated cellulose), a HPLC pump and a sampler. The reaction mixture consisted of: 5-methyl-2,3-hexanedione (100 mM), BcBDH (20 U), FDH (60 U), formate (300 mM), NADH (0.3 mM)



and 5% methanol in MES–NaOH buffer (50 mM; pH 6.8) in a volume of 300 ml. The flow rate was set to 0.2 ml min<sup>-1</sup>. The reactor outflow was fractionated (10 ml samples) and analysed by GC.

### 3 Results and discussion

#### 3.1 Cloning and expression of the (*R,R*)-butane-2,3-diol-dehydrogenase
























The cloning of the (*R,R*)-butane-2,3-diol dehydrogenase gene from *B. clausii* DSM 8716<sup>T</sup> (BcBDH) in a pASG vector was described in our previous publication.<sup>24</sup> In addition, the BcBDH gene with N-terminal StreptII-tag was cloned into a pET28a expression vector allowing expression by autoinduction. By using this system, no differences in BcBDH expression in shake flasks and 2 l fermentations were observed, but a higher biomass production and therefore more enzyme was achieved. After affinity chromatography purification, the enzyme was essentially pure with a specific activity of 70–100 U mg<sup>-1</sup>.<sup>24</sup>

#### 3.2 Extended substrate specificity of BcBDH

As reported in our previous publication BcBDH has the ability to reduce  $\alpha$ -hydroxy ketones, vicinal diketones, and hydroxyaldehydes, only<sup>24</sup> (Table 1; 1–9). Besides its natural substrates diacetyl and acetoin, BcBDH accepts several non-physiological molecules as substrates and is also able to catalyze the oxidation reaction, but solely of vicinal diols to the corresponding  $\alpha$ -hydroxy ketones. No oxidation of primary or secondary alcohols could be detected.

In this study we further elucidated the substrate scope of the enzyme. Additional molecules with a branched aliphatic structure or with a phenyl moiety were probed (7, 10–23). Activity tests were carried out as described before (10 mM substrate; 50 mM MES–NaOH; 0.3 mM NADH; pH 6.8 at 30 °C). Surprisingly the enzyme also exhibited some remarkable activity for some substrates which contain a phenyl moiety or a bulky aliphatic residue: 2,3-heptanedione (10), 5-methyl-2,3-hexanedione (7), (*R*)-HPP (13), (*S*)-HPP (14) 1-phenyl-1,2-propanedione (21), phenylglyoxal (15), 2-hydroxy-3-methoxy-1-(4-methoxyphenyl)-1-propanone (16), 1,2-cyclohexanedione (10). The compounds 3,5-dimethyl-1,2-cyclopentanedione (11), benzil (17), benzoin (18), 1-(2-chlorophenyl)-1-hydroxy-3-methyl-2-butanone (19), 1-(2-bromophenyl)-1-hydroxy-2-butanone (20) are no substrates of BcBDH. Surprisingly under these conditions the activity of BcBDH toward (*R*)-2-hydroxy-1-phenylpropan-1-one ((*R*)-HPP), a substrate with a phenyl moiety, is much higher than toward its natural substrate racemic acetoin. (*S*)-2-hydroxy-1-phenylpropan-1-one ((*S*)-HPP) is converted with 111.5% activity compared to acetoin. On the contrary no activity for both enantiomers of 1-hydroxy-1-phenylpropan-2-one (PAC; 22, 23) was observed (Table 1). This is the first time a (*R*)-butane-2,3-diol dehydrogenase is described to accept these aromatic molecules as substrates. In contrast to that, substrates with two phenyl moieties like benzil and benzoin, are not converted. Of particular interest is that substrates with at least one keto group next to the phenyl moiety, like HPP (13/14) or 2-hydroxy-3-methoxy-1-(4-methoxyphenyl)-1-

**Table 1** Substrates used for activity studies. Enzyme activities were measured with the standard reduction assay and compared to acetoin. 100% = 70–100 U mg<sup>-1</sup>

Substrate	Activity [%] (photometric assay)	Substrate	Activity [%] (photometric assay)
	100		0
	86.4 ± 3.1		0
	59.2 ± 3.4		194.6 ± 8.7
	55.7 ± 0.9		111.5 ± 4.9
	54.0 ± 5		46.8 ± 3.5
	48.5 ± 3.3		7.1 ± 0.4
	44.3 ± 2.3		0
	38.9 ± 2.1		0
	15.6 ± 0.6		0
	93 ± 9		0
			52.4 ± 5.3
			0
			0

propanone (16), are accepted, but not substrates with an external keto group (e.g. PAC (22), 1-(2-chlorophenyl)-1-hydroxy-3-methyl-2-butanone (19), 1-(2-bromophenyl)-1-hydroxy-2-butanone (20). The activity of BcBDH with both HPP enantiomers but not with the PAC enantiomers underlines the suggestion that this enzyme prefers  $\alpha$ -hydroxy ketones with the ketone group adjacent to a phenyl ring. While the reduction of “bulky” diketones or  $\alpha$ -hydroxy ketones is new for (*R*)-butane-2,3-diol dehydrogenases, there are already several oxidoreductases reported for the reduction of “bulky” aliphatic and aromatic  $\alpha$ -hydroxy ketones, diketones and ketones.<sup>33–36</sup>

#### 3.3 Influence of metal salts and EDTA on BcBDH activity

The enzyme BcBDH belongs to the superfamily of medium-chain dehydrogenases/reductases (MDR). Butane-2,3-diol dehydrogenases/acetoin reductases of that superfamily are mostly Zn<sup>2+</sup>-dependent and need at least one catalytical zinc



ion, bound to the highly conserved catalytic amino acids Cys<sub>37</sub>-His<sub>70</sub>-Glu<sub>71</sub> (BcBDH numbering). Additionally, some enzymes of that superfamily bind a structurally relevant zinc ion.<sup>37,38</sup> For that reason, we investigated the influence of Zn<sup>2+</sup> and other bivalent metal ions on BcBDH activity. Purified BcBDH without further pretreatment EDTA (note that the purification protocol is without addition of ZnCl<sub>2</sub>) incubated with 1 mM of the respective metal salts (22 °C, Tris-HCl buffer, 10 mM; pH 7.4) and the residual activity (standard reduction assay) was determined after one hour. As shown in Fig. 1 the metal ions strongly influenced the enzyme's activity. A loss of activity was detected with NiSO<sub>4</sub> (65%), with CuSO<sub>4</sub> (87%) and with FeCl<sub>2</sub> (100%). In contrast to that, KCl and MgCl<sub>2</sub> showed no effect. Most interestingly, addition of ZnSO<sub>4</sub> only slightly increased BcBDH activity (116%), whereas the addition of cobalt ions (CoCl<sub>2</sub>: 122%) and manganese(II)-ions (MnCl<sub>2</sub>: 217%) strongly activated BcBDH. As expected, the incubation of this enzyme with the chelator EDTA led to an activity loss (50%). By using EDTA for chelating the Zn<sup>2+</sup> ions a complete inactivation of this enzyme was not possible under the applied conditions. Additionally, no activity loss of this enzyme during purification without Zn<sup>2+</sup> ions was determined.

Motivated by the observed activation we further investigated the effect of manganese(II) and cobalt(II) ions on BcBDH for biocatalytic purposes. To elucidate the kinetics of that effect BcBDH (without any further pretreatment) was supplemented with 1 mM MnCl<sub>2</sub> and CoCl<sub>2</sub>, respectively, and the activity was compared to a reference sample for 25 hours (22 °C). BcBDH without any supplement (reference sample) rapidly lost activity over time. After 25 hours the residual activity was only 46% of the initial value. In contrast to that, BcBDH supplemented with 1 mM CoCl<sub>2</sub> or MnCl<sub>2</sub> showed an increase in activity over time. The supplementation with Co<sup>2+</sup> ions led to an activity increase of 174%, but the addition of Mn<sup>2+</sup> ions more than doubled the activity (245%) after 25 hours. Supplementation with Zn<sup>2+</sup> ions

showed only a slight activity increase after the first hour (130%) and resulted into an enhanced stability of this enzyme but no further activation with an activity of 100% after 25 hours (Fig. 2). For oxidation of (*R,R*)-2,3-butanediol and *meso*-butanediol (10 mM each; 0.3 mM NAD<sup>+</sup>; 50 mM Tris-HCl pH 8.0; 30 °C) the same effect was observed which resulted in a 3-fold higher enzyme activity and an enhanced stability (data not shown). (*S*)-Acetoin is still not a substrate of Mn<sup>2+</sup> "substituted" BcBDH.

Moreover, we investigated the influence of Zn<sup>2+</sup> and Mn<sup>2+</sup> ion addition on biocatalysis. The enzyme was preincubated with Zn<sup>2+</sup> and Mn<sup>2+</sup> ions, respectively, as described in Section 2.9. The reference was incubated with addition of the same amount of buffer without metal salts to maintain the same enzyme concentration. The same volumes of untreated (reference) BcBDH and of metal ion preincubated enzyme were used. The activity enhancement of Mn<sup>2+</sup>-supplemented BcBDH could also be observed in biotransformation experiments leading to a higher conversion rate. Whereas a total conversion of racemic acetoin (10 mM, pH 6.8, 30 °C, untreated BcBDH 0.5 U ml<sup>-1</sup>) to (*R,R*)-BD without metal ion addition could be achieved within 40 minutes, conversion BcBDH supplemented with 1 mM Mn<sup>2+</sup> was already completed after 10 minutes. The supplementation of Zn<sup>2+</sup> ions led to no effect in comparison to the reference. The increase of activity, by Mn<sup>2+</sup> and other bivalent transition metal ions, was already reported for other dehydrogenases (e.g. glycerol dehydrogenase, alcohol dehydrogenase from *Thermoanaerobacter brockii*) but is new for BDHs.<sup>39-41</sup> Another study showed that the substitution of the zinc-dependent *Thermoanaerobacter brockii* ADH with Co<sup>2+</sup> leads to an oxidation of the ion while forming an octahedral (instead of a tetrahedral like Zn<sup>2+</sup>) structure complex at the active site. The higher valence of the metal ion may contribute to enhanced substrate binding and reaction intermediate stabilization.<sup>42</sup> Furthermore, it could be shown that the substitution of the catalytic zinc ion in yeast ADH by cobalt or copper led to an enhancement of some biochemical properties, like thermo stability and pH stability.<sup>43,44</sup> The results shown here indicate that metal ion substitutions could be applied to improve the catalytic properties of BcBDH. Amino acid sequence analysis revealed no

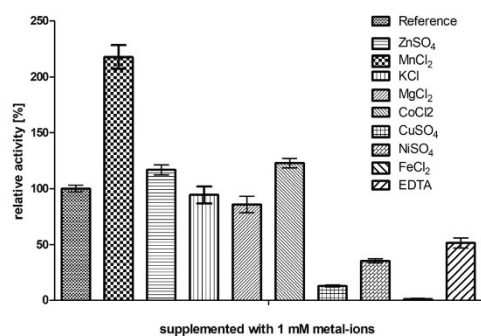


Fig. 1 Influence of several metal ions and the chelator EDTA on the enzyme activity. BcBDH was incubated in Tris-HCl buffer (10 mM; pH 7.4) with NaCl (150 mM) supplemented with several metal salts and EDTA (1 mM) for 1 hour at 22 °C. Activity was measured with the standard photometric assay. Reference was treated the same way without any addition.

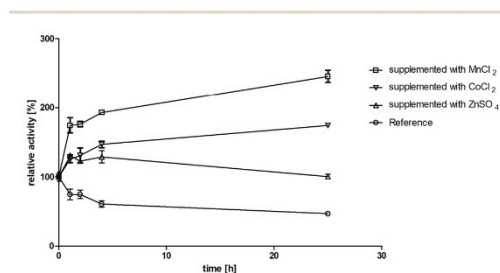


Fig. 2 Effect of MnCl<sub>2</sub>, CoCl<sub>2</sub> and ZnSO<sub>4</sub> (1 mM each) on incubation in Tris-HCl buffer (10 mM; pH 7.4, 150 mM NaCl) for 25 hours at 22 °C. Activity was measured after 1, 2, 4, 25 hours with the standard photometric assay. Reference was treated the same without metal ion addition.

additional binding motives for  $Mn^{2+}$  ions which could probably explain the activation. We suggest that the catalytic zinc ion is substituted over time by the  $Mn^{2+}$  ion. However, this is an intriguing effect and more research is needed to gain a deeper mechanistic insight.

Mutagenesis experiments involving the catalytic amino acids C37, H70 and E151 (C37A, H, S; H70A, C, Q; E151A, C, Q), which are crucial for binding the catalytic zinc ion, resulted in the loss of expression or in inactive enzyme variants (data not shown). Same results were published for the alcohol dehydrogenase of *Thermoanaerobacter brockii*.<sup>41</sup> Here, the mutagenesis of the catalytic amino acids led to inactive variants, too.

### 3.4 Effect of organic solvents on BcBDH activity

Residual activity (standard assay) was tested after addition of 0 to 60% (v/v) of different solvents after 1 hour of incubation (22 °C; Tris-HCl buffer, 10 mM; pH 7.4 with NaCl, 150 mM). The following organic solvents were tested: dimethyl sulfoxide (DMSO), acetone, methanol, ethanol, and acetonitrile (Fig. 3). It turned out that this enzyme shows its highest tolerance toward DMSO. Addition of up to 60% (v/v) DMSO resulted in a residual activity of 53% after incubation. In contrast, BcBDH seems to be quite sensitive toward acetonitrile and ethanol. A nearly complete loss of activity after the addition of 30% (v/v) of these organic solvents was determined. Organic solvent resistant enzymes are preferred for practical biocatalytic purposes. Until now, butanediol dehydrogenases are not well studied in that respect. The (*R,R*)-2,3-butanediol dehydrogenase from *Rhodococcus erythropolis* WZ010, for example, showed a good resistance against DMSO with a relative activity of 84% by adding 30% (v/v) DMSO to the assay (but without pre-incubation), but loses much activity at higher concentrations.<sup>45</sup> On the other hand, there are alcohol dehydrogenases, e.g. from *Paracoccus pantotrophus* DSM 11072 (up to 50% (v/v) DMSO) and *Rhodococcus ruber* DSM 44541 (e.g. up to 50% (v/v) in acetone and 80% (v/v) in 2-propanol) reported, which perform extremely well in aqueous/organic solvents or even micro aqueous-systems.<sup>46,47</sup>

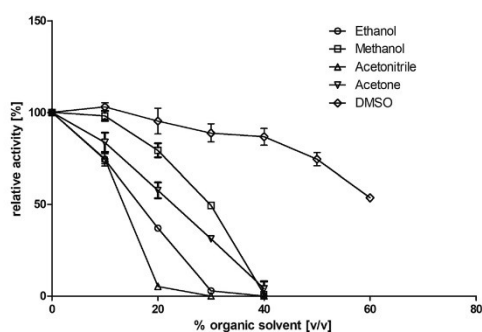


Fig. 3 Influence of several water miscible organic solvents on enzyme activity. BcBDH was incubated for 1 hour (22 °C) with 0 to 60% (v/v) solvent in Tris-HCl buffer (10 mM; pH 7.4) with NaCl (150 mM). Activity was measured with the standard photometric assay.

For the biocatalysis of hydrophobic substrates we decided to use methanol as a solubilizer, because of its low boiling point and therefore easier evaporation from preparative-scale biocatalytic samples, and the enzyme's good tolerance toward this solvent.

### 3.5 Biotransformations

The stereoselective synthesis of  $\alpha$ -hydroxy ketones and vicinal diols is an intriguing field, because of the broad applications of these substances. They can be used either as natural flavours, pheromones, or as building blocks in organic syntheses.<sup>3,4,48</sup> Often, it is beneficial to synthesize these molecules through biocatalysis, because of the helpful properties of enzymes (e.g. regio- and stereoselectivity) as biocatalysts. The butanediol dehydrogenase from *B. clausii* is (*R*)-selective toward its physiological substrate diacetyl, which leads to the vicinal diol (*R,R*)-2,3-butanediol via (*R*)-acetoin. Racemic acetoin is converted to *meso*- and (*R,R*)-2,3-butanediol by BcBDH. Oxidation of (*R,R*)-2,3-butanediol and *meso*-butanediol lead to (*R*)- and (*S*)-acetoin, respectively.<sup>24</sup> Acetoin is not a substrate of BcBDH with respect to the oxidation reaction. Nevertheless, there is still the unsolved question whether this enzyme exhibits the same stereoselectivity for its unphysiological substrates, which we investigated with the following experiments. The reduction of vicinal diones and hydroxy ketones was performed in small batch experiments with 10 mM substrate.

Starting from a diketone a dehydrogenase catalyzed reduction may lead to up to four  $\alpha$ -hydroxyketone intermediates and subsequently to up to four diols (or three  $\alpha$ -hydroxy ketones and diols if *meso*-forms are possible). To elucidate that question we investigated the selectivity of BcBDH for various  $\alpha$ -diketones and  $\alpha$ -hydroxy ketones. In the case of the molecules 2, 3, 6, 7, 10, 13, 14, 15 a total conversion after one hour could be achieved. For molecules 8, 9 and 21 a conversion of 90%, 88% and 97% after one-hour reaction time was achieved. No conversion of 2-hydroxy-3-methoxy-1-(4-methoxyphenyl)-1-propanone (16; 5% methanol (v/v)) could be detected. Although, a low activity for this substrate in the photometric assay was found. Probably, the addition of methanol as solubilizer led to a further decrease of the enzyme's activity for this already poorly accepted molecule. Even though no activity was detected for benzoin (5% DMSO (v/v)) in the photometric assay, biotransformation was performed to see whether some conversion could be detected over a prolonged period of time. However, no conversion took place.

Furthermore, this enzyme revealed a high selectivity for several substrates 2, 9, 10, 13, 14, which resulted in the formation of only one reduction product. In the batch reactions of 3 and 8 traces of a second product were present and in the case of 7 also a third product was detectable in traces. Reduction of phenylglyoxal (15) resulted in the formation of three products. The reduction of racemic 6 led to the generation of two diol products, of which one has the same retention time in GC, like the diol generated by the reduction of 3,4-hexanedione. The product formed by the reduction of substrate 13 could be identified as (*R,R*)-phenyl-1,2-propanediol by the use of a respective GC analysis (compared to standard). The retention

time of the product formed by the reduction of compound **14** could neither be matched with the retention times of (*R,R*)-phenyl-1,2-propanediol nor (*S,S*)-phenyl-1,2-propanediol. Therefore, we presume the formation of (*R,S*)-phenyl-1,2-propanediol.





















The reduction of **21** led to the generation of mainly (*R*)-1-hydroxy-1-phenylpropan-2-one ((*R*)-PAC) with (*S*)-1-hydroxy-1-phenylpropan-2-one ((*S*)-PAC) as a side product (ee: 86%).

Besides these three molecules no suitable references of the potentially produced diols of the here tried molecules were obtainable. To overcome this limitation, we reduced the diketones with NaBH<sub>4</sub> to obtain the racemic diols as a reference. To further investigate the stereoselectivity of the enzyme for aliphatic molecules, we focused on biotransformations of compounds **3**, **7**, **8**, **9**, **10**, because of the good performance BcBDH demonstrated for these substrates and in order to use a variety of aliphatic (symmetric, asymmetric, branched) molecules. The retention times of the chemically synthesized diols were compared to the products of the biotransformations. Combining this with the GC-MS analysis, we could verify that asymmetric molecules like **8**, **3**, **7**, are surprisingly reduced to the corresponding  $\alpha$ -hydroxy ketones (see ESI†). By reduction of **8** we obtained 3-hydroxy-2-pentanone. Reduction of molecule **3** resulted in of 3-hydroxy-2-hexanone with small amounts of 2-hydroxy-3-hexanone. Reduction of **10** gave solely the 3-hydroxy-2-heptanone, and reduction of **7** resulted mainly in 5-methyl-3-hydroxy-2-hexanone with small amounts of 5-methyl-2-hydroxy-3-hexanone and traces of its isomer of 5-methyl-3-hydroxy-2-hexanone (Table 2 and ESI†) was detected. No further reduction of molecules **3**, **7**, **10** to the corresponding diols was observed (within the reaction time of one hour and the chosen conditions). Only for substrate **8**, with the smallest aliphatic chain of the tested molecules, traces of the diol were detected, too. On the other hand, the reduction of the symmetric molecule **9** resulted in the generation of only one 3,4-hexanediol, but no  $\alpha$ -hydroxy ketone intermediate was detectable over the reaction time (Table 2). It seems the enzyme is able to distinguish between symmetric and asymmetric molecules, which induces the generation either of a diol, or in the latter case, of an  $\alpha$ -hydroxy ketone.

At last, the identification of the absolute configuration of the obtained products was investigated. Therefore, the products from the reduction of **3**, **9**, **7** were produced in preparative-scale biotransformations (200 mM; 48 h; 30 °C; 20 ml), extracted with diethyl ether and after solvent evaporation applied to circular dichroism spectroscopy. Preparative biotransformations of substrate **8** gave the mixed production of 3-hydroxy-2-pentanone as the main product and a small amount of the corresponding diol and was for that reason not further analyzed by CD spectroscopy.

The obtained spectra (Fig. 5) were compared with the CD spectrum of acetoin, which has the closest similar structure. As a result, the produced  $\alpha$ -hydroxy ketones could be identified as the (*R*)-enantiomers, because our produced products show the same maximum negative peak at 280 nm as (*R*)-acetoin in CD measurements.<sup>32</sup> Thus, BcBDH shows the same stereoselectivity for the chosen substrates as for its natural substrate.

**Table 2** Biocatalytic conversion of  $\alpha$ -diketones and  $\alpha$ -hydroxy ketones substrates by (*R,R*)-BDH of *B. clausii* and comparison to the specific activity toward reduction of acetoin. Substrates (10 mM) were reduced by 1 U ml<sup>-1</sup> BDH using formate (30 mM) and formate dehydrogenase (5 U ml<sup>-1</sup>) to regenerate NADH (0.3 mM) at 30 °C in MES-NaOH buffer (50 mM, pH 6.8; 1 ml, 60 min). Data for acetoin and diacetyl data were taken from Muschallik *et al.*<sup>24</sup>

Substrate	Biocatalytic conversion [%]	Identified products	ee/de [%]
	99	 main product	11
	99		99
	90	 main product	—
	99	 main product	89
	88		99
	99	 main product	97
	99		99
	99		99
	99		99
	97	 main product	86

Surprisingly, the compound 3,4-hexanediol generated with this enzyme gave no CD spectrum. Therefore, we compared the product 3,4-hexanediol obtained by BcBDH with reduction products of the *meso*-BDH from *Bacillus licheniformis* (unpublished data). The reduction of racemic 4-hydroxy-3-hexanone with the *meso*-BDH from *B. licheniformis* led to the synthesis of (*S,S*)-3,4-hexanediol and *meso*-3,4-hexanediol. GC analysis revealed different retention times of the diols produced by both BDHs. Because of that we conclude that BcBDH catalyzes the synthesis the (*R,R*)-3,4-hexanediol.

This enzyme showed the same reduction pattern like the previous reported butanediol dehydrogenase BudC from *Serratia marcescens* CECT 977.<sup>19</sup> The only difference is the conformation of the introduced stereo center. Given that BudC



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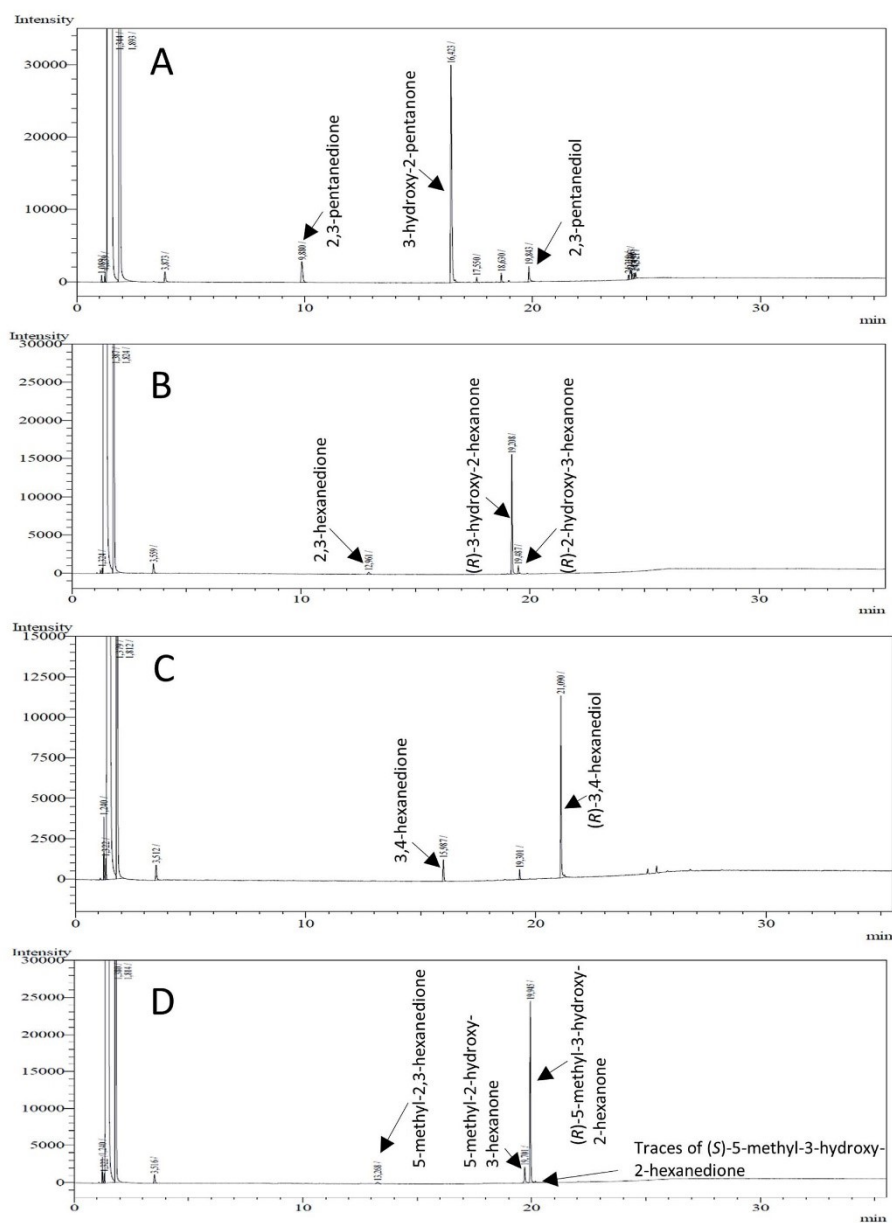


Fig. 4 Gas chromatograms of the formation of  $\alpha$ -hydroxy ketones and vicinal diols from  $\alpha$ -diketones by (*R,R*)-BDH of *B. clausii*. Substrates (10 mM each) were reduced by 1 U BDH (activity measured for each specified substrate) using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in MES–NaOH buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min. Biocatalytic reduction of (A) 2,3-pentanedione; (B) 2,3-hexanedione; (C) 3,4-hexanedione; (D) 5-methyl-2,3-hexanedione.

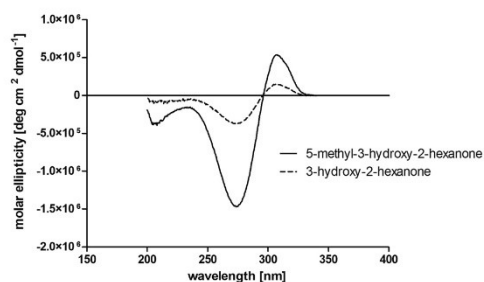


Fig. 5 Near UV circular dichroism measurements of the products 5-methyl-3-hydroxy-2-hexanone and 3-hydroxy-2-hexanone obtained by BcBDH catalyzed reduction of 5-methyl-2,3-hexanedione (7) and 2,3-hexanedione (3).

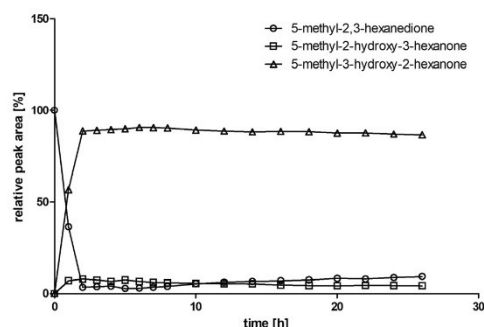


Fig. 6 Relative peak area over time for the conversion of 100 mM 5-methyl-2,3-hexanedione with 20 U BcBDH, 0.3 mM NADH, 60 U FDH, 300 mM formate in an enzyme membrane reactor system (10 ml, flow rate 0.2 ml min<sup>-1</sup>) over 26 hours.

belongs to the SDR superfamily it generates an *S*-configured stereocenter. Whereas BcBDH is a member of the MDR superfamily and introduces an *R*-configured stereocenter. In contrast to that, the reduction of 2,3-pentanediol, 2,3-hexanediol and 3,4-hexanediol with the butanediol dehydrogenase from *Saccharomyces cerevisiae* led exclusively to the corresponding *R*-configured diols.<sup>18</sup>

In conclusion, BcBDH is able to distinguish between asymmetric and symmetric substrate molecules. This leads to a stop of the reduction reaction either at the  $\alpha$ -hydroxy ketone intermediate, (in case of asymmetric substrates, like 2,3-hexanedione or 5-methyl-2,3-hexanedione) or the diol (in case of symmetric substrates, like diacetyl or 3,4-hexanedione), respectively. Important to mention is that the keto group next to the phenyl moiety is essential for the reaction to take place. Molecules with such an keto group configuration like, **13**, **16**, are reduced. In contrast to that PAC and derivatives such as **19**, **20** are not accepted by BcBDH (see Section 3.2). We also demonstrated that this enzyme exhibits the same (*R*)-stereoselectivity for unphysiological substrates, like for its natural substrates.

### 3.6 Preparative production of (*R*)-3-hydroxy-2-hexanone using an enzyme membrane reactor

Some  $\alpha$ -hydroxy ketones can act as flavouring compounds or as pheromones for insects.<sup>14,47,49</sup> Furthermore, the biocatalytic reduction of 2,3-octanedione by resting cells of *S. cerevisiae* and *B. subtilis* could already be demonstrated, yielding the  $\alpha$ -hydroxy ketone intermediates that can act as pheromones.<sup>50</sup> Here, we choose 5-methyl-2,3-hexanedione (**7**) as a substrate. The corresponding  $\alpha$ -hydroxyketone intermediates may act as flavouring compounds.<sup>51</sup>

Since BcBDH catalyzes the stereoselective synthesis of these compounds, we investigated a continuous stirred tank reactor setup for the conversion of larger volumes. An enzyme membrane reactor (EMR) was applied for this first preparative BcBDH catalyzed biotransformation setup (volume: 300 ml; 5-methyl-2,3-hexanedione: 100 mM). To enhance the solubility of the substrate 5% (v/v) methanol was used, although this was not

sufficient to dissolve the substrate completely. But higher methanol concentrations were deleterious for the enzyme. The EMR was operated with 20 U BcBDH, a flow rate of 0.2 ml min<sup>-1</sup> at 30 °C with gentle stirring (50 rpm) over 26 hours. The reactor outflow was fractionated (10 ml sample) and analyzed by GC (cofactor regeneration see Section 2.15). After an equilibration phase of 2 hours, a conversion of 96% could be achieved, yielding (*R*)-5-methyl-3-hydroxy-2-hexanone as the main product (88% relative peak area) and small amounts of 5-methyl-2-hydroxy-3-hexanone (7% relative peak area). After 26 hours, the conversion declined to 90% (Fig. 6). All in all, a volume of 260 ml substrate solution (30 mmol substrate) was converted which equates to 4 g product.

This experiment is a proof of principle for the application of BcBDH as a biocatalyst in a reactor setup. Further optimization of this system is envisaged.

## 4 Conclusions

We investigated the butanediol dehydrogenase BcBDH from *B. clausii* DSM 8716<sup>T</sup> for its biocatalytic applicability. BcBDH shows a high stereoselectivity for several  $\alpha$ -diketones in addition to its physiological substrates acetoin and diacetyl. Depending on the symmetry of a potential substrate and the length of the aliphatic chain, it is possible to generate selectively the  $\alpha$ -hydroxy ketone intermediate or one diol with high stereoselectivity (Table 2). Further, BcBDH accepts molecules with a phenyl moiety as a substrate and reduces these molecules stereoselectively. Surprisingly, this enzyme shows nearly double the activity (U mg<sup>-1</sup> standard assay) for (*R*)-2-hydroxy-1-phenylpropan-1-one ((*R*)-HPP) compared to acetoin. Moreover, it also reveals good activity for the other here tested "bulky" substrates, which is appealing for the application of this enzyme as a biocatalyst. The good resistance of BcBDH against organic solvents, especially DMSO, underlines the usage of this enzyme for such purposes (Fig. 4). Investigation of the stereoselectivity of BcBDH for several diketones and  $\alpha$ -hydroxy





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ketones revealed the formation of an *R*-configured stereo-center for the main product. Likewise, for its physiological substrates acetoin and diacetyl, this enzyme shows the same stereoselectivity for several unnatural substrates. BcBDH belongs to the family of zinc-dependent alcohol dehydrogenases. Because of this, we tested the influence of metal ions on the enzyme activity. Even though, this enzyme is zinc-dependent we could observe a high activity boost (more than double the activity) by supplementing this enzyme with  $Mn^{2+}$  ions. This effect could also be observed in biotransformation experiments by enhancing the conversion rate by using the enzyme previously pre-incubated with  $Mn^{2+}$  ions.

In summary, BcBDH is a suitable biocatalyst for the stereoselective synthesis of  $\alpha$ -hydroxy ketones and vicinal diols. With its ability to distinguish between asymmetric and symmetric molecules, the product outcome can be predicted. Furthermore, the ability of accepting "bulky" substrates and the stereoselective reduction of these molecules makes this enzyme even more appealing.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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Electronic Supplementary Material (ESI) for RSC Advances.  
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## Electronic Supplementary Information

### Synthesis of $\alpha$ -hydroxy ketones and vicinal (*R,R*)-diols by *Bacillus clausii*

#### DSM 8716<sup>T</sup> butanediol dehydrogenase

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Martina Pohl<sup>b</sup>, Torsten Wagner<sup>a</sup>, Michael J. Schöning<sup>a</sup>, Thorsten Selmer<sup>a</sup>, Petra Siegert<sup>\*a</sup>

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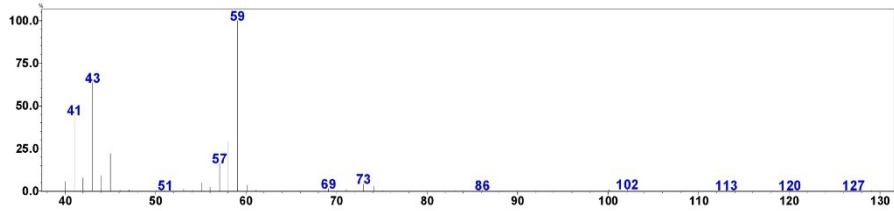
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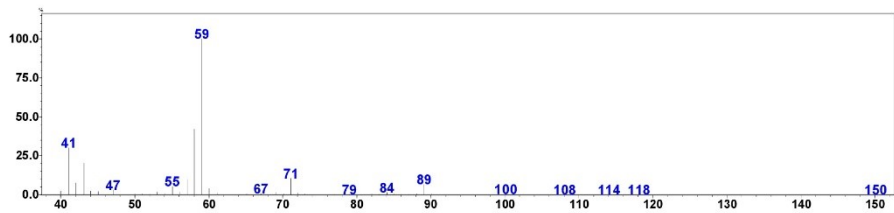
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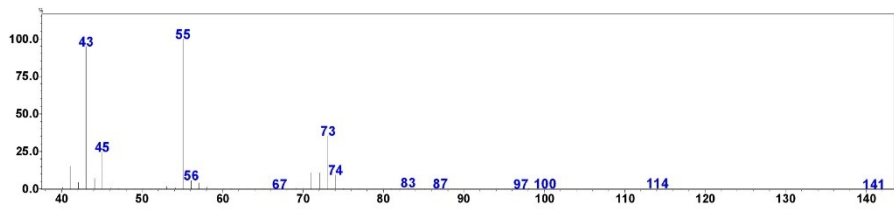
## 1 GC-MS spectra



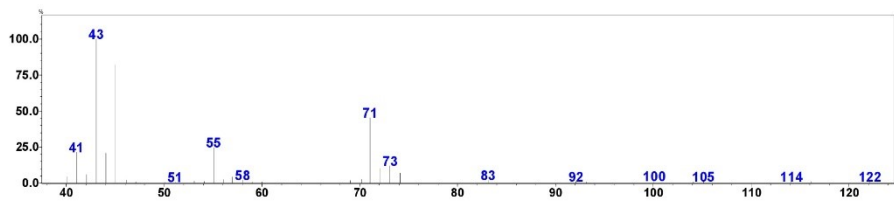
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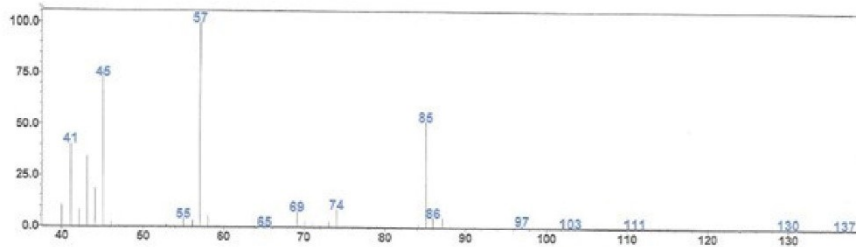
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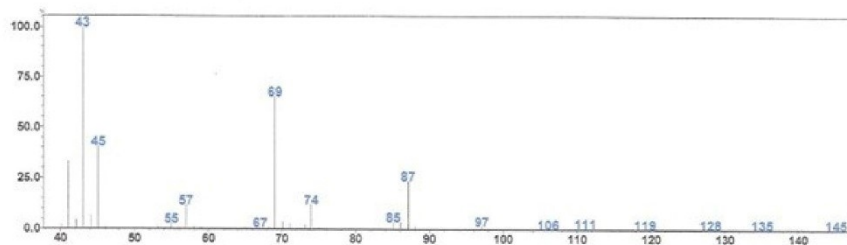
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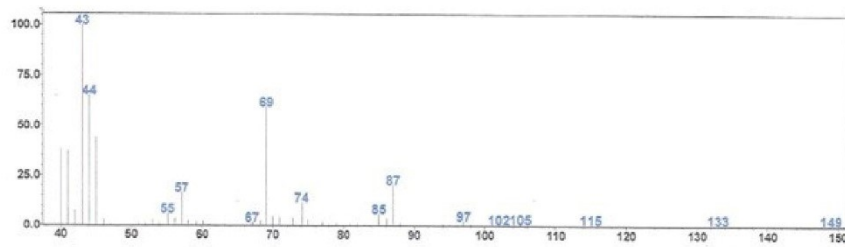
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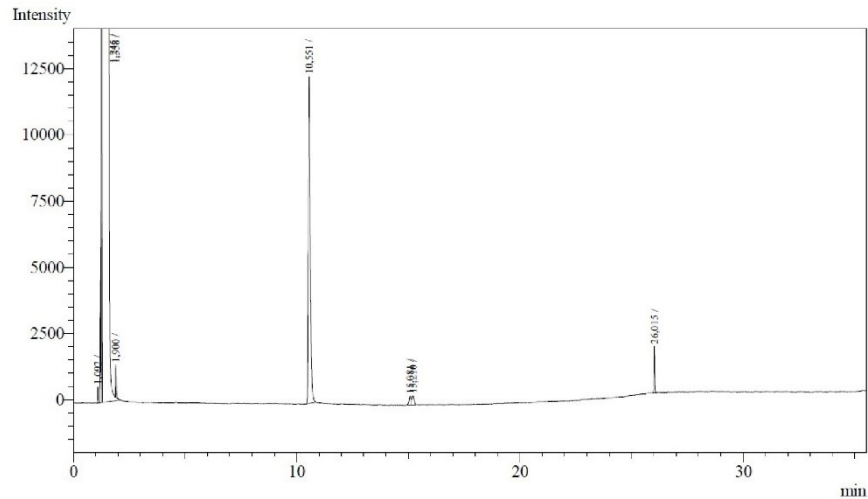


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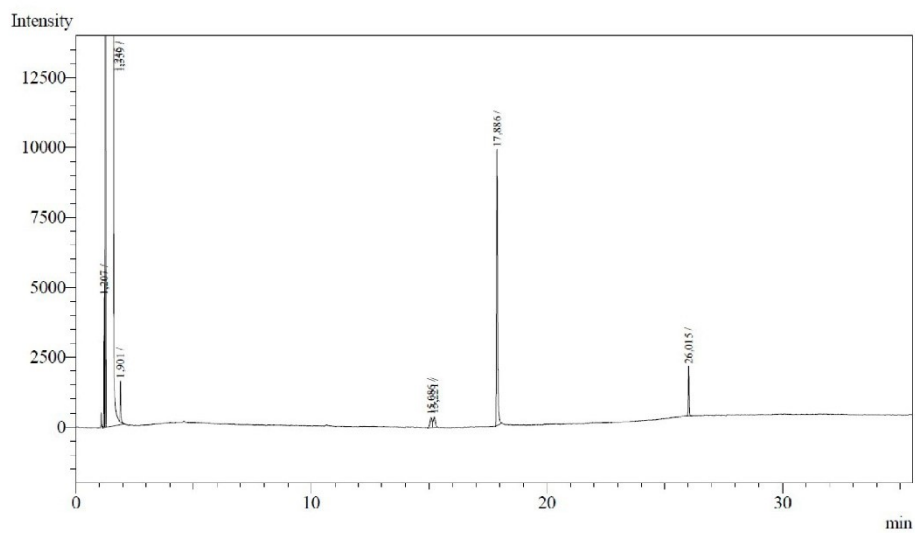


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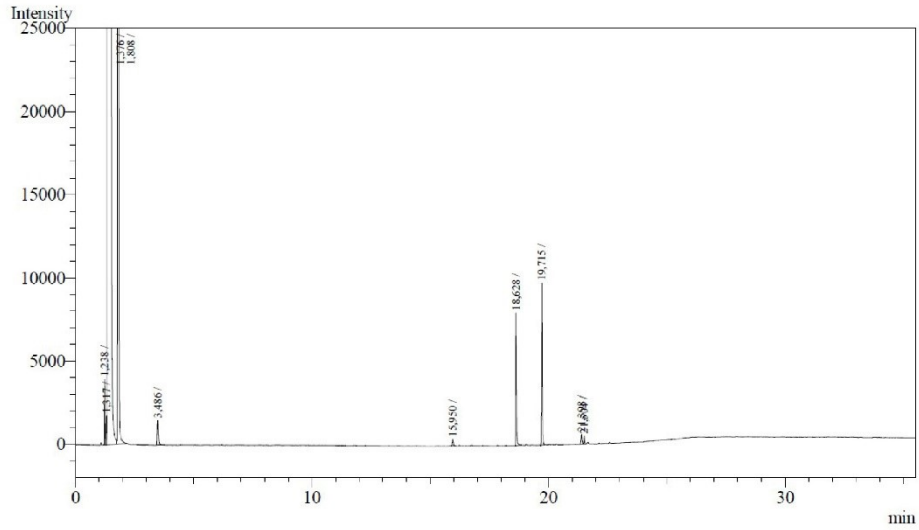
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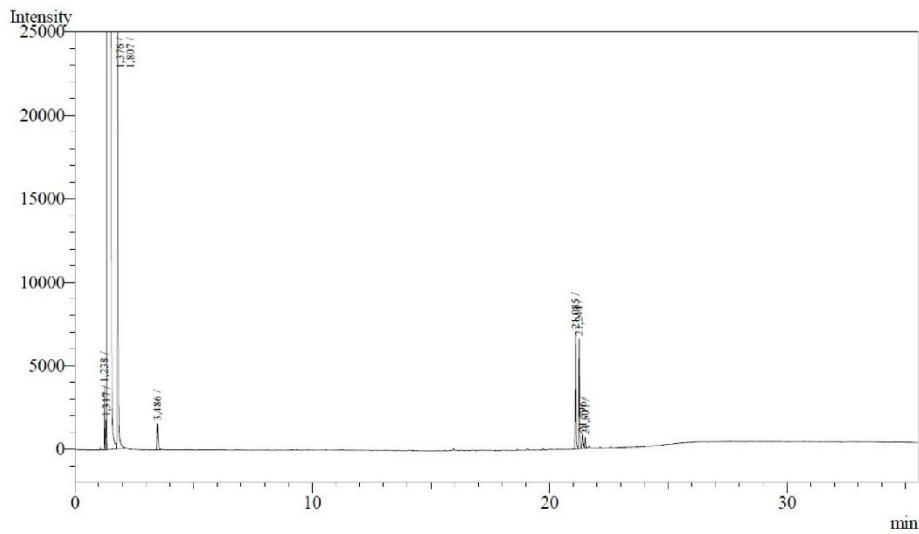
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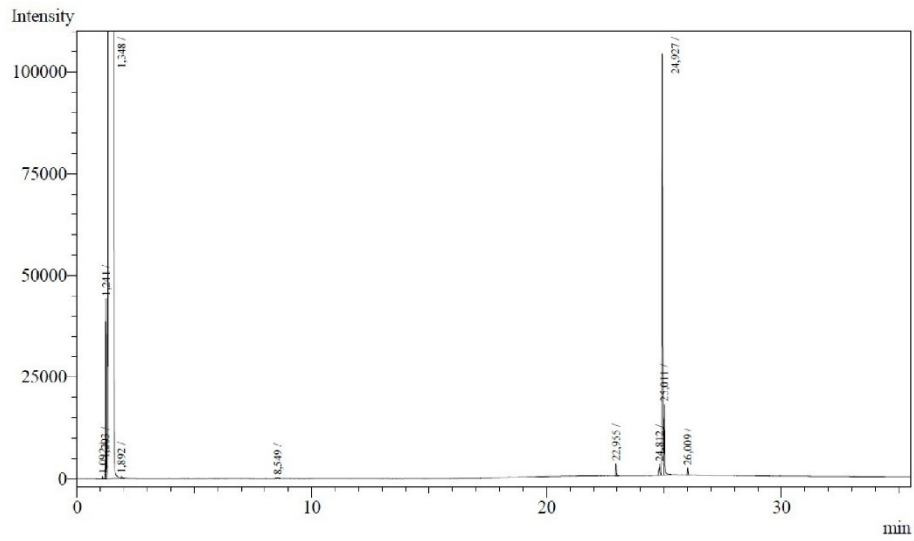
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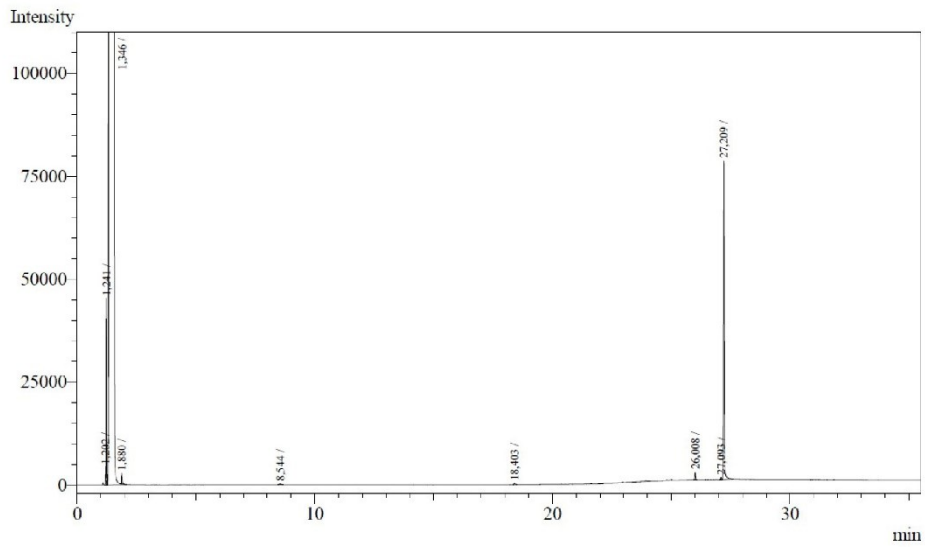
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Supplementary Figure 11: Gas chromatogramm of the reduction of *Rac* 4-hydroxy-3-hexanone by BcBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in MES-NaOH buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min.

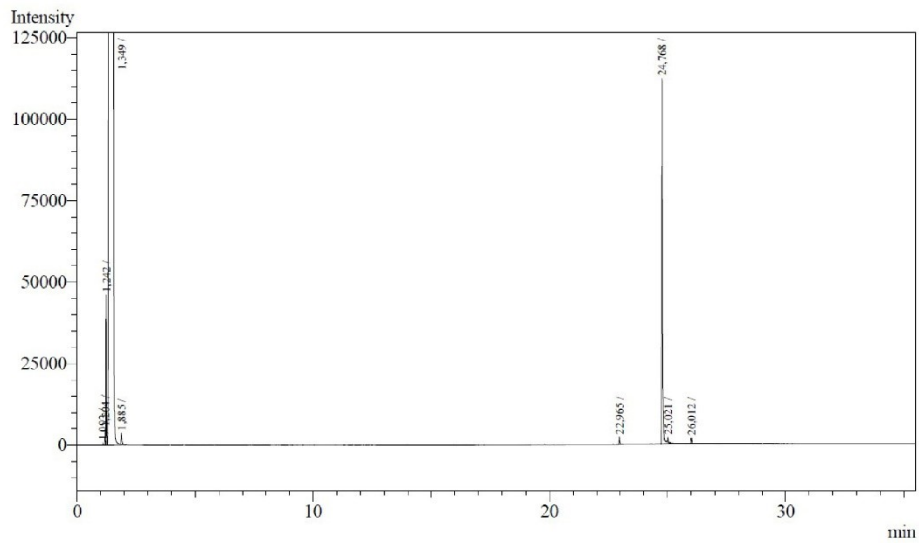


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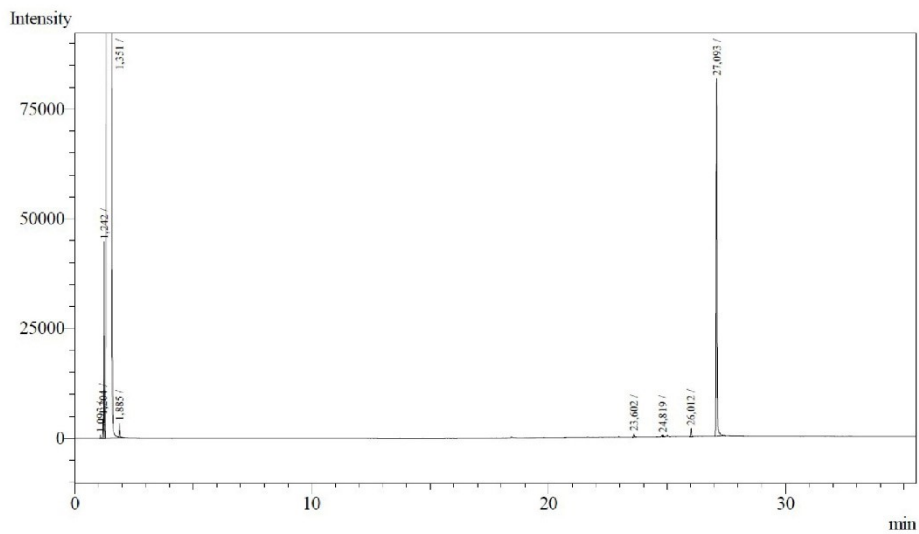


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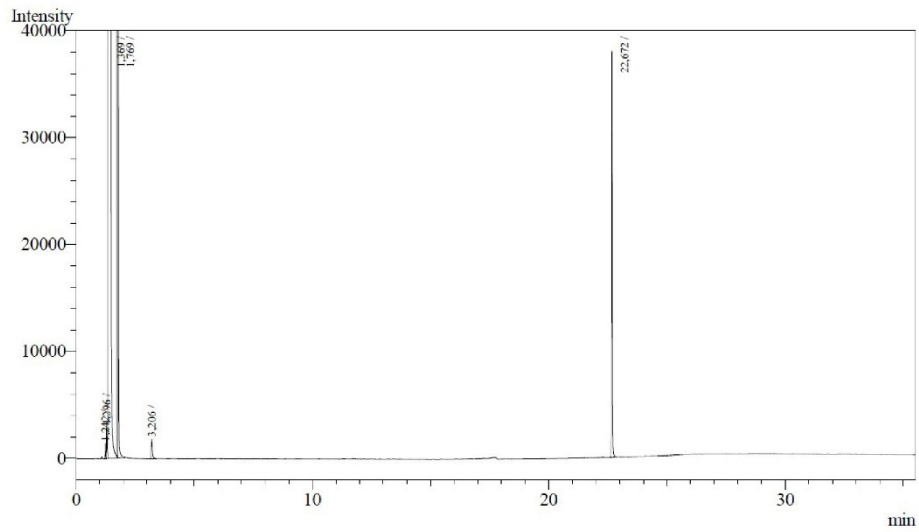




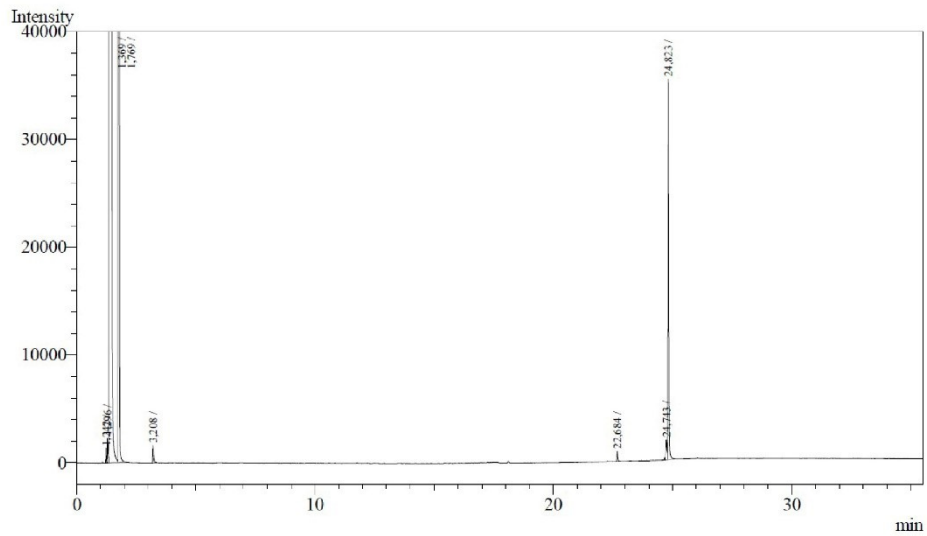
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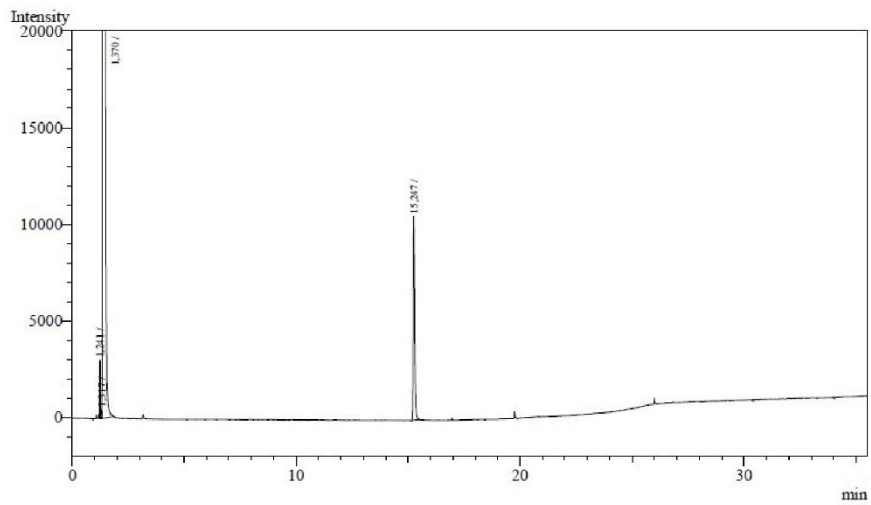
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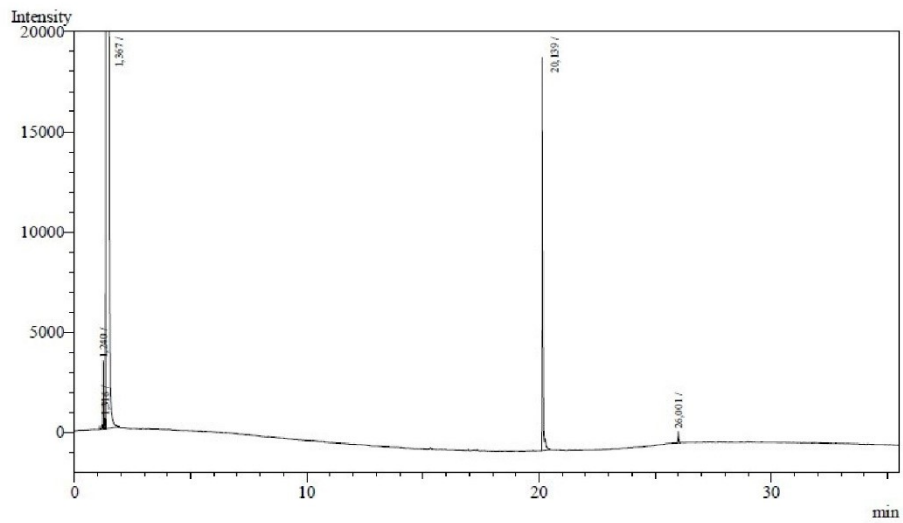
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Supplementary Figure 17: Gas chromatogramm of the reduction of 1-phenyl-1,2-propanedione by BcBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in MES-NaOH buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min.

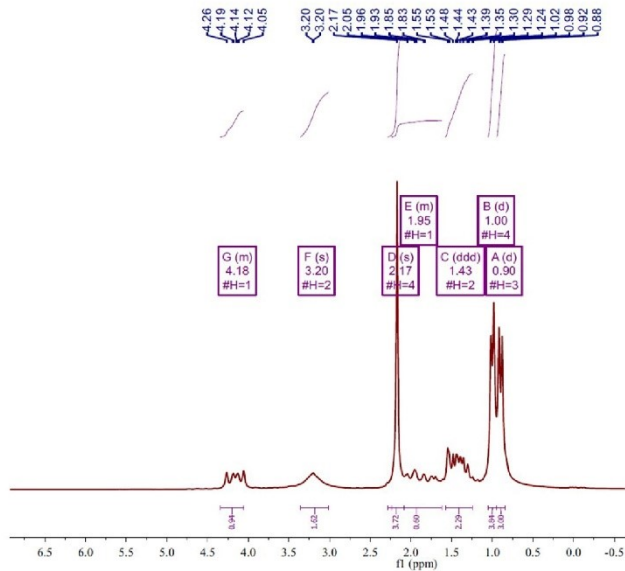


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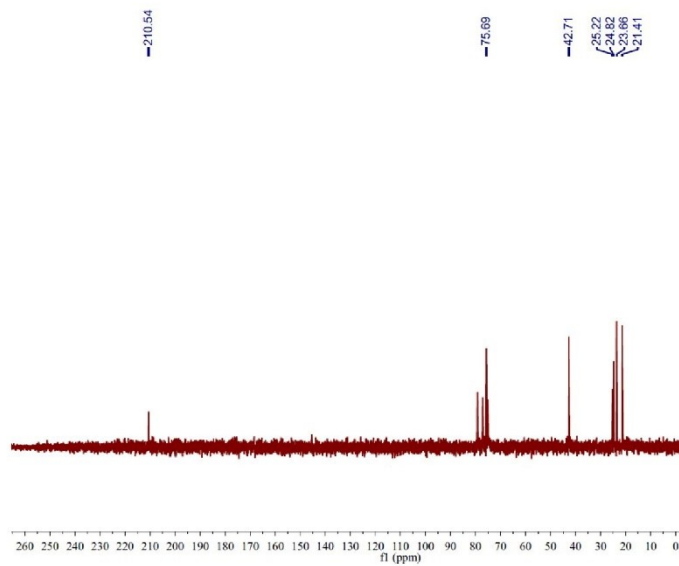


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### 3 NMR



Supplementary Figure 20:  $^1\text{H}$ -NMR of biocatalytic product (*R*)-5-methyl-3-hydroxy-2-hexanone, containing small amounts of 5-methyl-2-hydroxy-3-hexanone. ( $\text{CDCl}_3$ )  $\delta$  0.90 (d, 3H), 1.00 (d, 4H), 1.43 (ddd, 2H), 1.95 (m, 1H), 2.17 (s, 4H), 3.20 (s, 2H), 4.18 (m, 1H). s = singlet; d = doublet; ddd = doublet of doublet of doublet; m = multiplet.



Supplementary Figure 21:  $^{13}\text{C}$ -NMR of biocatalytic product (*R*)-5-methyl-3-hydroxy-2-hexanone, containing small amounts of 5-methyl-2-hydroxy-3-hexanone. ( $\text{CDCl}_3$ )  $\delta$  21.41; 23.66; 24.82; 25.22; 42.71; 75.69; 210.54.

### 2.3 Publikation 3

## **Synthesis of $\alpha$ -hydroxy ketones and vicinal diols with the *Bacillus licheniformis* DSM 13<sup>T</sup> butanediol dehydrogenase**

Lukas Muschallik, Carina Ronja Kipp, Inga Recker, Johannes Bongaerts, Martina Pohl, Torsten Wagner, Michael J. Schöning, Thorsten Selmer, Petra Siegert

Dieses Manuskript wurde eingereicht in: Journal of Biotechnology

#### Zusammenfassung:

Die Butandioldehydrogenase aus *Bacillus licheniformis* DSM 13<sup>T</sup> (BIBDH) wurde insbesondere auf seine biokatalytischen Eigenschaften hin untersucht. Hierfür wurde die Reduktion diverser Diketone und  $\alpha$ -Hydroxyketone im Hinblick auf die Produktselektivität betrachtet. Zusätzlich wurde eine detailliertere Untersuchung des Substratspektrums durchgeführt. Auch erfolgte ein tieferer Einblick in die biochemischen Parameter des Enzyms für nicht physiologische Substrate.

#### Anteile:

L. Muschallik plante und führte die Experimente durch. C. Kipp und I. Recker unterstützten in der Durchführung von Biotransformationen und Aufnahme kinetischer Parameter zur Charakterisierung des Enzyms. J. Bongaerts, M. Pohl, T. Wagner, M. J. Schöning und T. Selmer gaben wertvolle fachspezifische Beratung zur Analytik, Biotransformationen und mögliche Anwendungen z.B. in einem Biosensor System. P. Siegert führte die konzeptionelle Planung und Betreuung des Projekts durch. Alle Autoren beteiligten sich am Entwurf des Manuskripts.

# Synthesis of $\alpha$ -hydroxy ketones and vicinal diols with the *Bacillus licheniformis* DSM 13<sup>T</sup> butane-2,3-diol dehydrogenase

Lukas Muschallik<sup>a</sup>, Carina Ronja Kipp<sup>a</sup>, Inga Recker<sup>a</sup>, Johannes Bongaerts<sup>a</sup>, Martina Pohl<sup>b</sup>, Melanie Gellissen<sup>a</sup>, Michael J. Schöning<sup>a</sup>, Thorsten Selmer<sup>a†</sup>, Petra Siegert<sup>a\*</sup>

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<sup>†</sup> deceased

**Keywords:** Acetoin reductase/butane-2,3-diol dehydrogenase, *meso*-butane-2,3-diol dehydrogenase

SDR, *Bacillus licheniformis*, biocatalysis, pheromones

**Abbreviations:** BIBDH, SDR

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## Abstract

The enantioselective synthesis of  $\alpha$ -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<sup>T</sup> (BIBDH). The encoding gene was cloned with an N-terminal StrepII-tag and recombinantly overexpressed in *E. coli*. BIBDH is highly active towards several non-physiological diketones and  $\alpha$ -hydroxyketones with varying aliphatic chain lengths or even containing phenyl moieties. By adjusting the reaction parameters in biotransformations the formation of either the  $\alpha$ -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  $\alpha$ -acetolactate, acetoin and 2,3-butanediol as the main intermediates <sup>1,2</sup>. 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 <sup>3-5</sup>. Besides these

natural substrates, butanediol dehydrogenases catalyze the stereoselective reduction of different diketones to  $\alpha$ -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<sup>3,6-8</sup>. For instance, beetles from the family of *Cerambycidae* (longhorn beetles) highly react on mixtures of 3-hydroxy-2-hexanone and 2,3-hexanediols<sup>9,10</sup>. Furthermore, longer chain  $\alpha$ -hydroxy ketones and diols like 3-hydroxy-2-octanone or 2,3-octanediols, proved to be efficient as pheromones and can be used in traps for pest control<sup>11-13</sup>. To access these molecules different chemical approaches were reported, such as asymmetric  $\alpha$ -aminooxylation of aldehydes to generate enantiopure 1,2-diols or the use of cyclic ruthenates for regioselective oxidation of vicinal diols to  $\alpha$ -hydroxy ketones<sup>14,15</sup>. Chemoenzymatic routes reported the synthesis of  $\alpha$ -hydroxy ketones by CalB lipase<sup>16</sup>. Moreover, enantioselective biocatalytic synthesis of  $\alpha$ -hydroxy ketones and vicinal diols by thiamine diphosphate-dependent lyases (ThDP lyases) that catalyze the carbonylation of aldehydes to  $\alpha$ -hydroxy ketones<sup>17-19</sup> and oxidoreductases<sup>20-25</sup> proved to be efficient.

The butanediol dehydrogenase from *Bacillus licheniformis* DSM 13<sup>T</sup> catalyzes the NADH-dependent enantioselective reduction of diacetyl via acetoin to the corresponding *meso*- and (*S,S*)-2,3-butanediol stereoisomers<sup>26</sup>. The focus of this study is a deeper understanding of the BIBDH's enzymatic characteristics, in particular its substrate range, stereoselectivity for non-natural substrates and its use as a biocatalyst for the enantioselective synthesis of  $\alpha$ -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<sup>27,28</sup>. 1-Hydroxy-1-[2-(trifluoromethyl)phenyl]-2-propanone and 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-butanone 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<sup>®</sup> cloning vectors and streptactin columns were from IBA GmbH (Germany).

## 2.2 Bacterial strains and plasmids

Cloning was done in *Escherichia coli* DH5 $\alpha$  by using the Stargate<sup>®</sup>-pENTRY vector (kanamycin resistance). *Escherichia coli* BL21(DE3) was used for protein production. The Stargate<sup>®</sup> pASG.5 vector (pASG-BI02066-5; ampicillin resistance) and pASG.3 (pASG-BI02066-3; 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<sup>T</sup>

The primers (BI02066-fw: AGCGGCTCTTCAATGAGTAAAGTATCTGGAAAATTGC and BI02066-rev: AGCGGCTCTTCTCCATTAATACCATTCCGCCATCA) were deduced using the known sequence of the *B. licheniformis* DSM 13<sup>T</sup> 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<sup>®</sup> 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 <sup>29</sup>.

SDS-PAGE was carried out according to Laemmli <sup>30</sup> using 12-% polyacrylamide gels and Roti<sup>®</sup>-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  $\mu$ g/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  $\times$  g, 4 °C, 40 min). Recombinantly produced BDHs were purified using Strep-Tactin<sup>®</sup> 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 mM, pH 10.0) with substrate (10 mM) and NAD<sup>+</sup> (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 BIBDH. A correction was made by measuring a control without enzyme. Variability is expressed as standard deviation (in triplicates).

One unit of BIBDH is defined as the amount of enzyme that oxidizes one  $\mu\text{mol}$  of NADH per minute at 30 °C under the given conditions.  $k_{cat}$  was calculated per enzyme subunit.

## 2.7 Substrate spectrum of BIBDH

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 BIBDH

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 BIBDH 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<sup>+</sup> (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 BIBDH

Stereoselectivity and conversion were analyzed by carrying out the reduction reaction of selected diketones and  $\alpha$ -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 BIBDH (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  $\mu$ l) were taken at different points in time during the reaction, extracted with diethyl ether (300  $\mu$ l) and applied to GC analysis (GC-2010 Plus (Shimadzu) with a flame ionization detector) equipped with a Hydrodex  $\gamma$  - 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 ( $\text{NaBH}_4$ , 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  $\text{H}_2\text{O}$  the reaction was stirred for another 10 min. The reaction products were extracted with diethyl ether, neutralized, dried with  $\text{MgSO}_4$  (anhydrous), filtrated and the solvent was removed under vacuum <sup>21</sup>.

### 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<sup>T</sup> and purification of the StrepII-tagged enzyme

The BIBDH 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 *Lgl*I restriction sites yielding the pE-BI02066 vector. Sequencing verified a 100% identity with the previously published *meso*-2,3-BDH <sup>31</sup> from *Bacillus licheniformis* DSM 13 on nucleotide level.

In order to perform further biochemical studies, the enzyme here was sub-cloned from pE-BI02066 into the vectors pASG.3 and pASG.5, respectively. By using *Esp*3I 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 proved 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<sup>31</sup>. 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<sup>31</sup>. However, our conditions ensured that  $V_{max}$  was reached.

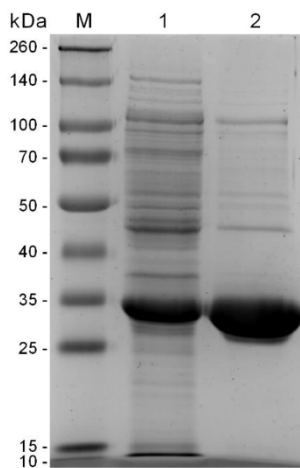


Figure 1: Purification of recombinant BIBDH. Cell-free extract (10 µg protein, lane 1) and purified enzyme (10 µg, lane 2) were analyzed by SDS-PAGE and stained with Coomassie blue.

Sequence alignment with the biochemically characterized *meso*-BDH from *Klebsiella pneumoniae*<sup>26</sup> and *Serratia marcescens* H30<sup>32</sup> 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 BIBDH and the *meso*-BDHs from *Klebsiella pneumoniae* (accession number: JN865245.1)<sup>26</sup> and *Serratia marcescens* H30 (accession number: AFH00999.1)<sup>32</sup>.

### 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. BIBDH shows higher reductase activities for acetoin over diacetyl like the *meso*-BDH from *Serratia marcescens* H30<sup>32</sup>. Instead, the acetoin reductase from *Rhodococcus erythropolis* shows a two times higher activity for diacetyl over acetoin, under the given conditions<sup>33</sup>. To further elucidate the biocatalytic properties of BIBDH, we extended the studies on several aliphatic and alkylaryl  $\alpha$ -diketones such as  $\alpha$ -keto acids and  $\alpha$ -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  $\alpha$ -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  $\alpha$ -keto aldehydes such as methylglyoxal or  $\alpha$ -hydroxy aldehydes such as (*R*)-lactaldehyde were measured. Additionally, moderate to low activities were observed with bulky vicinal diketones and  $\alpha$ -hydroxy ketones, like 1-phenyl-1,2-propanedione. While most BDHs struggle with molecules with longer alkyl chains, BIBDH 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<sup>34,35</sup>. Instead, the acetoin reductase from *Enterobacter aerogenes* exhibits a higher activity for this molecule with 85% (in comparison to diacetyl)<sup>36</sup>. 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<sup>22</sup>. For the reduction of 1-phenyl-1,2-propanedione both enzymes exhibit a similar activity under the given reaction conditions<sup>22</sup>. Instead, with bulky substrates like benzoin or benzil no activity of BIBDH 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 BIBDH 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<sup>T</sup> with no activity detected for primary and secondary alcohols like ethanol, 2-propanol and ethyleneglycol<sup>37,38</sup>. 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<sup>32</sup>.

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<sup>+</sup>-reducing substrates (50 mM glycine-NaOH buffer, pH 10.0, 30 °C, 0.3 mM NAD<sup>+</sup> and 10 mM substrate (100% = 73.4 U/mg)). Data for enzymes from this study, *S. marcescens*<sup>32</sup> (assay conditions: potassium phosphate buffer (50 mM, pH 8.0); NAD<sup>+</sup> (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*<sup>26</sup> (assay conditions: sodium pyrophosphate (33 mM, pH 8.0); NAD<sup>+</sup> (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*<sup>31</sup> (assay conditions: glycine-NaOH buffer (100 mM, pH 10.0); NAD<sup>+</sup> (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.*<sup>31</sup> n.d., no data.

Entry No.	Substrate	<i>meso</i> -butanediol dehydrogenase from			
		<i>B. licheniformis</i> activity [%]	<i>B. licheniformis</i> <sup>31</sup> activity [%]	<i>S. marcescens</i> H30 <sup>32</sup> activity [%]	<i>E. pneumoniae</i> <sup>26</sup> activity [%]
<b>Reductive reaction</b>					
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.
<b>Oxidative reaction</b>					
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 BIBDH

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). BIBDH 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 is 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.

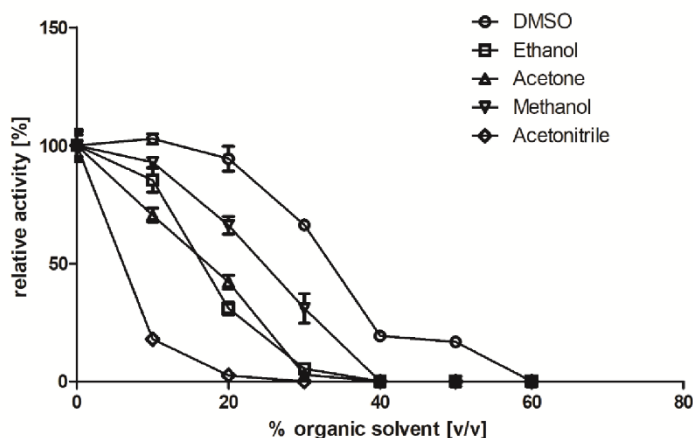


Figure 3: Effect of water-miscible organic solvents on the stability of BIBDH. BIBDH was incubated for 1 hour (25°C) with 0 to 60% (v/v) solvent in potassium phosphate buffer (50 mM, pH 6.8). Activity was determined with the standard photometric assay.

### 3.4 Biochemical properties of BIBDH

BIBDH 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.*<sup>31</sup> a pH optimum of 10.0 was measured for the oxidation of 2,3-butanediol (all stereoisomers; see supplements).

Similar pH optima for the oxidation and reduction reaction were observed with the *meso*-BDH from *Serratia marcescens* H30, the BDH from *B. stearothersophilus* and the BDH from *Leuconostoc pseudomesenteroides*<sup>32,39,40</sup>. 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, BIBDH 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.*<sup>31</sup>) and the  $K_m$  and  $k_{cat}$  values for acetoin and diacetyl are  $2.76 \pm 0.63$  mM;  $81.78$  s<sup>-1</sup> and  $77.5 \pm 6.2$ ;  $872.3$  s<sup>-1</sup>, 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, BIBDH 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  $\pm$   $6.5$  mM and  $535.8$  s<sup>-1</sup> for 2,3-hexanedione,  $42$  mM  $\pm$   $15.4$  and  $423.8$  s<sup>-1</sup> for 5-methyl-2,3-hexanedione,  $11.0$  mM  $\pm$   $1.6$  and  $306$  s<sup>-1</sup> 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<sup>-1</sup>). 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.

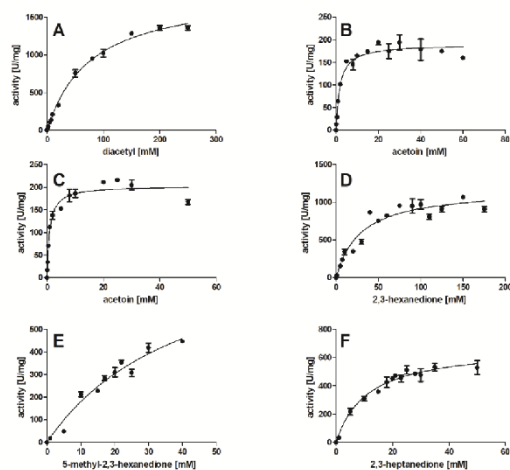


Figure 4: Michaelis-Menten plot for the reduction of diacetyl (A), acetoin with 5% (v/v) DMSO (B), acetoin without 5% (v/v) DMSO (C), 2,3-hexanedione (D), 5-methyl-2,3-hexanedione (E), 2,3-heptanedione (F). Activity was determined in potassium-phosphate buffer (50 mM; pH 6.8), with 5% (v/v) DMSO as solubilizer and NADH (300  $\mu$ M) 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 BIBDH for different substrates

Substrate	$K_m$ [mM]	$k_{cat}$ [ $s^{-1}$ ]	$V_{max}$ [U/mg]
Acetoin without 5% (v/v) DMSO	$0.9 \pm 0.2$	92.28	$203.3 \pm 6.7$
Acetoin with 5% (v/v) DMSO	$1.8 \pm 0.3$	81.78	$190.0 \pm 5.4$
Diacetyl	$77.5 \pm 6.2$	872.3	$1856.0 \pm 57.5$
2,3-Hexanedione	$29.0 \pm 6.5$	535.8	$1183.0 \pm 79.0$
5-Methyl-2,3-hexanedione	$42.0 \pm 15.4$	423.8	$941.7 \pm 218.1$
2,3-Heptanedione	$11.0 \pm 1.6$	306	$680.5 \pm 32.71$

### 3.5 Biocatalytic characterization of BIBDH

To gain a deeper insight into the biocatalytic potential of BIBDH, biotransformations with its physiological substrates as well as non-physiological vicinal diketones and  $\alpha$ -hydroxyketones as substrates (Figure 5) and BIBDH 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).

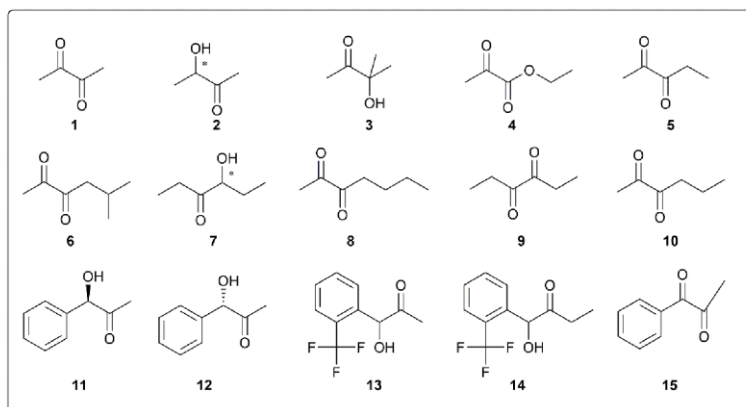
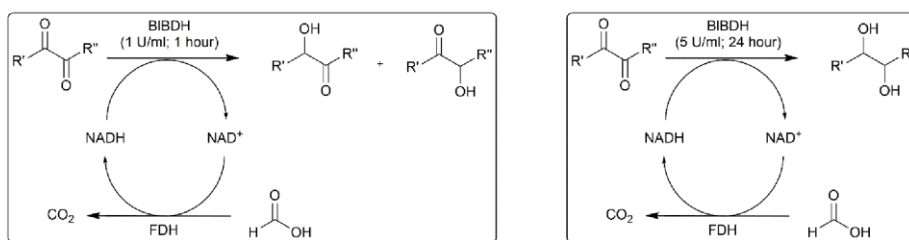


Figure 5: Vicinal diketones and  $\alpha$ -hydroxy ketones (10 mM) reduced by BIBDH (1 U/ml for the corresponding substrate). A cofactor regeneration system was applied by using formate dehydrogenase (5 U/ml) and formate (30 mM).



1: R'=R''=CH<sub>3</sub> 2: R'=R''=CH<sub>3</sub> 3: R'=CH<sub>3</sub>, R''=OH-CH<sub>2</sub>-CH<sub>3</sub> 4: R'=CH<sub>3</sub>, R''=O-CH<sub>2</sub>-CH<sub>3</sub> 5: R'=CH<sub>3</sub>, R''=CH<sub>2</sub>-CH<sub>3</sub> 6: R'=CH<sub>3</sub>, R''=CH<sub>2</sub>-(CH<sub>3</sub>)<sub>2</sub> 7: R'=R''=CH<sub>2</sub>-CH<sub>3</sub> 8: R'=CH<sub>3</sub>, R''=(CH<sub>2</sub>)<sub>3</sub>-CH<sub>3</sub> 9: R'=R''=CH<sub>2</sub>-CH<sub>3</sub> 10: R'=CH<sub>3</sub>, R''=(CH<sub>2</sub>)<sub>2</sub>-CH<sub>3</sub> 11: R'=Ph, R''=CH<sub>3</sub> 12: R'=Ph, R''=CH<sub>3</sub> 13: R'=Ph-(F)<sub>3</sub>, R''=CH<sub>3</sub> 14: R'=Ph-(F)<sub>3</sub>, R''=CH<sub>2</sub>-CH<sub>3</sub> 15: R'=Ph, R''=CH<sub>3</sub>

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

As there are no commercial sources of enantiomerically pure  $\alpha$ -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<sub>4</sub>)<sup>41</sup>. Thus, the reaction products of BIBDH and the constitutional isomer of the  $\alpha$ -hydroxy ketones were identified. By comparing the reaction products of BIBDH with our previous published (*R,R*)-2,3-butanediol dehydrogenase from *Bacillus clausii* DSM 8716<sup>T</sup> the identification of some enantiomers stereoisomers was possible<sup>38,41</sup>.

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<sup>31</sup>. In comparison to diacetyl, the BIBDH-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 BIBDH exhibits a much higher affinity to the (*R*)-enantiomer and introduces a (*S*)-configured 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 superfamily. It introduces an (*S*)-configured stereo center but favors (*R*)-acetoin<sup>22</sup>. The same holds true for the *meso*-butanediol dehydrogenase from *Serratia marcescens* H30<sup>32</sup>. Fermentative production of *meso*-2,3-butanediol was demonstrated by using the *Bacillus licheniformis* MW3 ( $\Delta$ gdh encoding for a (*R,R*)-2,3-butanediol dehydrogenase gene) strain with concentrations of 90.1 g/l after 32 hours<sup>42</sup>. This is a modified *Bacillus licheniformis* DSM 13 strain harboring the gene of the butanediol dehydrogenase investigated in the present study<sup>42,43</sup>.

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  $\alpha$ -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 BIBDH (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 BIBDH 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 BIBDH 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-butanediol enantiomer (conversion after 20 min: > 99%). Ethyl pyruvate was reduced to one product (conversion after 20 min, > 99%).

Surprisingly, BIBDH 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  $\alpha$ -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 BIBDH is similar to the *meso*-butanediol dehydrogenase from *Serratia marcescens* CECT 977<sup>22</sup>. 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, BIBDH 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  $\alpha$ -hydroxyketones with BIBDH. Substrate concentration 10 mM; potassium phosphate buffer (50mM, pH 6.8), reaction time 1 hour; 1 U/ml BIBDH (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 *Bacillus licheniformis* DSM 13<sup>T</sup>. 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 BIBDH 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 BIBDH introduces an (*S*)-configured stereo center preferably into (*R*)-configured  $\alpha$ -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 opens 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*)- $\alpha$ -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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## 5 References

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## Electronic Supplementary Information

### Synthesis of $\alpha$ -Hydroxy ketones and vicinal diols with the *Bacillus licheniformis* DSM 13<sup>T</sup> butanediol dehydrogenase

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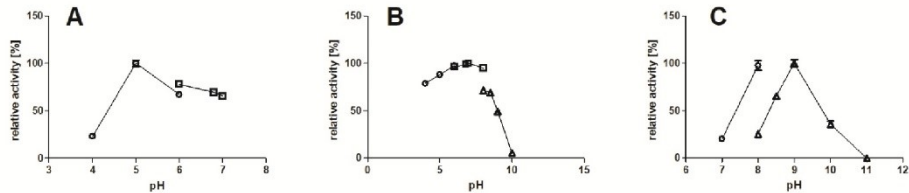
<sup>b</sup> *IBG-1: Biotechnology, Forschungszentrum Jülich, 52425 Jülich, Germany*

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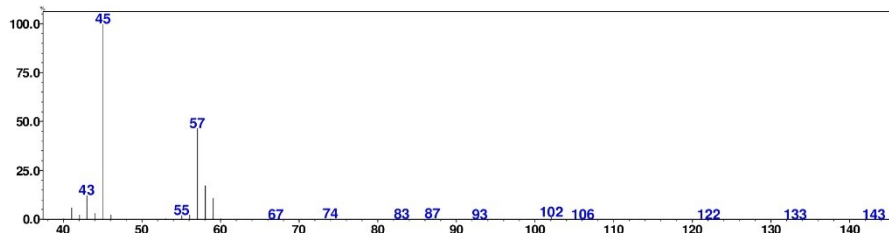
1. pH-optima
2. GC-MS spectra
3. GC-chromatograms

## 1 pH-optima

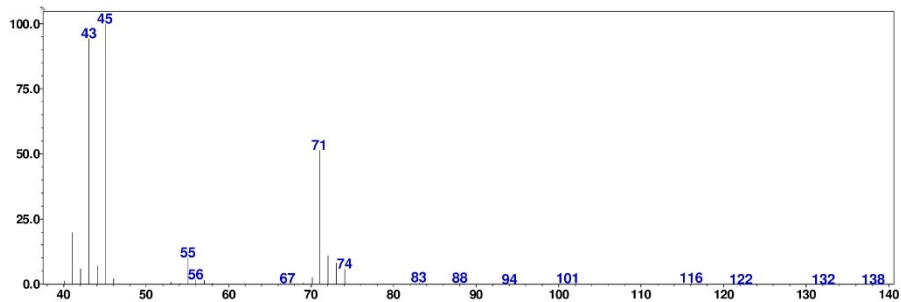


Supplementary Figure 1: pH-dependent activity of BIBDH for (A) the reduction of diacetyl, (B) reduction of acetoin, and (C) oxidation of *meso*-2,3-butanediol. The activity was measured with substrate concentrations of 10 mM (300  $\mu$ M NAD(H)) at 30 °C in sodium acetate (pH 4.0–6.0, open circles), potassium phosphate buffer (pH 6.0–8.0, open squares), glycine-NaOH buffer (pH 8.0–11.0, open triangles); buffer concentration 50 mM. Experiments were carried out in triplicates.

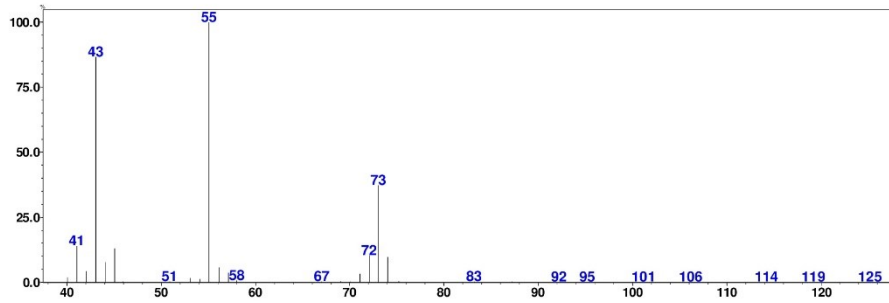
## 2 GC-MS spectra



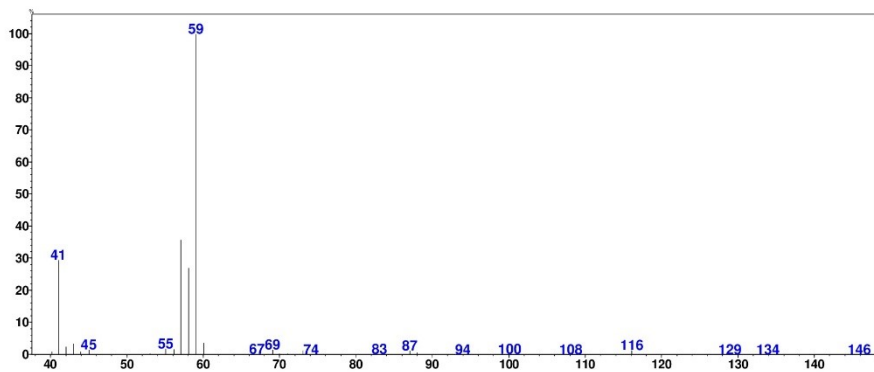
Supplementary Figure 2: Mass spectrum of 2-hydroxy-3-pentanone. Retention time: 19.9 min.



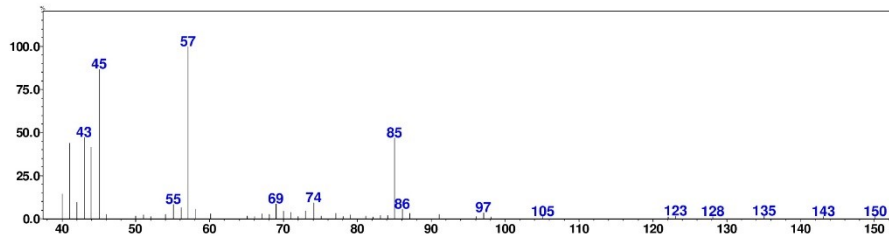
Supplementary Figure 3: Mass spectrum of 2-hydroxy-3-hexanone. Retention time: 21.5 min.



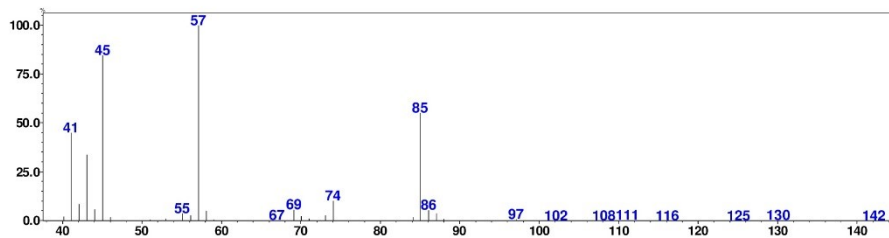
Supplementary Figure 4: Mass spectrum of 3-hydroxy-2-hexanone. Retention time: 22.4 min.



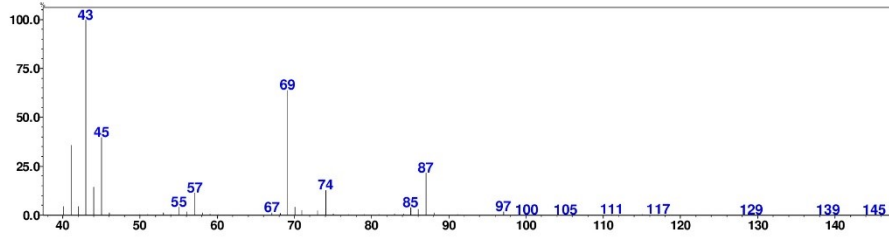
Supplementary Figure 5: Mass spectrum of 3-hydroxy-4-hexanone. Retention time: 22.3 min.



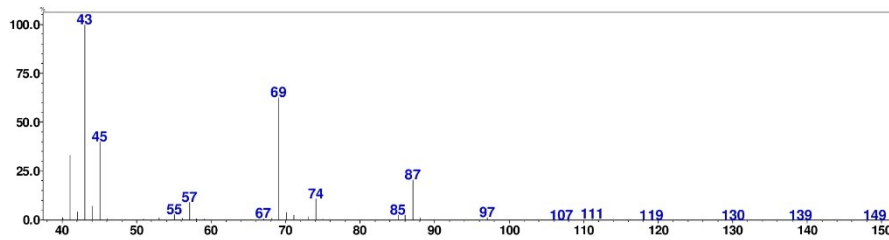
Supplementary Figure 6: Mass spectrum of 5-methyl-2-hydroxy-3-hexanone. Retention time: 21.32 min.



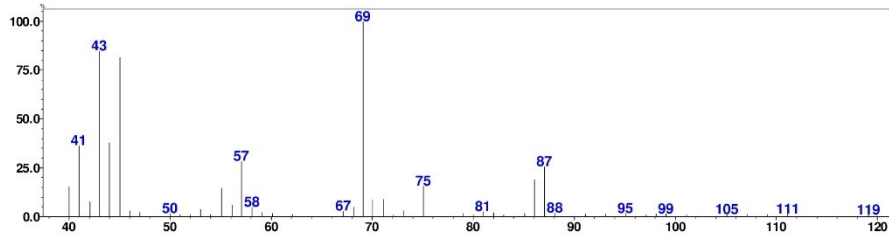
Supplementary Figure 7: Mass spectrum of 5-methyl-2-hydroxy-3-hexanone. Retention time: 21.39 min.



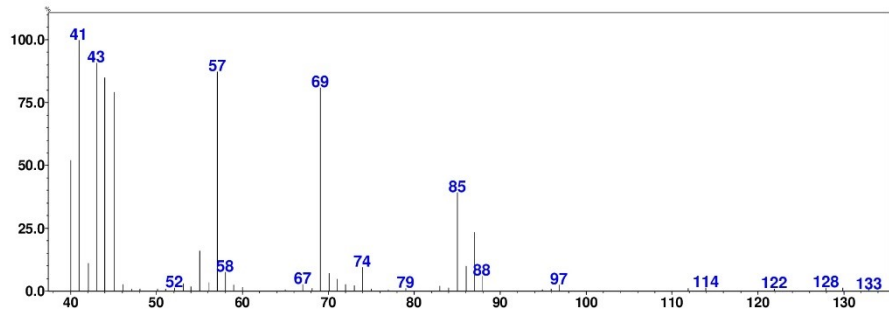
Supplementary Figure 8: Mass spectrum of 5-methyl-3-hydroxy-2-hexanone. Retention time: 21.8 min.



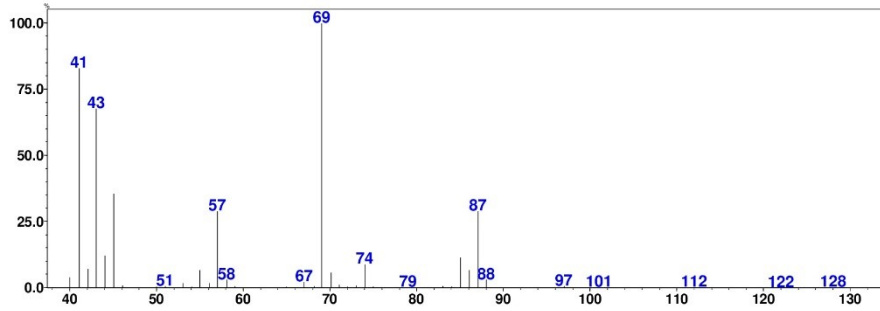
Supplementary Figure 9: Mass spectrum of 5-methyl-3-hydroxy-2-hexanone. Retention time: 22.4 min.



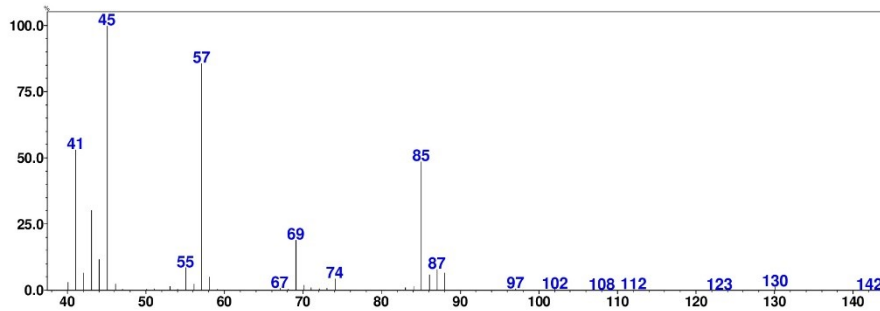
Supplementary Figure 10: Mass spectrum of 5-methyl-2,3-hexanediol. Retention time: 24.3 min.



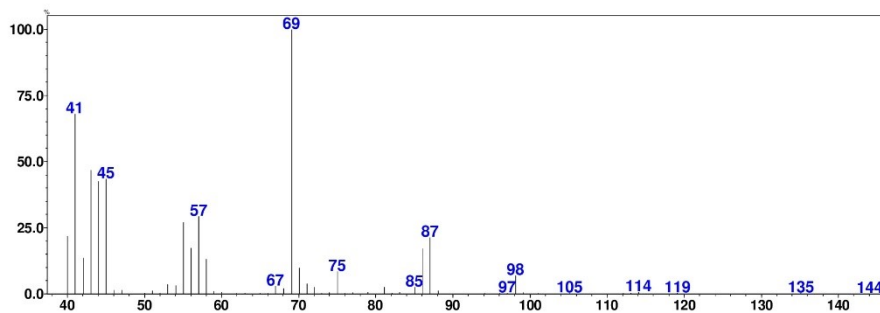
Supplementary Figure 11: Mass spectrum of 3-hydroxy-2-heptanone. Retention time: 22.4 min.



Supplementary Figure 12: Mass spectrum of 3-hydroxy-2-heptanone. Retention time: 23.0 min.

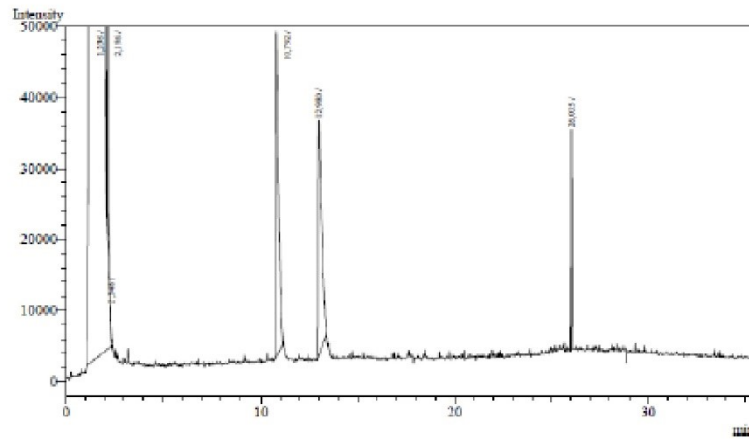


Supplementary Figure 13: Mass spectrum of 2-hydroxy-3-heptanone. Retention time: 22.9 min.

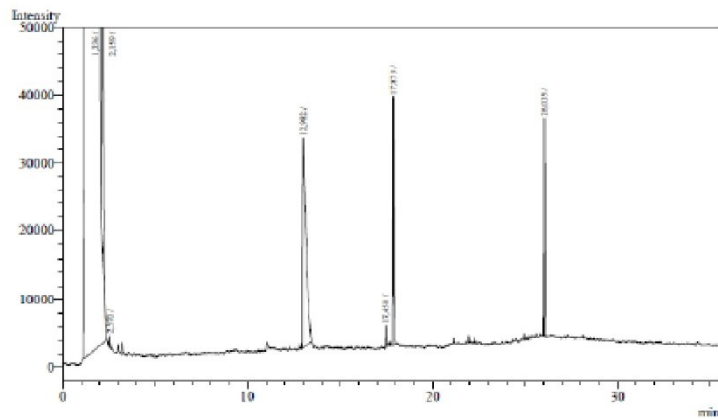


Supplementary Figure 14: Mass spectrum of 2,3-heptanediol. Retention time: 25.2 min.

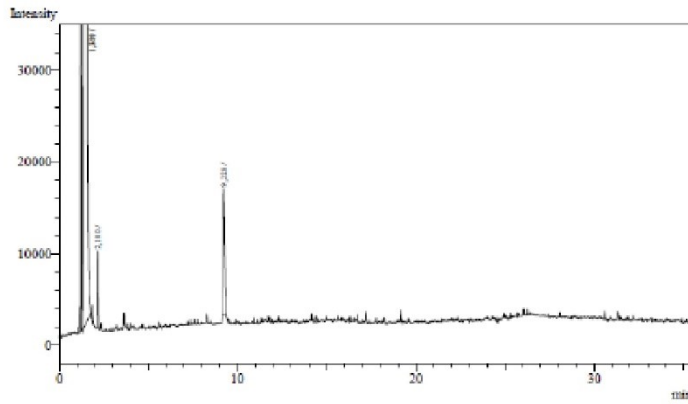
### 3 GC-Chromatograms



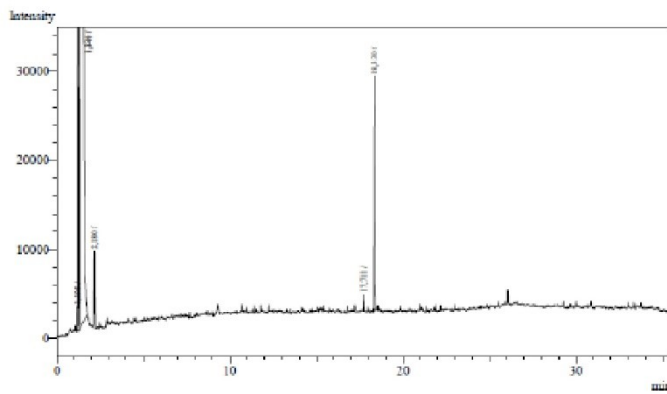
Supplementary Figure 15: Gas chromatogram of the reduction of racemic acetoin by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.



Supplementary Figure 16: Gas chromatogram of the reduction of racemic acetoin by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min.

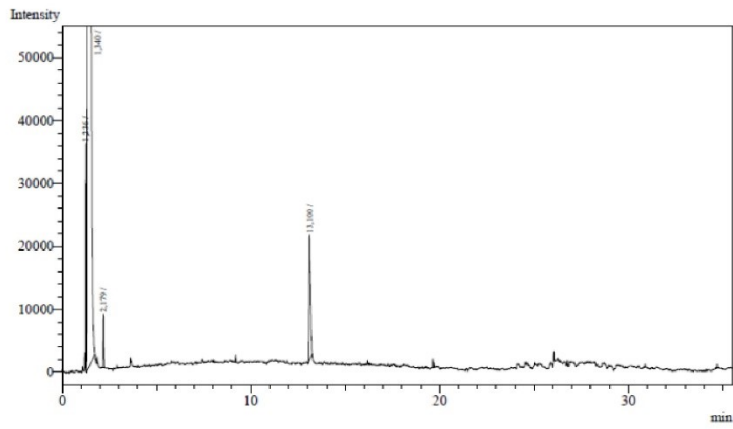


Supplementary Figure 17: Gas chromatogram of the reduction of 2,3-pentanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.

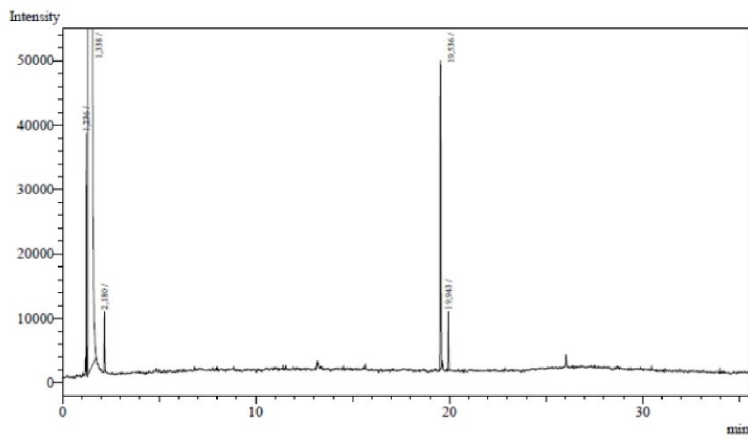


Supplementary Figure 18: Gas chromatogram of the reduction of 2,3-pentanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 20 min.

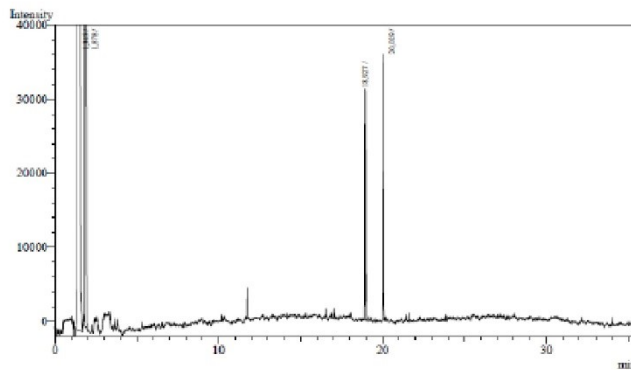




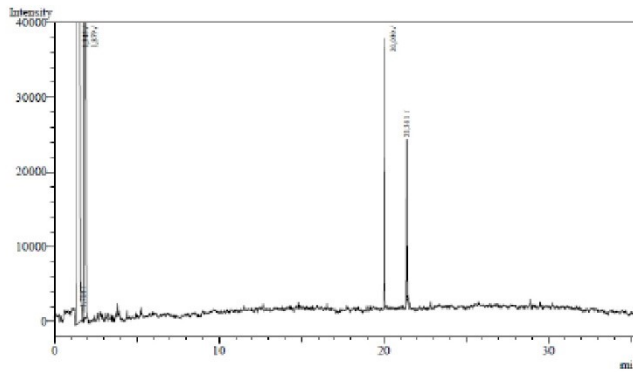
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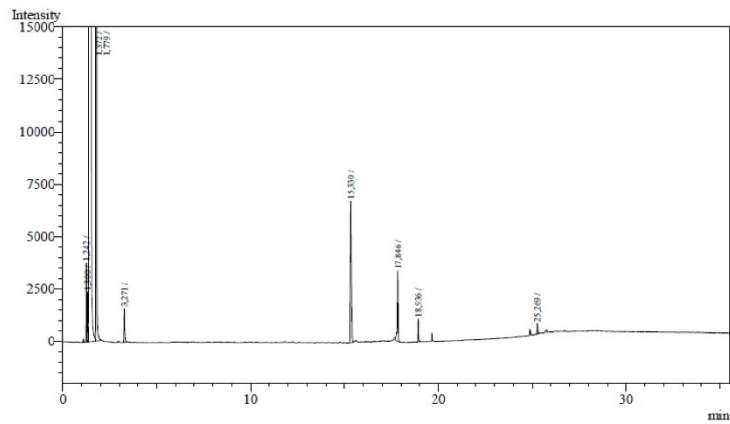
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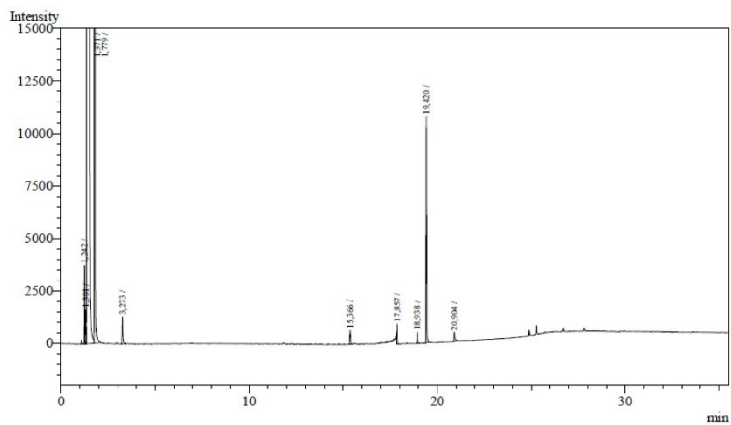
Supplementary Figure 21: Gas chromatogram of the reduction of 4-hydroxy-3-hexanone by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.



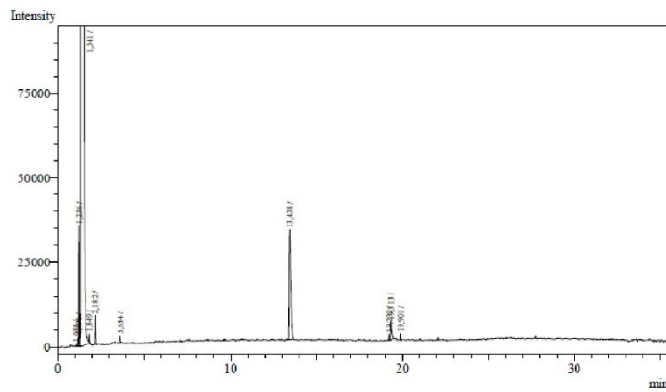
Supplementary Figure 22: Gas chromatogram of the reduction of 4-hydroxy-3-hexanone by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 20 min.



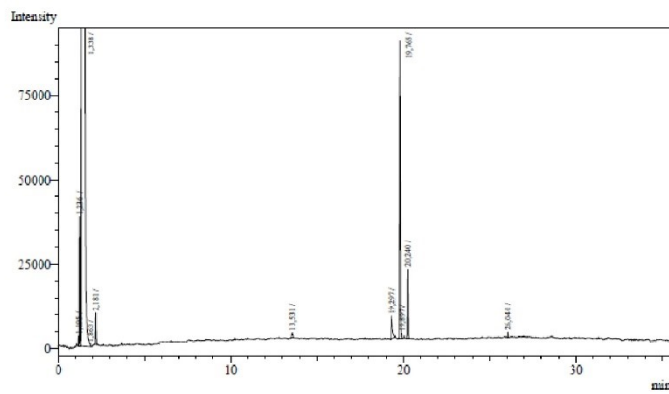
Supplementary Figure 23: Gas chromatogram of the reduction of 3,4-hexanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.



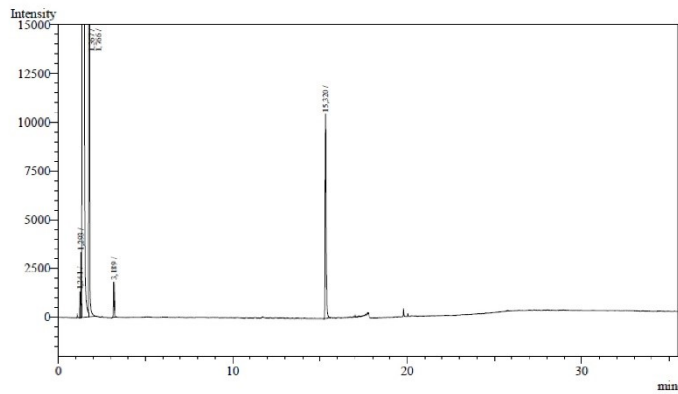
Supplementary Figure 24: Gas chromatogram of the reduction of 3,4-hexanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min.



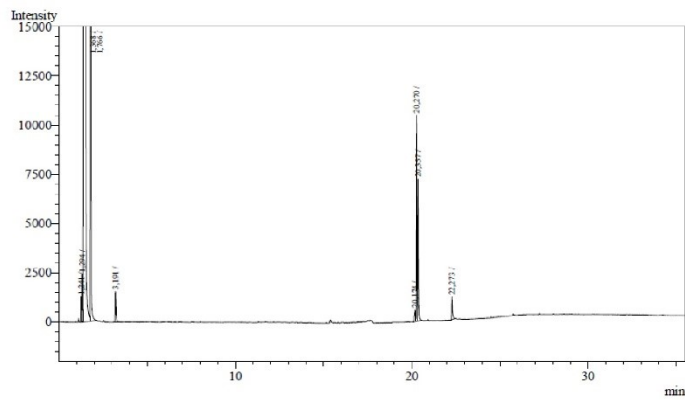
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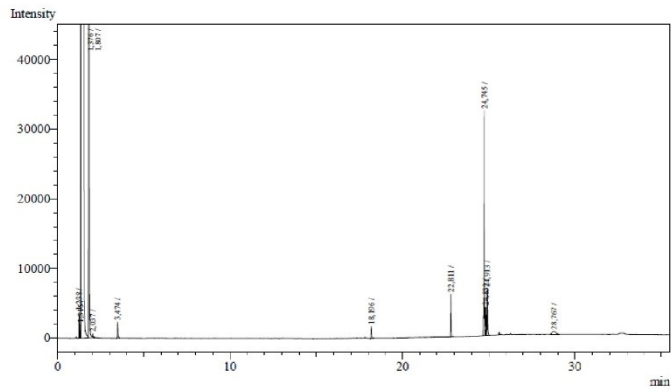
Supplementary Figure 26: Gas chromatogram of the reduction of 5-methyl-2,3-hexanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 20 min.



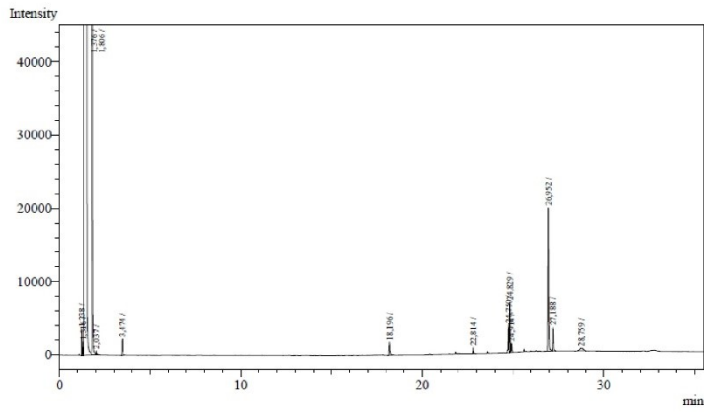
Supplementary Figure 27: Gas chromatogram of the reduction of 2,3-heptanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.



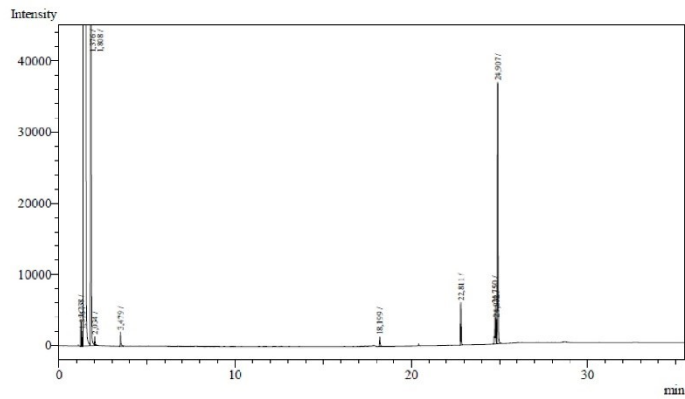
Supplementary Figure 28: Gas chromatogram of the reduction of 2,3-heptanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min.



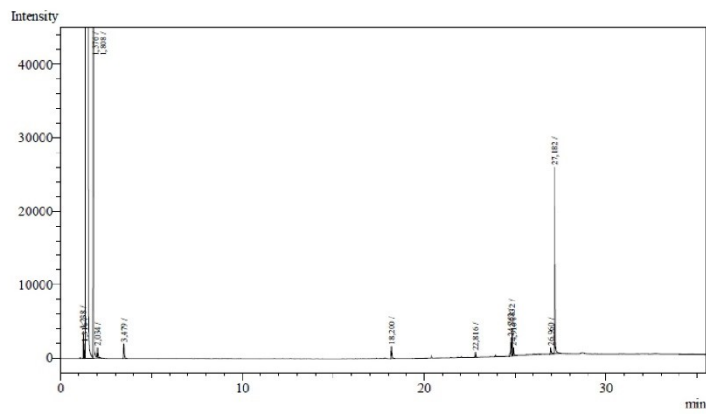
Supplementary Figure 29: Gas chromatogram of the reduction of (S)-1-hydroxy-1-phenyl-2-propanone ((S)-PAC) by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.



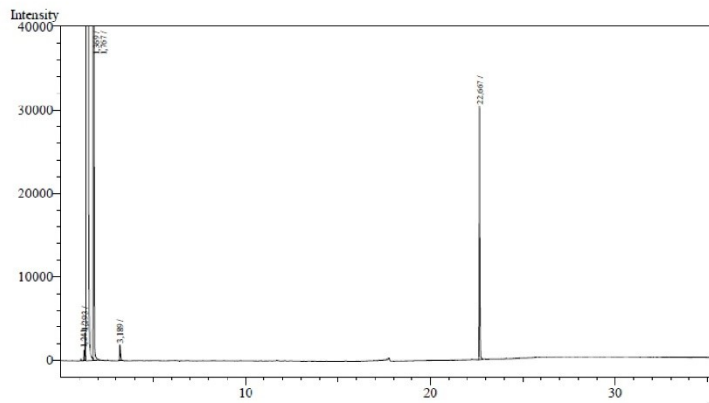
Supplementary Figure 30: Gas chromatogram of the reduction of (S)-1-hydroxy-1-phenyl-2-propanone ((S)-PAC) by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min.



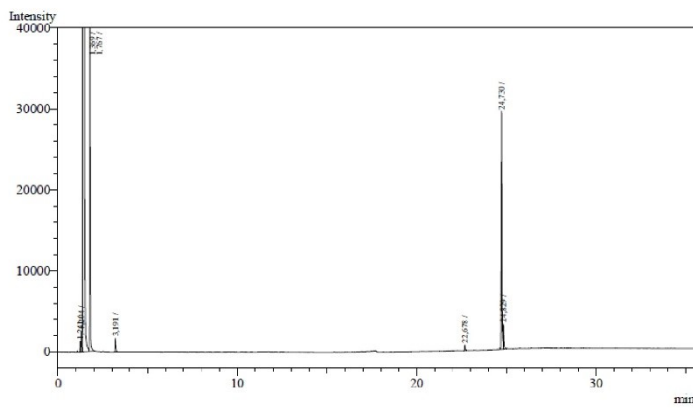
Supplementary Figure 31: Gas chromatogram of the reduction of (*R*)-1-hydroxy-1-phenyl-2-propanone ((*R*)-PAC) by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.



Supplementary Figure 32: Gas chromatogram of the reduction of (*R*)-1-hydroxy-1-phenyl-2-propanone ((*R*)-PAC) by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min.

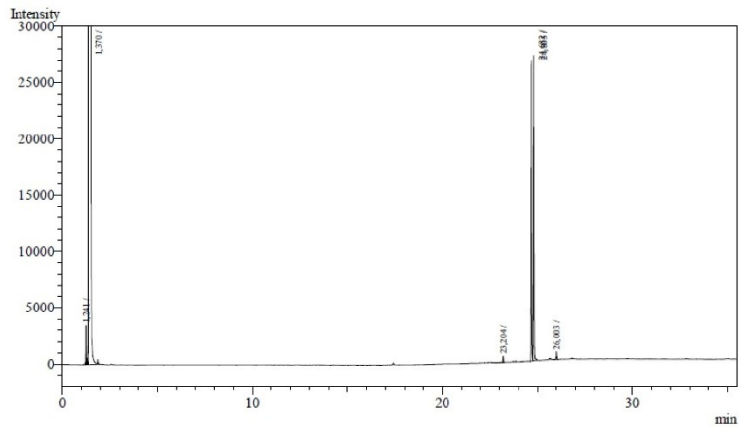


Supplementary Figure 33: Gas chromatogram of the reduction of 1-phenyl-1,2-propanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.

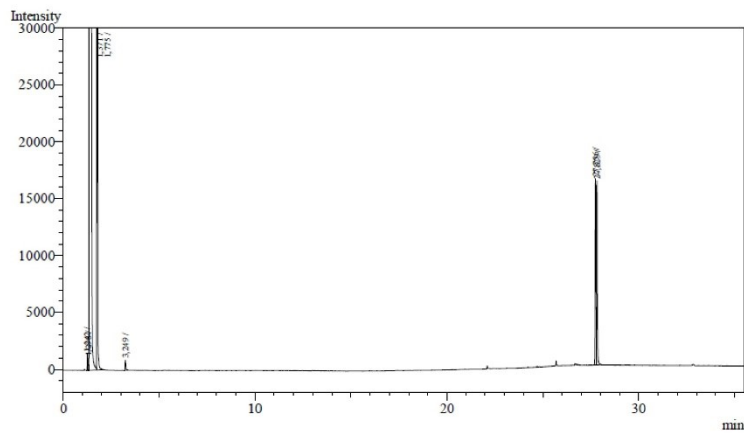


Supplementary Figure 34: Gas chromatogram of the reduction of 1-phenyl-1,2-propanedione by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 60 min.

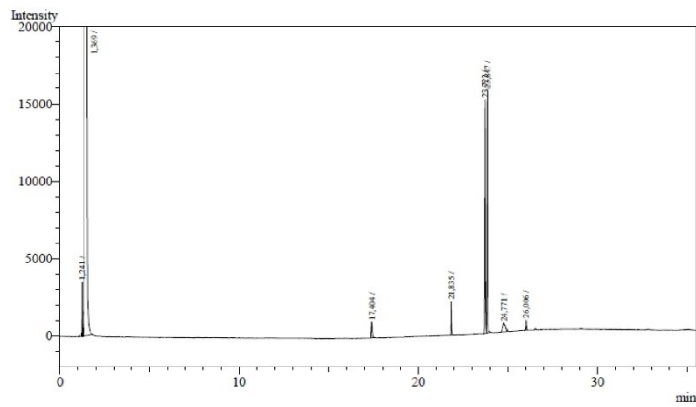




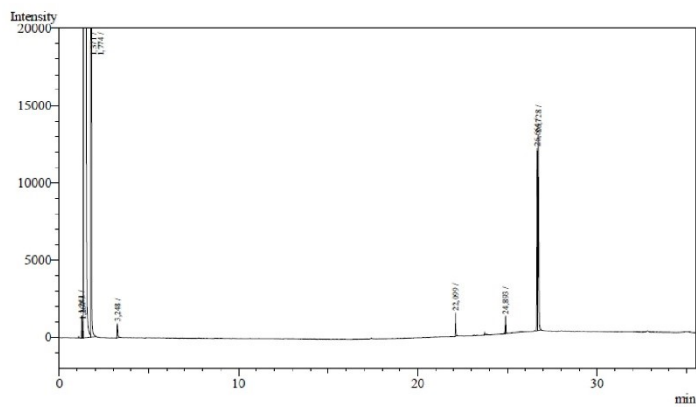
Supplementary Figure 35: Gas chromatogram of the reduction of 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-butanone by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.



Supplementary Figure 36: Gas chromatogram of the reduction of 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-butanone by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 24 hours.



Supplementary Figure 37: Gas chromatogram of the reduction of 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-propanone by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 0 min.



Supplementary Figure 38: Gas chromatogram of the reduction of 1-hydroxy-1-[2-(trifluoromethyl)phenyl]-2-propanone by BIBDH. Substrate (10 mM each) was reduced by 1 U BDH using formate (30 mM) and formate dehydrogenase (5 U) to regenerate NADH (0.3 mM) at 30 °C in potassium phosphate buffer (50 mM, pH 6.8; 1 ml). Reaction time 24 hours.

## 2.4 Publikation 4

### **Development and characterization of a field-effect biosensor for the detection of acetoin**

Denise Molinnus, Lukas Muschallik, Laura Osorio Gonzalez, Johannes Bongaerts, Torsten Wagner, Thorsten Selmer, Petra Siegert, Michael Keusgen, Michael J. Schöning  
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#### Zusammenfassung:

In dieser Publikation wurde zum ersten Mal ein kapazitiver Feld-Effekt Sensor für die Detektion von Acetoin und Diacetyl vorgestellt. Der Biosensor besteht aus Schichten von Al/p-Si/SiO<sub>2</sub>/Ta<sub>2</sub>O<sub>5</sub>. Auf der pH-sensitiven Tantalpentoxid Schicht wurde die (*R,R*)-Butandioldehydrogenase aus *B. clausii* DSM13<sup>T</sup> einmal über Quervernetzung mittels Glutardialdehyd und einmal adsorptiv immobilisiert. Die von der Butandioldehydrogenase katalysierten Reaktion induzierte pH-Änderung im Elektrolyten kann von dem hier vorgestellten Sensor Setup gemessen und in Korrelation mit der Acetoinkonzentration gebracht werden. Typische Sensor Parameter, wie optimaler pH Bereich, Sensitivität, Hysteresis, linearer Konzentrationsbereich und Langzeitstabilität wurden ermittelt. Zusätzlich konnten erste erfolgreiche Messungen in verdünnten Weinproben als Anwendungsbeispiel dargestellt werden.

#### Anteile:

D. Molinnus führte die Planung und Validierung der Experimente, das Entwerfen der Rohfassung des Manuskripts und Umsetzung der Korrektur durch. L. Muschallik führte die heterologe Expression, Reinigung und Charakterisierung der in diesem Sensor verwendeten Butandioldehydrogenase durch sowie Beteiligung am Lektorat des Manuskripts. L. O. Gonzalez unterstützte bei der Durchführung der Experimente. J. Bongaerts, T. Wagner, T. Selmer, P. Siegert, M. Keusgen beteiligten sich durch fachliche Unterstützung und Lektorat des Manuskripts. M. J. Schöning führte die konzeptionelle Planung, Validierung und Betreuung des Projekts sowie die Überarbeitung des Manuskripts durch.



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## Development and characterization of a field-effect biosensor for the detection of acetoin

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## ARTICLE INFO

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## ABSTRACT

A capacitive electrolyte-insulator-semiconductor (EIS) field-effect biosensor for acetoin detection has been presented for the first time. The EIS sensor consists of a layer structure of Al/p-Si/SiO<sub>2</sub>/Ta<sub>2</sub>O<sub>5</sub>/enzyme acetoin reductase. The enzyme, also referred to as butane-2,3-diol dehydrogenase from *B. clausii* DSM 8716<sup>T</sup>, has been recently characterized. The enzyme catalyzes the (R)-specific reduction of racemic acetoin to (R,R)- and meso-butane-2,3-diol, respectively. Two different enzyme immobilization strategies (cross-linking by using glutaraldehyde and adsorption) have been studied. Typical biosensor parameters such as optimal pH working range, sensitivity, hysteresis, linear concentration range and long-term stability have been examined by means of constant-capacitance (ConCap) mode measurements. Furthermore, preliminary experiments have been successfully carried out for the detection of acetoin in diluted white wine samples.

## 1. Introduction

Acetoin and diacetyl are widely distributed in various beverages, and are also used in foods and cosmetics as flavouring and fragrance, as well as in chemical synthesis (Hahn et al., 1987; Lawson et al., 1995; Vasavada and White, 1977; Xiao et al., 2012). These compounds are products of fermentative metabolism in different microorganisms. Acetoin can be formed, e.g., in most bacteria from pyruvate and is thus a product of carbohydrate metabolism, or from diacetyl while NAD(P)H serves as a cofactor of the enzyme acetoin reductase (Cogan et al., 1981; Huang et al., 1999).

During the fermentation process of alcoholic beverages, such as beer or wine, acetoin plays an important role in their quality due to its buttery-like taste, although acetoin is not pungent smelling (Romano and Suzzi, 1996). Typical acetoin concentrations in alcoholic beverages are in the range of 10–50 µM in beer, ~500 µM in white wine and ~150 µM in red wine (Haukeli and Lie, 1975; Reed and Nagodawithana, 1991; Romano and Suzzi, 1996). The detection of acetoin content during the fermentation process could control the quality of alcoholic beverages due to its involvement in the wine bouquet or its influence in the beer flavor. Furthermore, the acetoin concentration during beer storage is used as a parameter to establish the degree of the beer's maturity (Haukeli and Lie, 1975). Precise detection

of the acetoin level can be used to avoid unnecessary maturation time (Romano and Suzzi, 1996; Vann and Sheppard, 2005). Hence, its control of concentration change during the fermentation course could help assess the fermentation processes, as well as the maturation process.

Several methods have already been described for the detection of acetoin, mainly colorimetric techniques, like the Voges-Proskauer test, which is the most commonly applied procedure for the detection of acetoin in analytical microbiology or gas chromatography (Levine, 1916; Speck and Freese, 1973). However, none of these techniques provide the advantages that can be achieved by using a biosensor which offers a faster analytical approach and that does not need additional trained staff.

Capacitive EIS sensors are field-effect devices that are used for the detection of surface potential changes, e.g., due to pH alterations (Poghossian et al., 2004). These changes can also be induced by, e.g., enzymatic reactions (Poghossian et al., 2001b; Schöning et al., 2005b; Siqueira et al., 2014; Thust et al., 1996), and binding of charged molecules, such as DNA (Abouzar et al., 2012; Bronder et al., 2015; Poghossian et al., 2001a; Veigas et al., 2015). These sensors can also be applied for the development of enzyme logic gates (Molinnus et al., 2017b; Poghossian et al., 2011, 2015). Furthermore, EIS sensors have many advantages over conventional analytical methods such as small size, low weight and fast response time, and they are easy and cost-

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effective in fabrication (Poghossian and Schöning, 2014; Schöning et al., 2005a). Furthermore, due to the miniaturized sensor layout, a small sample volume is necessary for the measurement. Additionally, with an array of different modified EIS sensors, several analytes can be detected simultaneously.

In this study, we report on the development of a chip-based biosensor for the acetoin detection for the first time. The sensor is based on a pH-sensitive capacitive EIS field-effect structure consisting of an Al/p-Si/SiO<sub>2</sub>/Ta<sub>2</sub>O<sub>5</sub> layer set-up. The chip was modified using a recently introduced acetoin reductase from *B. clausii* DSM 8716<sup>T</sup> (Muschallik et al., 2017) accountable for the reduction of acetoin in the presence of NADH as a cofactor (Molinnus et al., 2017a). The local pH shift induced by the enzymatic reaction resulted in the modulation of the flat-band potential of the field-effect biosensor, arising in a shift of the capacitance-voltage (C-V) curve of the EIS structure. Two immobilization strategies, namely adsorptive and cross-linking by forming cross-linkages between the enzyme molecules, have been investigated to attach the acetoin reductase to the pH-sensitive Ta<sub>2</sub>O<sub>5</sub> surface of the EIS sensor chip. Characteristic biosensor parameters such as linear concentration range, pH optimum of the field-effect sensor with the immobilized enzyme, sensitivity, lower and upper detection limit, hysteresis and long-term stability will be discussed. In addition, the newly developed acetoin biosensor was applied for measurements in real samples such as white wine.

## 2. Experimental

### 2.1. Materials

Acetoin and the cofactor NADH were acquired from Sigma-Aldrich (USA), as well as glutaraldehyde, glycerol and NaCl. TRIS-HCl buffer (0.2 mM) was purchased from Carl Roth (Germany). The respective pH values of the buffer solution were adjusted by addition of 0.1 M NaOH or 0.1 M HCl. The enzyme acetoin reductase from *B. clausii* (~380 U/mL) is produced in our institute as described before (Muschallik et al., 2017).

### 2.2. Preparation of the sensor structures

The applied capacitive EIS sensor consists of the following layer stack: a p-doped silicon substrate with a thickness of ~400 μm and a specific resistance of ρ = 5–10 Ωcm, a 30 nm thermally grown SiO<sub>2</sub> insulating layer and a 60 nm thick Ta<sub>2</sub>O<sub>5</sub> gate insulator layer (for that 30 nm Ta is deposited by electron-beam evaporation, followed by a thermal oxidation step). A rear side contact, consisting of a 300 nm thick aluminum layer is deposited by electron-beam evaporation and annealed afterwards. As a final step, the wafer is separated into 1 cm × 1 cm chips with a diamond saw. Fig. 1 shows the EIS-sensor set-up with the different layers. Detailed information about the sensor's fabrication process is described in Ref. (Schöning et al., 2005a). The Ta<sub>2</sub>O<sub>5</sub> layer as gate insulator has been selected because of its well-known excellent pH behavior and high permittivity but also because of its chemical stability (Atanassova and Spassov, 1998; Chaneliere et al., 1998).

The acetoin EIS biosensor was developed by modifying the Ta<sub>2</sub>O<sub>5</sub> surface with the enzyme acetoin reductase. Immediately before modification, each sensor was cleaned in acetone, isopropanol and deionized water for 5 min, respectively. Two different immobilization strategies have been investigated. As first immobilization method, the enzyme acetoin reductase is adsorptively bound to the sensor surface. For this, 80 μL of acetoin reductase solution was dropped onto the sensor surface. For the second immobilization procedure, cross-linking is performed by formation of cross-linkages between the enzyme molecules, where the membrane cocktail consisting of 48 μL glutaraldehyde (2 vol%) / glycerol (10 vol%) solution and 32 μL of enzyme solution was mixed. 80 μL of the membrane cocktail was pipetted onto the Ta<sub>2</sub>O<sub>5</sub> surface. After drying of the different prepared EIS sensors,

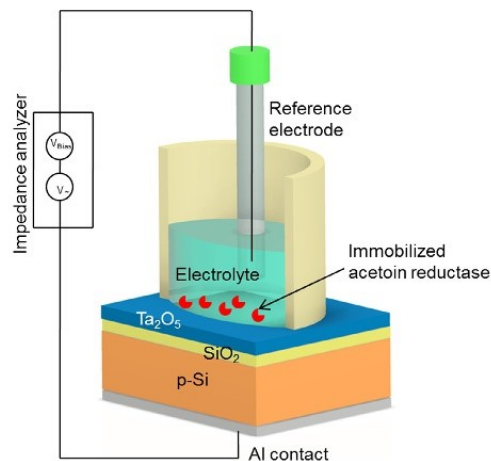


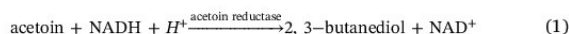
Fig. 1. Schematic of the measurement set-up with the Al-p-Si-SiO<sub>2</sub>-Ta<sub>2</sub>O<sub>5</sub> EIS sensor modified with the enzyme acetoin reductase for the detection of acetoin.

they were mounted into a homemade measuring cell, sealed by an O-ring to protect the rear side contact and to define the contact area of the EIS sensor with the analyte solution (~0.5 cm<sup>2</sup>). Before measurements, the sensors were stored at 4 °C in the dark.

## 3. Measurement principle

Fig. 1 illustrates the measurement set-up with the developed acetoin field-effect biosensor. For the electrochemical characterization of the acetoin biosensor chip, C-V (capacitance / voltage) and ConCap (constant capacitance) measurements were performed by connecting the EIS chip with an impedance analyzer IM6 (Zahner Elektrik, Germany). Before performing ConCap measurements, C-V curves of each sensor chip, in a gate voltage range between -2 V and 2 V with steps of 100 mV were recorded to define a fixed capacitance value (in the linear range of the depletion region, ~60% of the maximum capacitance) using a feedback-control circuit. With the help of ConCap measurements, potential and/or charge changes at the Ta<sub>2</sub>O<sub>5</sub> surface can be detected in real time. An external liquid-junction Ag/AgCl electrode (Metrohm, Germany) filled with 3 M KCl was applied as the reference electrode. The C-V and ConCap measurements were carried out at a frequency of 120 Hz. A 20 mV ac (alternating current) voltage has been applied between the Ag/AgCl reference electrode and the rear side Al contact, to measure the capacitance.

The measurement principle for the detection of acetoin using the capacitive field-effect sensor is based on the enzymatic reaction as depicted in Eq. (1). (R)- and (S)-acetoin will be reduced by the R-specific enzyme acetoin reductase to (R,R)-2,3-butanediol and *meso*-butanediol, respectively, while NADH serves as a cofactor and will be oxidized to NAD<sup>+</sup>.



As a result of this enzymatic reaction, the hydrogen ion concentration decreases, and this pH change can be detected by the pH-sensitive Ta<sub>2</sub>O<sub>5</sub> transducer surface of the field-effect sensor. The resulting change in the flat-band potential of the sensor is recorded and corresponds to the measured acetoin concentration. All measurements were performed in a dark Faraday cage at room temperature. Before starting the measurements, the sensor chip was incubated in 0.2 mM TRIS-HCl buffer solution (pH 7.1) containing 150 mM NaCl for 2 h. All solutions contained 500 μM of the cofactor NADH and all measurements were performed in 1 mL analyte solution containing different acetoin

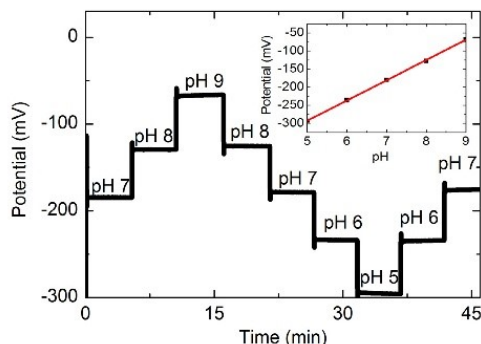


Fig. 2. Typical ConCap measurement for different pH values of Titrissol buffer solution of a bare EIS sensor; inset figure depicts the resulting calibration curve with an average slope of 55.9 mV/pH between pH 5 and pH 9.

concentrations varying from 1  $\mu\text{M}$  to 500  $\mu\text{M}$ . The pH value of the solutions used was additionally controlled by a pH meter (Mettler-Toledo, Germany).

#### 4. Results and discussions

##### 4.1. Electrochemical characterization of the capacitive acetoin biosensor

Before surface modification, the functionality and pH sensitivity of the bare EIS sensor chip was studied. Therefore, the pH sensitivity of each sensor was determined in the ConCap measurement mode with standard pH buffer solutions (Titrissol, Merck, Germany). The result of a typical ConCap measurement of a bare EIS sensor chip is presented in Fig. 2, for the pH values of 7-8-9-8-7-6-5-6-7, respectively. Each pH value has been recorded for 5 min. As the pH buffer solution is changed, an immediate signal step is perceived. This result shows that the EIS sensor is highly reproducible as demonstrated for pH 7.0, which was measured three times within this measurement cycle, yielding a potential at this pH value that is always  $-179 \pm 5$  mV. The corresponding calibration curve is shown in the inset figure of Fig. 2 and demonstrates a nearly Nernstian pH sensitivity of 55.9 mV/pH, as described in literature (Schöning et al., 2005a).

Additionally, different acetoin concentrations in the range between 30  $\mu\text{M}$  and 90  $\mu\text{M}$  were tested with the bare sensor chip (i.e., without the immobilized enzyme) (data not shown). The variation of the acetoin concentration only resulted in negligible potential changes of less than 3 mV, which can be related to slight pH variations of the analyte and drift effects of the sensor chip itself. In a further experiment, the EIS sensor chip modified with the enzyme acetoin reductase was also examined with regard to its original pH sensitivity in Titrissol buffer solutions of different pH values (identical procedure as performed in Fig. 2). The sensor modified by the enzyme possesses a similar sensitivity of 55.7 mV/pH as for the bare EIS sensor. Thus, the immobilized enzyme has no influence on the pH response of the pH-sensitive  $\text{Ta}_2\text{O}_5$  transducer layer.

The immobilization of the enzyme onto the sensor surface is always an essential step in the development of a biosensor. Several immobilization methods such as adsorptive, covalent bonding, entrapment or cross-linking have been discussed in literature (Sassolas et al., 2012). For the immobilization of the enzyme acetoin reductase, two different immobilization methods were investigated, in particular, adsorptive and cross-linking by using glutaraldehyde to form a stable membrane which is drop-coated onto the sensor surface. Biosensor chips were prepared with each immobilization method. The influence of the enzyme immobilization on the sensitivity of the capacitive acetoin EIS biosensor chip was studied at different acetoin

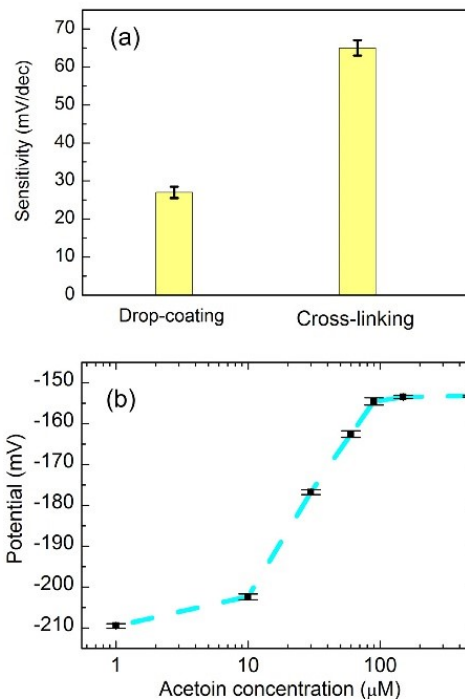
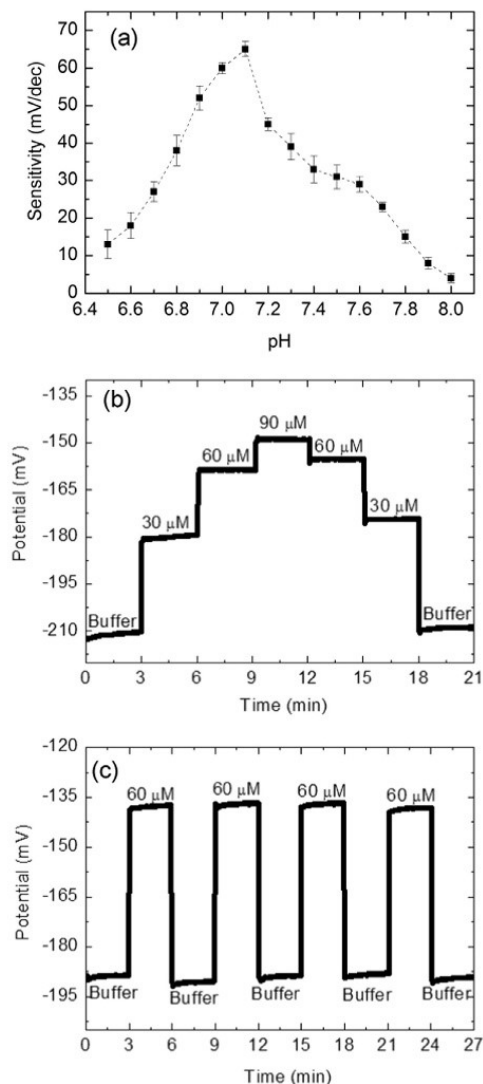


Fig. 3. (a) Mean values of the sensitivity with standard deviation (set of three series of measurements for each immobilization method) for the capacitive acetoin EIS biosensor chip recorded in buffer solution with different acetoin concentrations from 30  $\mu\text{M}$  to 90  $\mu\text{M}$ . (b) Calibration plot with standard deviation obtained with the capacitive, cross-linked acetoin EIS biosensor chip measured in an acetoin concentration range between 1  $\mu\text{M}$  and 500  $\mu\text{M}$ .

concentrations ranging between 30  $\mu\text{M}$  and 90  $\mu\text{M}$  in 0.2 mM TRIS-HCl buffer solution, containing 150 mM NaCl and 500  $\mu\text{M}$  NADH at pH 7.1. Fig. 3(a) shows the results of the mean values of the sensitivities. The two bars depict the mean values of the sensitivities obtained from each three individual sensors modified using adsorptive and cross-linking, respectively. The highest sensitivity has been observed with the sensors modified by means of cross-linking with a mean sensitivity of 65 mV/dec, while the sensors modified using adsorptive immobilization only have a mean sensitivity of 27 mV/dec. These results demonstrate that although the adsorption method causes little to no enzyme inactivation, the enzymes are probably loosely attached to the sensor surface resulting in less amount of enzymes, which are fixed on the surface and hence, a lower acetoin sensitivity was achieved. Therefore, for the subsequent experiments, the sensor was modified by applying the cross-linking method for the immobilization of the enzyme.

To investigate the lower and upper detection limit of the developed biosensor chip towards acetoin, ConCap measurements were further performed in the acetoin concentration range between 1  $\mu\text{M}$  and 500  $\mu\text{M}$ . Fig. 3(b) shows an S-shaped calibration curve, as typically expected for electrochemical biosensors. The sensor signal is plotted versus the logarithmic acetoin concentration. A linear behavior in the acetoin concentration range between 10  $\mu\text{M}$  and 90  $\mu\text{M}$  with a sensitivity of 65 mV/dec is given. A saturation effect is resulting for acetoin concentrations higher than 150  $\mu\text{M}$ . Due to the enzymatic reaction, the pH increase close to the sensor surface might lead to an enzyme inhibition due to the pH dependence of acetoin reductase's activity (see also Fig. 4). Note that a shift in the biosensor signal of about 65 mV for varying acetoin concentrations corresponds to a pH shift from originally



**Fig. 4.** (a) Mean values of the sensitivity with standard deviation (set of three series of measurements) for three fabricated capacitive acetoin EIS biosensors recorded in TRIS-HCl buffer containing 150 mM NaCl in the pH range between pH 6.5 and pH 8.0 with different acetoin concentrations from 30  $\mu$ M to 90  $\mu$ M. (b) Typical ConCap calibration measurement of different acetoin concentrations in the range from 30  $\mu$ M to 90  $\mu$ M measured by the acetoin biosensor chip. (c) Reproducibility measurement of buffer solution alternating with 60  $\mu$ M acetoin.

pH 7.1 to about pH 8.2 at the biosensor surface. This is defined by the pH sensitivity of the Ta<sub>2</sub>O<sub>5</sub> layer with immobilized enzyme of about 56 mV/pH as described above. On the other side, the curve starts to get flat at low acetoin concentrations (< 10  $\mu$ M) that defines the detection limit of the biosensor set-up.

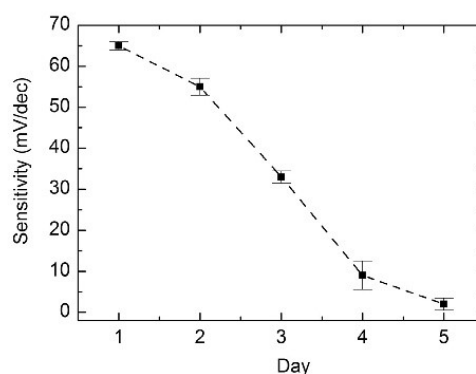
To study the pH influence on the sensitivity of the developed capacitive acetoin EIS biosensor in more detail, measurements in the ConCap mode were performed by variation of the pH of the buffer solution over a range from pH 6.5 to pH 8.0. Different acetoin concentrations ranging from 30  $\mu$ M to 90  $\mu$ M (buffer solution) were taken

into account for the measurement. Fig. 4(a) summarizes the mean values and their standard deviations of the sensitivity obtained for three individual acetoin biosensors determined at each pH value. Maximum response with a sensitivity of  $65 \pm 4$  mV/dec could be reached at a pH value of  $\sim 7.1$ , which is also consistent with the pH optimum of the used acetoin reductase ( $\sim$ pH 7.0) for the given reaction (Muschallik et al., 2017).

Fig. 4(b) demonstrates an example of a ConCap measurement of the acetoin biosensor with regard to its hysteresis behavior and response time. Acetoin concentrations were varied starting with a concentration of 30  $\mu$ M up to 90  $\mu$ M and then decreasing the concentration again down to 30  $\mu$ M. Each concentration was recorded for 3 min and was varied in steps of 30  $\mu$ M. A clear dependence of the sensor signal on the acetoin concentration is observed. The sensor signal is increasing with an increase of the acetoin concentration due to the decrease of the H<sup>+</sup> ion concentration as discussed in Eq. (1) affected by the biocatalytic reaction. Furthermore, the sensor output is almost the same in the upward and downward series of the performed measurement with a small hysteresis of less than 5 mV. In contrast to standard measurement techniques for the detection of acetoin (Speck and Freese, 1973), the response time  $t_{90\%}$  is only about 25 s by using the developed acetoin EIS biosensor.

To proof the reproducibility of the developed acetoin biosensor chip, the sensor signal of the measurement performed in TRIS-HCl buffer solution at pH 7.1 was recorded in alternation with an acetoin concentration of 60  $\mu$ M in the ConCap mode. Fig. 4(c) shows exemplarily the results obtained with one sensor, when this cycle is repeated four (60  $\mu$ M acetoin) and five (buffer) times, respectively. The sensor signal is increased to a value of  $-137 \pm 1$  mV with each acetoin concentration step and again decreased when measured in buffer solution to a value of  $-189 \pm 1$  mV. This experiment again underlines that the sensor delivered reproducible and reversible results independent of the titration direction, only with a small hysteresis of  $\sim 1$  mV.

The stability of a biosensor often limits its use to a certain number of measurements, and losses of sensitivity are caused due to either the decrease of the applied enzyme activity or the enzyme leakage out from the sensor surface after certain time. Therefore, the biosensor's stability has been studied over a time period of 5 days. At each day, different acetoin concentrations between 30  $\mu$ M and 90  $\mu$ M were measured in buffer solution. Three individual biosensor chips were tested. Between the measurements, the sensors were stored under dry conditions in the refrigerator at 4  $^{\circ}$ C. The results of mean values of the obtained sensitivities with their standard deviations are presented in Fig. 5. Within



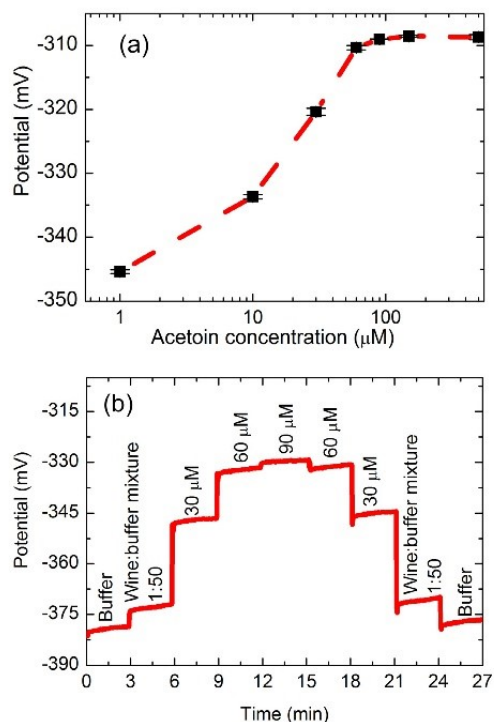
**Fig. 5.** Long-term stability of the acetoin biosensor. Mean values of the sensitivity with standard deviations of three individual biosensors recorded in buffer solution at different days (day 1 to day 5) with different acetoin concentrations from 30  $\mu$ M to 90  $\mu$ M.

the first two days, the biosensors show good sensitivities of about 65 mV/dec and 55 mV/dec, respectively. However, after two performed measurement days, the sensitivity starts to decrease rapidly to a value of 3 mV/dec at day 5. These results are comparable to results obtained, when the pure enzyme activity was investigated in solution without immobilization (Muschalik et al., 2017).

#### 4.2. Application of the acetoin biosensor chip in wine

The aim of the newly developed capacitive acetoin EIS biosensor is to control and monitor fermentation processes to avoid unnecessary storage of beer or wine. Therefore, such biosensor chip should be able to detect acetoin in real fermentation samples.

In this study, preliminary experiments have been performed in diluted white wine samples (alcohol 10 vol%) to verify the ability of the acetoin biosensor. The pH value of white wine was about pH 3.3. After dilution with TRIS-HCl buffer solution (pH 7.1) containing 150 mM NaCl (wine to buffer mixture of 1:50), the pH value was about pH 3.8. Therefore, the pH value was adjusted to pH 7.1, the optimum working pH of the field-effect sensor with the immobilized enzyme, by addition of 0.1 M NaOH. Different acetoin concentrations between 1  $\mu\text{M}$  and 500  $\mu\text{M}$  were spiked to the wine-buffer mixture. As already demonstrated for the buffer solution containing varying acetoin concentrations (see Fig. 3(b)), Fig. 6(a) depicts again a typical S-shaped calibration curve obtained in the wine-buffer mixture with the potential recordings depending on the logarithmic acetoin concentration. Here, a linear behavior was reached in the acetoin concentration range between 10  $\mu\text{M}$  and 90  $\mu\text{M}$  with an average sensitivity of 40 mV/dec. Note



**Fig. 6.** (a) Calibration plot with standard deviation obtained with the capacitive acetoin EIS biosensor measured in a concentration range between 1  $\mu\text{M}$  and 500  $\mu\text{M}$  in a wine-buffer mixture (1:50). (b) ConCap measurement of different acetoin concentrations in a wine-buffer mixture (1:50) in the range from 30  $\mu\text{M}$  to 90  $\mu\text{M}$  measured by the acetoin biosensor chip.

that the average sensitivity is somewhat lower than for the acetoin measurements in buffer without wine (65 mV/dec). On the other hand, the sensitivity and detection limit of the developed biosensor is sufficient to detect acetoin in the industrially relevant concentration range. For the detection of higher concentrations, the sample might be diluted if necessary.

Fig. 6(b) demonstrates exemplarily a ConCap response of the acetoin biosensor in buffer solution and wine-buffer mixture, both at pH 7.1, without acetoin or spiked acetoin concentrations (30–90  $\mu\text{M}$ ). The sensor signal obtained in each solution was recorded for 3 min. Again, with increasing acetoin concentrations, the biosensor signal raises, clarifying the clear dependence of the biosensor signal in wine-buffer mixtures with spiked acetoin content. Interestingly, an increase in the biosensor signal was also observed for the pure wine-buffer mixture (without additionally spiked acetoin). The shift in contrast to the sensor signal in buffer might be explained due to the naturally present acetoin content in that sample. These experiments in wine-buffer mixture underline that the developed acetoin biosensor can detect different acetoin concentrations even in real samples.

#### 5. Conclusions

A capacitive EIS field-effect biosensor has been modified by a recently introduced acetoin reductase. Due to the immobilized enzyme, racemic acetoin will be converted to (*R,R*)-2,3-butanediol and *meso*-butanediol, respectively, in a  $\text{H}^+$ -consuming reaction resulting in a pH change that can be detected by the EIS sensor. The acetoin biosensor has been characterized regarding the immobilization method and the pH optimum. The developed biosensor depicted the highest sensitivity through immobilization of the enzyme via cross-linking by using glutaraldehyde at a pH value of 7.1. Furthermore, reproducible measurement results could be achieved at different acetoin concentrations in the range between 10  $\mu\text{M}$  and 100  $\mu\text{M}$  with an average acetoin sensitivity of 65 mV/dec. The sensor shows a long-term stability of around two days, which should be further improved. Ongoing experiments are dealing with the stabilization of the enzyme's activity. Moreover, first experiments in white wine have been carried out where acetoin concentrations between 10  $\mu\text{M}$  and 90  $\mu\text{M}$  could be successfully detected, giving a hint that the sensor can be applied to real fermentation solutions. In future experiments, possible cross-sensitivity effects towards diacetyl and acetylbutanediol shall be investigated, especially for diacetyl, which is also an important flavor in beer and responsible for the buttery taste (Wainwright, 1973). Additionally, the obtained results in real samples, such as wine or beer, should be compared with already established measurement methods, like gas chromatography.

Thus, both substances (acetoin and diacetyl) can be used as indicator for different fermentation processes and could prove the quality of alcoholic beverages. By applying Boolean operations (such as AND, OR, NAND,...) (Katz et al., 2017; Katz and Minko, 2015) and by defining a threshold voltage of the particular biosensor signal (depending on the fermentation process), the overall sensor read-out could describe the status of the fermentation course. By simultaneously detecting diacetyl and acetoin, the fermentation process could be stopped at the right moment to avoid unnecessary fermentation or maturation time and finally, to accelerate the process. Furthermore, the unpleasant buttery-like flavor, which occurs due to exceeded fermentation/maturation process, can be avoided.

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### 3 Diskussion

Der Fokus dieser Arbeit liegt in der Evaluierung von ausgewählten Butandioldehydrogenasen für den Einsatz in der Biokatalyse. Obwohl diese Enzyme als Schlüsselement in der fermentativen Produktion von 2,3-Butandiol bekannt sind<sup>67,72</sup>, wurden sie für den isolierten Einsatz in Biotransformationen vor Beginn dieser Arbeit kaum untersucht. Physiologisch reduzieren diese Enzyme das vicinale Diketon Diacetyl zum  $\alpha$ -Hydroxyketon Acetoin und dieses weiter zum vicinalen Diol 2,3-Butandiol. Ebenfalls ist die Rückreaktion, die Oxidation vom 2,3-Butandiol zum Acetoin, möglich. Die Reduktion ausgehend vom einen vicinalen Diketon kann dabei strikt enantioselektiv durchgeführt werden. So werden diese Enzyme in (*R*)-, (*S*)- und *meso*-Butandioldehydrogenasen eingeteilt. Aufgrund dieser Eigenschaften ist die Verwendung dieser Enzymklasse für die enantioselektive biokatalytische Synthese von diversen nicht physiologischen  $\alpha$ -Hydroxyketonen und vicinalen Diolen denkbar. Die stereoisomerenreine Synthese, besonders von  $\alpha$ -Hydroxyketonen, ist vor allem aufgrund deren breiten Einsatzspektrums interessant. So können diese Moleküle als Synthesebausteine für Feinchemikalien und Pharmazeutika, als Geschmacksstoffe in Nahrungsmitteln oder als Pheromone für bestimmte Insektenarten eingesetzt werden<sup>53,57,73</sup>. 5-Methyl-2-hydroxy-3-hexanon und 5-Methyl-3-hydroxy-2-hexanon können zum Beispiel als „röstbraun“-Aroma verwendet werden<sup>74</sup>. Andere lineare aliphatische  $\alpha$ -Hydroxyketone und vicinale Diole, wie 3-Hydroxy-2-hexanon, 3-Hydroxy-2-oktanon oder 2,3-Hexandiol, werden in Pheromonfallen in der Schädlingsbekämpfung eingesetzt<sup>53,56,75</sup>. Da die rein chemische Synthese dieser Moleküle oft harsche Reaktionsbedingungen oder die Verwendung von schwermetallhaltigen Katalysatoren beinhaltet, ist die biokatalytische Synthese oft wünschenswert. Auf biokatalytischem Wege ist die Synthese von  $\alpha$ -Hydroxyketonen vor allem über Thiamindiphosphat-abhängige Enzyme, oder über dynamische kinetische Racematspaltung mittels Lipasen beschrieben<sup>64,66,76–78</sup>. Butandioldehydrogenasen können hier eine sinnvolle Erweiterung der biokatalytischen Möglichkeiten zur Synthese solcher Moleküle darstellen. Im Folgenden sollen die in dieser Arbeit verwendeten Enzyme im Hinblick auf ihre biochemischen Eigenschaften eingeordnet und für die Verwendung in der biokatalytischen Synthese bewertet werden.

### 3.1 Überblick der Ergebnisse

Die Ergebnisse zur biochemischen und biokatalytischen Charakterisierung der Enzyme, die in dieser Arbeit untersucht wurden, wurden in der AG Biokatalyse der FH Aachen im Institut für Nano- und Biotechnologie (INB) erstellt.

In **Publikation 1** (Kap. 2.1) wurde die initiale biochemische Charakterisierung der neuartigen Butandioldehydrogenase aus *Bacillus clausii* DSM 8716<sup>T</sup> (BcBDH) berichtet. Grundlegende biochemische Parameter wie Temperatur, pH-Optima sowie kinetische Daten zu den physiologischen Substraten Acetoin, Diacetyl und 2,3-Butandiol wurden ermittelt. Eine Einordnung des Enzyms in die Gruppe der MDR-Dehydrogenasen war aufgrund von konservierten Motiven in der Aminosäuresequenz möglich. Neben der Bestimmung der Stereoselektivität für die physiologischen Substrate lag ein besonderer Fokus auf der Untersuchung des Substratspektrums.

**Publikation 2** (Kap. 2.2) baut auf den Ergebnissen von Publikation 1 auf und beschreibt die biokatalytische Charakterisierung der BcBDH. Es wurde der Einfluss von diversen wassermischbaren Lösungsmitteln sowie von Metallionen auf die Enzymaktivität untersucht. Zudem wurde das Substratspektrums im Hinblick auf Moleküle, die einen Phenylrest beinhalten, vertieft. Der Hauptfokus lag in der Untersuchung der Reduktion diverser Diketone und  $\alpha$ -Hydroxyketone mit diesem Enzym. Die Bestimmung der Stereoselektivität und Produktidentifikation erfolgte mittels Gaschromatographie, Massenspektroskopie und Circular dichroismus-Spektroskopie.

In **Publikation 3** (Kap. 2.3) wurde, das bereits durch Xu *et al.* initial beschriebene Enzym<sup>34</sup>, die Butandioldehydrogenase aus *Bacillus licheniformis* DSM 13<sup>T</sup> (BIBDH) auf seine biokatalytischen Eigenschaften untersucht. Hierfür wurde die Reduktion diverser Diketone und  $\alpha$ -Hydroxyketone im Hinblick auf die Produktselektivität betrachtet. Zusätzlich wurde eine detailliertere Untersuchung des Substratspektrums durchgeführt. Auch erfolgte ein tieferer Einblick in die biochemischen Parameter des Enzyms für nicht physiologische Substrate.

Im Rahmen dieser Promotionsarbeit erfolgte, in Kooperation mit der Arbeitsgruppe für Chemo- und Biosensorik der FH Aachen, die Beteiligung an einem Projekt zur Entwicklung eines potentiometrischen Biosensors für die Detektion von Acetoin und Diacetyl in Fermentationsprozessen (**Publikation 4**, Kap. 2.4). Butandioldehydrogenasen dienen dabei in diesem Sensor als elektrochemischer Transduktor. Die grundlegende Funktion des Sensor, auch in realen Weinproben, konnte bereits publiziert werden<sup>79</sup>.

Zudem konnten erfolgreich zwei Bachelorarbeiten in diesem Projekt beendet und im Rahmen dieser Promotion betreut werden<sup>80,81</sup>.

### **3.2 Butandioldehydrogenasen aus *Bacillus clausii* DSM 8716<sup>T</sup> und *Bacillus licheniformis* DSM 13<sup>T</sup>**

In dieser Arbeit wurden die Butandioldehydrogenasen aus *Bacillus clausii* DSM 8716<sup>T</sup> (BcBDH) und *Bacillus licheniformis* DSM 13<sup>T</sup> (BIBDH) untersucht. Ein wesentlicher Unterschied beider Enzyme ist die Zugehörigkeit in die jeweilige Superfamilie. So wird die BDH aus *Bacillus clausii* DSM 8716<sup>T</sup> in die Superfamilie der MDR-Dehydrogenasen eingeordnet. Die BDH aus *Bacillus licheniformis* DSM 13<sup>T</sup> gehört hingegen in die Superfamilie der SDR-Dehydrogenasen. Wie in Kap. 1.3 bereits erwähnt, weisen BDHs unterschiedlicher Superfamilien-Zugehörigkeit teils starke biochemische Unterschiede auf. Die markantesten sind dabei die Metallionenabhängigkeit, die Quartärstruktur sowie meist die Stereoselektivität. Im Folgenden sollen nun die beiden BDHs auch vergleichend im Hinblick auf eine Evaluierung der Enzyme für den biokatalytischen Einsatz und im Kontext der Literatur verglichen werden.

#### **3.2.1 Vergleichende biochemische Charakterisierung der BcBDH und BIBDH**

Die Gene beider Enzyme konnten erfolgreich kloniert und in *E. coli* BL21(DE3) mit sehr guten Ausbeuten (ca. 50% in der lösl. Fraktion) überexprimiert werden. Die Reinigung erfolgte in beiden Fällen über eine Strep-Tag Affinitätschromatographie. Dazu wurden Gen-Varianten mit jeweils einem N-terminalen und C-terminalen StrepII-Tag kloniert. Jedoch zeigte es sich, dass in beiden Fällen nur die Variante mit N-terminalen Strep-Tag aktiv hergestellt wurde. Ein Sequenzvergleich ist aufgrund der großen strukturellen Unterschiede beider Enzyme nicht sinnvoll.

So besitzt die BIBDH pro Untereinheit 260 Aminosäuren und bildet als aktives Enzym ein Homotetramer. Die BcBDH hingegen bildet mit 351 Aminosäuren pro Untereinheit ein Homodimer. Enzyme aus der Klasse der MDR-Dehydrogenasen bestehen aus zwei Domänen, wobei die C-terminale Domäne für die Cofaktorbindung verantwortlich ist (Kap. 1.2, Abbildung 2)<sup>22</sup>.

Für Enzyme aus der Superfamilie der SDR-Dehydrogenasen ist die C-terminale Region verantwortlich für die Substratbindung<sup>82</sup>, was vermutlich der Grund für die Inaktivität der

Variante mit C-terminalen StrepII-Tag ist, aufgrund möglicher sterischer Komplikationen im aktiven Zentrum. In einer SDS-PAGE Analyse konnte eine Bande bei der erwarteten Größe verifiziert werden. Jedoch besteht auch die Möglichkeit einer durch den C-terminalen StrepII-Tag induzierte Fehlfaltungen.

Im Hinblick auf das pH-Optimum und das Temperaturoptimum sind die beiden Enzyme vergleichbar (Publikation 1 und 3). Wie in Tabelle 3 dargestellt, liegt der größte Unterschied in einem breiteren pH-Optimum von BIBDH für die Reduktion von Acetoin, sowie einem niedrigeren Temperaturoptimum im Vergleich zu der BcBDH. Eine initiale biochemische Charakterisierung der BIBDH wurde bereits von Xu *et al.* publiziert<sup>34</sup>. Das pH-Optimum wurde in dieser Arbeit im Rahmen der biokatalytischen Charakterisierung erneut bestimmt, da sich die Aktivitätstests deutlich voneinander unterscheiden. So wurde in dieser Arbeit eine Substratkonzentration von 10 mM statt 5 mM verwendet, da erst ab dieser Substratkonzentration  $V_{max}$  erreicht wird. Auch wurde eine geringere NADH Konzentration (0,3 mM statt 1 mM) eingesetzt. Es konnte gezeigt werden, dass eine Cofaktorkonzentration von 0,3 mM bereits zur maximalen Aktivität ( $V_{max}$ ) führt. Vorversuche deuteten sogar auf eine potenzielle Überschussinhibierung bei Konzentrationen über 0,5 mM NADH bei der BIBDH hin. Eine NADH Cofaktorinhibierung bei Konzentrationen über 0,3 mM und einer kompletten Inaktivierung bei 1 mM NADH wurde auch für die BDH aus *Serratia marcescens* CECT 977 berichtet<sup>27</sup>, ein Enzym, das ebenfalls in die Superfamilie der SDR-Dehydrogenasen gehört.

Tabelle 3: Temperatur- und pH-Optima der BcBDH und BIBDH im Vergleich (Publikation 1 und 3). \*entnommen aus Xu *et al.*<sup>34</sup>

Enzym	pH-Optimum			Temperaturoptimum
	Reduktion Diacetyl	Reduktion Acetoin	Oxidation 2,3-Butandiol	
BcBDH	6,0	7,0	8,0	50 °C
BIBDH	5,0	6,0 – 8,0	9,0	37 °C*

Diese Parameter gliedern sich gut in die Werte der bisher beschriebenen BDHs ein. Bei den MDR-BDHs wurde das pH-Optimum für die Diketon-Reduktion in einem Bereich von pH 6,4 – 6,7 und für die Diol-Oxidation in einem Bereich von pH 10 – 11 für die Enzyme aus *Clostridium beijerinckii*, *Enterobacter aerogenes*, *Rhodococcus erythropolis*, und *Saccharomyces cerevisiae* beobachtet<sup>26, 33,83,84</sup>. Im Segment der SDR-BDHs sind die pH-Optima etwas breiter gefächert. So liegt das pH-Optimum für die Diketon-Reduktion im

Bereich von pH 5,5 – 8,0 für die Enzyme aus *Leuconostoc pseudomesenteroides*, *Bacillus stearothermophilus*, *Brevibacterium saccharolyticum* C-1012 und *Serratia marcescens* H30<sup>85-88</sup>. Wobei das Optimum für die Reduktion von Diacetyl eher im niedrigeren pH Bereich und das pH-Optimum für die Reduktion von Acetoin auch bei pH 8,0 liegen kann. Die pH-Optima für die Diol-Oxidation liegen im Bereich von pH 7,5-10,5.

### 3.2.1.1 Substratspektrum im Vergleich

Ein wesentlicher Aspekt für den biokatalytischen Einsatz dieser Enzyme ist deren Substratspektrum. So ist generell ein breites Substratspektrum verbunden mit einer hohen Enantioselektivität biokatalytisch besonders interessant. BDHs sind in der biokatalytischen Synthese noch nicht gut etabliert. So beschränken sich viele Charakterisierungen auf die physiologischen Substrate Diacetyl, Acetoin und 2,3-Butandiol. In dieser Arbeit wurden neben den physiologischen Substraten diverse weitere Ketone, Diketone, Alkohole und Diole als mögliche Substrate getestet. Dabei zeigt sich, dass beide Enzyme neben den natürlichen Substraten auch diverse nicht physiologische vicinale Diketone,  $\alpha$ -Hydroxyketone und Diole umsetzen. Die BIBDH ist zusätzlich in der Lage,  $\alpha$ -Ketosäuren und  $\alpha$ -Ketoester umzusetzen. Im Gegensatz zu den nah verwandten klassischen Alkoholdehydrogenasen ist eine Reduktion von einfachen Ketonen, wie auch die Oxidation von primären und sekundären Alkoholen für die hier untersuchten BDHs nicht beobachtet worden (Publikation 2 und 3). Das Fehlen von einfachen Ketonen oder primären sowie sekundären Diolen im Substratspektrum ist für die meisten BDHs charakteristisch<sup>27, 83,89-91</sup>. Als Ausnahme kann jedoch die *S*-selektive BDH aus *Rhodococcus erythropolis* WZ010 genannt werden. Hier werden teils sehr hohe Aktivitäten für die Oxidation von primären und sekundären Alkoholen, wie 1-Butanol (42,8% relativ zu 2,3-Butandiol (Mischung aus allen der Stereoisomeren)), 2-Butanol (71,4% relativ 2,3-Butandiol (Mischung aus allen der Stereoisomeren)) und 2-Pentanol (257% relativ zu 2,3-Butandiol (Mischung aus allen der Stereoisomeren)) gemessen<sup>68</sup>. Auch die *meso*-Butandiol-Dehydrogenase aus *Klebsiella pneumoniae* XJ-Li zeigt Aktivitäten für das Keton 4-Methyl-2-pentanon (21 U/mg)<sup>92</sup>. Bei der *R*-selektiven BDH aus *Saccharomyces cerevisiae* und der *meso*-BDH aus *Serratia marcescens* CECT 977 konnte ein Trend hin zu verringerter Aktivität beziehungsweise niedriger  $k_{cat}$ -Werten bei zunehmender Alkylseitenkette des Substrats beobachtet werden<sup>27,70</sup>. Diesen Trend zeigen die in dieser Arbeit verwendeten BDHs nicht. Beide Enzyme zeigen unter Standardbedingungen teils sehr gute Aktivitäten für Moleküle mit längeren oder verzweigten Alkylketten wie 2,3-Heptandion und 5-Methyl-2,3-hexandion.

Überraschend ist hierbei die Akzeptanz von Substraten mit einem Phenylrest. So zeigen beide in dieser Arbeit untersuchten Enzyme Aktivität gegenüber Molekülen mit einem Phenylrest, jedoch in unterschiedlichem Ausmaß (Publikation 2 und 3). Unter Standardbedingungen (10 mM Substrat; 0,3 mM NADH; 50 mM MES-Puffer (BcBDH) / 50 mM Kpi-Puffer (BIBDH) pH 6,8; bei 30°C) zeigt die BIBDH generell nur niedrige Aktivitäten für Moleküle mit Phenylrest und hier auch nur hauptsächlich für das Diketon 1-Phenyl-1,2-propandion und das  $\alpha$ -Hydroxyketon 1-Hydroxy-1-phenylpropan-2-on (PAC). Hingegen wird das  $\alpha$ -Hydroxyketon 2-Hydroxy-1-phenylpropan-1-on (HPP) gar nicht angenommen. Bei der BcBDH verhält es sich umgekehrt. Hier wird nur HPP reduziert, aber nicht PAC. Bemerkenswert ist hier die fast doppelt so hohe Aktivität, die die BcBDH unter Standardbedingungen gegenüber (*R*)-HPP zeigt, relativ zum Standardsubstrat Acetoin (Publikation 2 und 3). Die Biokatalyse im Detail wird im Kapitel 3.3 diskutiert. Der Umsatz von diesen sterisch anspruchsvollen Molekülen wurde für BDHs bisher kaum untersucht und ist besonders interessant für die biokatalytische Synthese von Feinchemikalien. Eine Ausnahme bildet die *meso*-BDH aus *Serratia marcescens* CECT 977, mit der der Umsatz von 1-Phenyl-1,2-propandion zum (*S*)-2-Hydroxy-1-phenyl-1-propanon als Hauptprodukt und (*S*)-1-Hydroxy-1-phenyl-2-propanon sowie (*S,S*)-1-Phenyl-1,2-propandiol als Nebenprodukte beschrieben wurde<sup>27</sup>. Ebenfalls zeigt dieses Enzym, wie die BcBDH, geringe Aktivitäten für 1,2-Cyclohexandion<sup>27</sup>. Im Rahmen der Bestimmung eines Substratspektrums konnte auch für die *R*-selektive BDH aus *Rhodococcus erythropolis* WZ010 gezeigt werden, dass diese in der Lage ist vicinale Dirole mit Phenylrest wie (*R*)-1-Phenyl-1,2-ethandiol zu oxidieren<sup>26</sup>. Hingegen können sogenannte „bulky-bulky“ vicinale Diketone oder  $\alpha$ -Hydroxyketone, also Moleküle mit zum Beispiel zwei Phenylresten wie Benzoin und Benzil, von beiden in dieser Arbeit untersuchten Enzymen nicht umgesetzt werden. Dies scheint möglicherweise charakteristisch für diese Enzymgruppe zu sein. Hingegen sind durchaus Dehydrogenasen bekannt, die in der Lage sind, besonders sterisch anspruchsvolle Moleküle wie Benzil und Benzoin umzusetzen. So zeigt die Ketoreduktase aus *Pichia glucozyma* einen Umsatz von 50 mg Benzil zu (*S*)-Benzoin in zwei Stunden mit einer Ausbeute von >95% und einem *ee*-Wert von >98%<sup>93</sup>. Desweiteren ist für die zu den SDR-Dehydrogenasen gehörende Alkoholdehydrogenase aus *Sulfolobus acidocaldarius*, ein Umsatz von 5 mM Benzil zu (*R*)-Benzoin von >98% und einen *ee*-Wert von 98% nach 24 Stunden berichtet worden<sup>94</sup>. Interessanterweise weist dieses Enzym jedoch nur geringe Aktivitäten für lineare aliphatische Ketone auf.

### 3.2.1.2 Kinetische Parameter der BcBDH und BIBDH

Um detailliertere Aussagen bezüglich der Aktivitäten gegenüber diversen Substraten treffen zu können, ist die Bestimmung der kinetischen Parameter erforderlich. Für beide Enzyme wurden Kinetiken für die physiologischen Substrate Diacetyl und Acetoin aufgenommen, diese folgen der Michaelis-Menten-Kinetik. BcBDH zeigt dabei eine höhere Affinität für Acetoin und Diacetyl mit  $K_m$  Werten von 0,52 mM und 2,5 mM im Vergleich zur BIBDH (0,9 mM für Acetoin und 77,5 mM für Diacetyl). Überraschend ist hier auch der hohe  $K_m$  Wert der BIBDH für Diacetyl (77,5 mM) wenn man in Betracht zieht, dass es sich um ein physiologisches Substrat handelt. Hingegen zeigt BIBDH aber eine besonders hohe  $V_{\max}$  von  $1856,0 \pm 57,5$  U/mg für Diacetyl im Vergleich zu  $74,0 \pm 3,4$  U/mg bei der BcBDH. Auch bei Acetoin ist eine höhere  $V_{\max}$  bei der BIBDH im Vergleich zur BcBDH zu beobachten ( $115,2 \pm 5,2$  U/mg zu  $190,0 \pm 5,4$  U/mg). Da gerade die BIBDH unter Standardbedingungen Aktivitäten für die Moleküle 2,3-Hexandion, 2,3-Heptandion und 5-Methyl-2,3-hexandion gezeigt hat, die über die Aktivität für das physiologische Substrat Acetoin hinausgingen, wurden zusätzlich die kinetischen Parameter für diese Moleküle bestimmt (Publikation 3). Dabei zeigte sich, dass die berechneten maximalen Aktivitäten für diese Moleküle in einem Bereich von 680 – 1155 U/mg liegen. Dies ist deutlich über der maximalen Aktivität für Acetoin (174 U/mg). Die BIBDH zeigt aber auch eine geringere Affinität für diese Moleküle mit einem (scheinbaren)  $K_m$  von 11 – 42 mM im Vergleich zu einem  $K_m$  von 2,7 mM für Acetoin. Dabei ist eine höhere Affinität für die hier getesteten Hexandione im Vergleich zum 2,3-Heptandion zu beobachten. Jedoch konnte experimentell, aufgrund der Hydrophobizität der Substrate, die Substratsättigung zumindest bei 5-Methyl-2,3-hexandion nicht erreicht werden. Nichtsdestotrotz ist eindeutig eine Tendenz zu hohen maximalen Aktivitäten für diese Moleküle bei hohen Substratkonzentrationen bei der BIBDH zu erkennen. Die höchste maximale Aktivität konnte dabei beim 2,3-Hexandion erreicht werden mit einer  $V_{\max}$  von  $1183,0 \pm 79,0$  U/mg. Dies liegt jedoch noch unter der maximalen Aktivität von Diacetyl ( $V_{\max}$  von  $1856,0 \pm 57,5$  U/mg) bei diesem Enzym (Publikation 3).

### 3.2.1.3 Einfluss von Metallionen auf die Enzymaktivität

Ein wesentlicher Unterschied der beiden in dieser Arbeit untersuchten BDHs ist ihre grundlegende Metallionenabhängigkeit bzw. -unabhängigkeit. So benötigt die BDH aus *Bacillus licheniformis*, wie andere Vertreter der SDR-Familie, keine Metallionen<sup>34</sup>. Der Einfluss auf die Enzymaktivität von diversen bivalenten Metallionen wurde in dieser Arbeit für die BDH aus *Bacillus clausii* untersucht. Die BcBDH benötigt für die Katalyse ein Zinkion,



welches von den katalytischen Aminosäuren Cys37-His70-Glu71 komplexiert wird. Dies ist, wie eingangs erwähnt, charakteristisch für Enzyme, die der MDR-Dehydrogenasen Superfamilie zugehörig sind. Zusätzlich wird oft bei Enzymen dieser Gruppe eine zweite Zinkionen-Bindungsstelle beobachtet, bestehend aus vier konservierten Cysteinen (Cys<sub>100</sub>, Cys<sub>103</sub>, Cys<sub>106</sub> und Cys<sub>114</sub>; Nummerierung basierend auf der 2,3-Butandioldehydrogenase aus *Clostridium beijerinckii* NCIMB 8052<sup>33</sup>), die verantwortlich für die Stabilisierung der Struktur ist<sup>95</sup>. Diese muss nicht zwingend vorhanden sein und BcBDH weist diese Bindungsstelle auch nicht auf. Bei der zu den MDR zählenden BDH aus *Clostridium beijerinckii* NCIMB 8052 wurde dies ebenfalls beobachtet<sup>33</sup>. Die Zugabe von einigen Metallionen (1 mM) wie Cu<sup>2+</sup> und Fe<sup>2+</sup> führte zu einem hohen Aktivitätsverlust von 65% und 99%. Auch die Zugabe von 1 mM des Komplexbildners Ethylendiamintetraessigsäure (EDTA) bewirkte, einen Verlust der Enzymaktivität (50%). Da EDTA Chelatkomplexe mit zweiwertigen Metallionen bildet und diese somit aus dem Enzym entfernt werden, verifiziert der Aktivitätsverlust, dass dieses Enzym Metallionen (höchstwahrscheinlich Zn<sup>2+</sup>) zur Katalyse benötigt. Hingegen führte die Zugabe von Cobalt-(II)-Ionen zu einer Aktivitätssteigerung von 50% und die Zugabe von Mangan-(II)-Ionen führt sogar zu einer Aktivitätssteigerung von 117% (Publikation 2). Zusätzliche Zugabe von Zinkionen hingegen hat nur einen geringfügig aktivierenden Effekt (Steigerung von 26%). Aktivitätsmessungen über 25 Stunden von Enzympräparationen mit und ohne Mn<sup>2+</sup> Ionen zeigen eine fallende Aktivität bei der Präparation ohne Mn<sup>2+</sup> Ionen und eine zunehmend steigende Aktivität bei der Präparation mit Mn<sup>2+</sup>-Ionen bis auf mehr als das doppelte (245% rel. Aktivität; Publikation 2). Dieser Effekt konnte auch in Biotransformationen beobachtet werden. Dies könnte auf einen zunehmenden Austausch des katalytischen Zinkions mit dem Manganion hindeuten. Weitere Bindungsmotive für Mn<sup>2+</sup>-Ionen konnten nicht gefunden werden. Dieses Phänomen ist für BDHs aus der MDR-Superfamilie völlig neu. Bisher wurde lediglich für die (2R,3R)-2,3-Butandioldehydrogenase aus *Rhodococcus erythropolis* WZ010 bei Zugabe von 10 mM KCl eine Aktivitätssteigerung von 38,9 % berichtet. Andere Ionen (z.B. Mn<sup>2+</sup> und Mg<sup>2+</sup>) zeigten keinen oder nur einen geringen Einfluss auf die Aktivität (19 % Verlust bei Mn<sup>2+</sup>, Inkubation mit 1 mM, für eine Stunde; 4 % Verlust bei Mg<sup>2+</sup>, Inkubation mit 10 mM, für eine Stunde)<sup>26</sup>. Hingegen ist die Steigerung der Aktivität und Verbesserung der Stabilität durch eine Substitution des katalytischen Zink-Ions mit anderen zweiwertigen Metallionen für andere Dehydrogenasen aus der MDR-Superfamilie bereits beschrieben. So zeigt die Glyceroldehydrogenase aus *Klebsiella pneumonia* nach Substitution mit Mangan-(II) - und Magnesiumionen eine erhöhte katalytische Effizienz, auch ist die Aktivität für nicht natürliche Substrate und die Thermostabilität erhöht<sup>96</sup>. Auch

Alkoholdehydrogenasen z.B. aus dem thermophilen Bakterium *Thermotoga hypogea* oder aus *Thermoanaerobacter brockii* zeigen eine Erhöhung der Aktivität bei Substitution des Zink-Ions durch Mangan-(II)- oder Kobalt-(II)-Ionen<sup>97-99</sup>. Eine mögliche Erklärung für diesen Effekt ist, dass das Mangan-(II)-Ion ( $Mn^{2+}$ ) den harten Lewis-Säuren zugeordnet werden kann. Das Zink-Ion ( $Zn^{2+}$ ) ist eher ein Grenzfall, der zwischen den harten und weichen Lewis-Säuren liegt. Als Liganden dienen die Aminosäuren Cystein, Histidin und Glutaminsäure. Die Hydroxygruppe der Glutaminsäure lässt sich den harten Lewis-Basen zuordnen und auch die Aminogruppe des Imidazol-Rings von Histidin zählt zu den harten Lewis-Basen. Harte Lewis-Säuren bilden stabilere Komplexe mit harten Lewis-Basen als mit weichen Lewis-Basen. Nach dieser Überlegung wäre der Komplex mit  $Mn^{2+}$  stabiler als der mit  $Zn^{2+}$ . Auch in der Publikation von Dunn *et al.* wird die Lewis-Säurestärke des Metallions in Verbindung mit der Enzymaktivität gebracht<sup>100</sup>. Jedoch lässt sich auch beobachten, dass die Inkubation des Enzyms mit Kupfer-(II)- oder Eisen-(II)-Ionen zu einem starken Aktivitätsverlust führt. Sowohl  $Fe^{2+}$  wie auch  $Cu^{2+}$  sind, wie  $Zn^{2+}$ , zwischen harten und weichen Lewis-Säuren einzuordnen. Daher müssen auch andere Effekte für die Aktivitätssteigerung oder den Aktivitätsverlust verantwortlich sein, wie z.B. die unterschiedliche Ionengröße und Komplexgeometrie. Der Austausch des Zinkions gegen ein Cobaltion bei der Alkoholdehydrogenase aus *Thermoanaerobacter brockii* führt zu einem oktaedrischen anstatt eines tetraedrischen Metallkomplex. Die daraus resultierende höhere Valenz des Ions könnte positive Eigenschaften auf die Stabilisierung des Substrats und Reaktionsintermediats haben<sup>97</sup>.

Anders ist dies bei der in dieser Arbeit untersuchten BDH aus *Bacillus licheniformis* DSM 13<sup>T</sup>. Als Mitglied der SDR-Dehydrogenase Superfamilie ist dieses Enzym nicht metallionen-abhängig, benötigt diese also nicht für den Reaktionsmechanismus<sup>34</sup>. In der Charakterisierung durch Xu *et al.* hatte die Zugabe von EDTA auch kaum negative Auswirkungen auf die Enzymaktivität. Interessant ist jedoch, dass diese BDH ebenfalls stark durch Eisen- und Kupferionen inhibiert wird. Auch für die *meso*-BDH aus *Serratia marcescens* H30 wurde eine besonders starke Inhibierung der Enzymaktivität durch Eisenionen gezeigt<sup>88</sup>.

#### 3.2.1.4 Einfluss von Lösungsmitteln auf die Enzymaktivität

Gerade in biokatalytischen Synthesen ist die Stabilität gegenüber organischen Lösungsmitteln oft ein wichtiger Parameter für den praktischen Einsatz eines Enzyms, da viele Substrate und Produkte schlecht wasserlöslich sind. Hier wurde die Lösungsmittelstabilität der Enzyme BcBDH und B1BDH mit einigen wassermischbaren organischen Lösungsmitteln untersucht

(Publikation 2 und 3). Der Einsatz von organischen Lösungsmitteln wirkt sich jedoch oft negativ auf die Enzymaktivität aus. So sind organische Lösungsmittel in der Lage, intramolekulare hydrophobe Wechselwirkungen im Protein zu stören und dieses dadurch partiell zu entfalten. Im Hinblick auf biokatalytische Synthesen wäre auch der Einsatz von nicht mit Wasser mischbaren Lösungsmitteln in Form eines Zwei-Phasen Systems denkbar. In Abhängigkeit seines Oktanol-Wasser-Verteilungskoeffizienten (LogP-Wert) löst sich ein solches Lösungsmittel nur in geringen Mengen in der wässrigen Phase (bei einem hohen LogP-Wert) und interagiert wenig mit dem Enzym. Aus biokatalytischer Sicht besteht der Vorteil darin, dass höhere Konzentrationen des hydrophoben Edukts in der organischen Phase gelöst werden können. Weiterhin kann sich das gebildete Produkt nach der Katalyse aus der wässrigen Phase wieder in der organischen Phase lösen, wodurch eine Produktinhibierung des Enzyms umgangen wird<sup>101</sup>. Dies wurde jedoch in dieser Arbeit nicht genauer untersucht.

Trotz der starken strukturellen Unterschiede der beiden hier untersuchten BDHs, weisen beide Enzyme ein ähnliches Stabilitätsprofil für die hier verwendeten organischen Lösungsmittel (DMSO, Ethanol, Aceton, Methanol und Acetonitril) auf. In beiden Fällen ist Acetonitril das am wenigsten tolerierte Lösungsmittel. Eine einstündige Inkubation mit 20% Acetonitril (v/v) führt praktisch zu einem vollständigen Aktivitätsverlust. Auch bei Ethanol ist in beiden Fällen ein schneller Aktivitätsverlust mit steigendem Lösungsmittelanteil festzustellen (Abbildung 16).

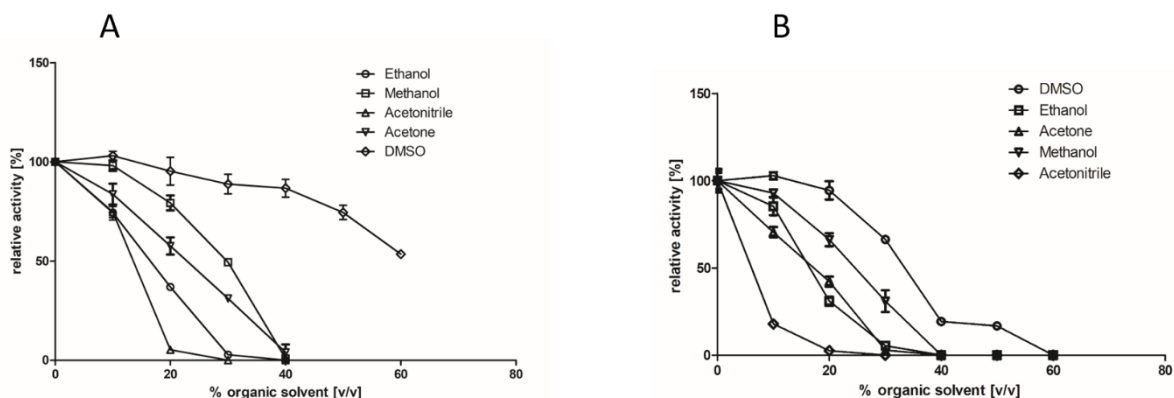


Abbildung 16: Vergleich der Lösungsmittelstabilitäten der BcBDH (A) und BIBDH (B) mit diversen Wasser mischbaren organischen Lösungsmitteln. Restaktivität bestimmt nach einstündiger Inkubationszeit. Entnommen aus Publikation 2 und 3.

Die besten Lösungsmittelstabilitäten konnten bei beiden Enzymen mit Methanol und vor allem mit DMSO gemessen werden. Hier weist die BcBDH eine höhere Stabilität im Vergleich zur BIBDH auf. So kann bei der BcBDH nach einer einstündigen Inkubation mit 60% DMSO (v/v) noch eine Restaktivität von ca. 50% gemessen werden. Die BIBDH hingegen zeigt bei einer

DMSO Konzentration von 60% (v/v) keine Aktivität mehr. Durch die höhere Stabilität der BcBDH in DMSO-haltigem Puffer ist hier das Potential gegeben, auch hydrophobe Substrate in höheren Konzentrationen in Biotransformationen einsetzen zu können. Nachteilig ist jedoch der hohe Siedepunkt des Lösungsmittels, wodurch eine spätere Abtrennung der Produkte vom Lösungsmittel unter Umständen erschwert wird. Eine Einschätzung der beiden hier untersuchten Enzyme im Vergleich zu anderen BDHs ist auch hier aufgrund der geringen Datenlage schwierig. Bei der BDH aus *Rhodococcus erythropolis* WZ010 konnte eine Aktivierung der Enzymaktivität bei Zugabe von 10% DMSO (v/v) in den Aktivitätstest beobachtet werden. Jedoch fällt die Enzymaktivität bei Zugabe von 40% DMSO (v/v) stark ab<sup>26</sup>. Die Zugabe von 10 mM DMSO in den Aktivitätstest der BDH aus *Bacillus thuringiensis* zeigte keinen merklich negativen Einfluss auf die Enzymaktivität<sup>102</sup>.

### 3.2.1.5 Biochemische Zusammenfassung

Zusammenfassend lässt sich feststellen, dass die beiden in dieser Arbeit untersuchten Enzyme sich biochemisch in vielerlei Punkten ähneln, obwohl sie unterschiedlichen Superfamilien angehören und damit auch strukturell verschieden sind. Auch im Vergleich zu bereits charakterisierten BDHs gliedern sich die beiden Enzyme gut ein. So zeigen beide Enzyme ein ähnliches pH-Optimum und Temperatur-Aktivitätsprofil für die natürlichen Substrate. Ungewöhnlich ist jedoch die geringe Affinität aber hohe maximale Aktivität der BIBDH für Diacetyl. Die verwandte und charakterisierte BDH aus *Serratia marcescens* CECT 977 zeigt zwar auch eine deutlich höhere maximale Aktivität für die Reduktion von Diacetyl ( $412 \pm 11,0$  U/mg), liegt aber um das 4,5-fache unter der maximalen Aktivität der BIBDH (Publikation 1 und 3). Hingegen hat dieses Enzym aber auch eine deutlich höhere Affinität für Diacetyl, als die hier untersuchte BIBDH ( $K_m$  von 1,7 mM zu 77,5 mM). Die BcBDH zeigt aber, auch im Vergleich zu bekannten BDHs, eine vergleichsweise hohe Stabilität gegenüber DMSO-Zusatz zum wässrigen Puffer. Wesentliche Unterschiede sind im Substratspektrum zu beobachten. Vor allem die Akzeptanz von sterisch anspruchsvollen Molekülen wie PAC und HPP ist unterschiedlich. Im Standardaktivitätstest zeigt die BcBDH überraschend hohe Aktivitäten für das  $\alpha$ -Hydroxyketon HPP jedoch keine Aktivität für das Strukturisomer PAC. Die BIBDH hingegen zeigt generell sehr geringe Aktivitäten für diese  $\alpha$ -Hydroxyketone mit Phenylrest unter diesen Bedingungen. Vorwiegend wird jedoch das PAC akzeptiert (Publikation 2 und 3).

### 3.3 Butandioldehydrogenasen in der Biokatalyse

Obwohl Dehydrogenasen im Allgemeinen (z.B. Alkoholdehydrogenasen) in der enzymatischen Synthese von Feinchemikalien bereits sehr gut etabliert sind, sind BDHs im Speziellen biokatalytisch bisher kaum untersucht. Wesentliche Erwähnung finden diese vor allem in der fermentativen Produktion von 2,3-Butandiol. Schon bei Hefen konnte die Bildung von 2,3-Butandiol aber auch Acetoin und Diacetyl nachgewiesen werden. Jedoch ist die Ausbeute oftmals gering<sup>72,103</sup>. In einem durch „metabolic engineering“ modifiziertem Hefestamm, indem drei Gene kodierend für Alkoholdehydrogenasen ausgeschaltet und der Syntheseweg von Butandiol aus *Bacillus subtilis* und *E. aerogenes* integriert wurde, konnte ein Titer von 2,29 g/L und eine Ausbeute von 0,113 g/g erreicht werden<sup>104</sup>. Als wesentliche Produktionsstämme haben sich vor allem Mikroorganismen aus der Gruppe *Klebsiella*, *Enterobacter*, *Bacillus* und *Serratia* herauskristallisiert<sup>67,72</sup>. Durch weitere Stamm- und Prozessoptimierung konnten hohe 2,3-Butandiol Ausbeuten erreicht werden. Eine Überproduktion der stammeigenen *Klebsiella oxytoca* M1 BDH und Optimierung der Prozessbedingungen ermöglichten in Fed-Batch Fermentationen die Bildung von 142 g/L 2,3-Butandiol (keine Angabe zur Stereoselektivität) mit einer Produktivität von 1,47g/L/h.<sup>105</sup>. In einem biokatalytischen Ansatz konnte eine Mischung von Glukose mit (*S,S*)-2,3-Butandiol und *meso*-2,3-Butandiol durch ganze Zellen von *Klebsiella pneumoniae* CICC 10011 umgesetzt werden. Der nachfolgende Einsatz von ganzen *Bacillus subtilis* 168 Zellen führte zur Umsetzung des *meso*-2,3-Butandiols zu (*S*)-Acetoin. Dies ermöglichte die enantioselektive Produktion von 12,5 g/L (*S,S*)-2,3-Butandiol und 56,7 g/L (*S*)-Acetoin<sup>106</sup>.

Durch die natürliche Affinität der BDHs zu vicinalen Diketonen,  $\alpha$ -Hydroxyketonen und vicinalen Diolen liegt es nahe, diese Enzyme als Biokatalysatoren für die enantioselektive Synthese dieser Molekülgruppen zu verwenden. Wie in der Einleitung bereits erwähnt, ist die rein chemische enantioselektive Synthese, gerade von  $\alpha$ -Hydroxyketonen, oft schwer und verbunden mit der Verwendung von schwermetallhaltigen Katalysatoren. Dennoch wurden BDHs bis jetzt, abseits der natürlichen Substrate, wenig Beachtung für den Umsatz von nicht physiologischen Substraten geschenkt. In dieser Arbeit wurden die Butandiol-Dehydrogenasen aus *Bacillus clausii* DSM 8716<sup>T</sup> und *Bacillus licheniformis* DSM 13<sup>T</sup> auf ihre Charakteristika gegenüber der Reduktion diverser vicinaler Diketone genauer untersucht und sollen nun vergleichend betrachtet werden. Die Reduktionsprodukte wurden über Gaschromatographie gekoppelt mit einer Massenspektroskopie identifiziert. Aufgrund der, in vielen Fällen, Nichtverfügbarkeit von enantiomeren Reinsubstanzen der Produkte konnte mit dieser

Analysemethode oft nur bestätigt werden, ob die Bildung eines Diols oder  $\alpha$ -Hydroxyketons erfolgt ist und welches Strukturisomer gebildet wurde. Um die Stereoselektivität für die nicht physiologischen Substrate dennoch bestätigen zu können, wurden die Circular dichroismus (CD) Spektren der Substrate 2,3-Hexandion und 5-Methyl-2,3-Hexandion mit den CD-Spektren von (*R*)-Acetoin aus der Arbeit von Vinogradov *et al.* verglichen wurden<sup>107</sup>. Hierzu wurden mit der BcBDH die Substrate 2,3-Hexandion und 5-Methyl-2,3-hexandion im präparativen Maßstab reduziert. Durch die hohe Stereoselektivität der BcBDH für diese Substrate (89% bzw. 97% *ee*-Wert) konnte im Wesentlichen ein Enantiomer des  $\alpha$ -Hydroxyketon Produkts im deutlichen Überschuss synthetisiert werden. Obwohl es sich hier um einen Analogieschluss handelt, liegt die Struktur von Acetoin und den hier untersuchten  $\alpha$ -Hydroxyketonen so nah beieinander, dass eine Einschätzung auf dieser Basis möglich ist. Die Synthese der (*R*)-Enantiomere der Produkte 5-Methyl-3-hydroxy-2-hexanon und 3-Hydroxy-2-hexanon mit der BcBDH konnte bestätigt werden. Somit zeigt das Enzym die gleiche Stereoselektivität, wie für die natürlichen Substrate. Eine Identifikation der Produkte der BIBDH auf gleicher Basis sollte in nachfolgenden Arbeiten durchgeführt werden.

### 3.3.1 Vergleich der biokatalytischen Charakteristika von BcBDH und BIBDH


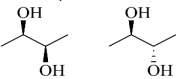
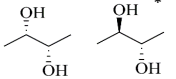








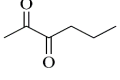
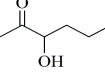
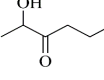
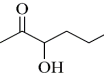
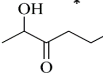
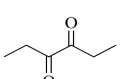
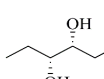
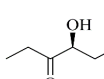
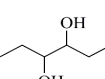
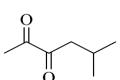
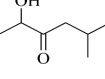
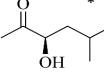
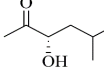
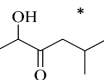
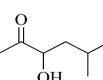
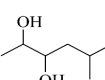
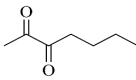
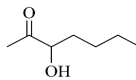
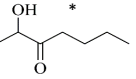
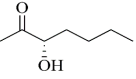
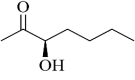
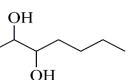
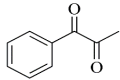
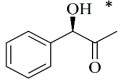
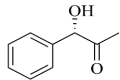
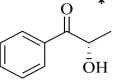
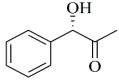
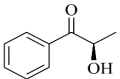
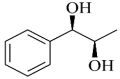
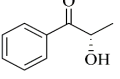
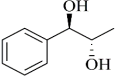
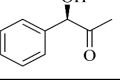
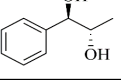
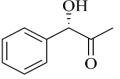
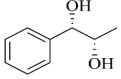
Werden die beiden Enzyme miteinander verglichen, so sind deutliche Unterschiede in der Produktbildung zu beobachten (Tab. 2). Bei der Reduktion von Diacetyl unter den hier gewählten Standardreaktionsbedingungen (1 U/ml BDH, Reaktionszeit 1 Stunde; Publikation 3) konnte mit der BIBDH ausschließlich die Bildung von (*S*)-Acetoin beobachtet werden. Dies steht nur scheinbar im Gegensatz zu den Daten aus Xu *et al.*, da hier die Bildung von (*S*)-Acetoin und die weitere Reduktion zu (*S,S*)-2,3-Butandiol beobachtet wurde<sup>34</sup>. Jedoch ist zu beachten, dass in dieser Studie deutlich höhere Aktivitäten (100 Units/ 5 ml) und eine viel längere Reaktionszeit von 12 Stunden gewählt wurden. Interessant ist die Reduktion von racemischen Acetoin mit der BIBDH. Hier kann unter den Standardreaktionsbedingungen beobachtet werden, dass vornehmlich das (*R*)-Enantiomer des Acetoin zu *meso*-2,3-Butandiol umgesetzt wird. Spuren eines Umsatzes von (*S*)-Acetoin zu (*S,S*)-2,3-Butandiol wurden ebenfalls beobachtet. Dies zeigt, dass die BIBDH tatsächlich eine *meso*-BDH ist. Augenscheinlich hat dieses Enzym eine viel höhere Affinität zum (*R*)-Enantiomer und fügt ein *S*-konfiguriertes Stereozentrum ein. Die Bestimmung der kinetischen Parameter für die einzelnen Enantiomere des Acetoin wäre hilfreich, um diese Beobachtung zu unterstützen, jedoch waren diese in enantiomerenreiner Form nicht verfügbar. Gleiche biokatalytische

Charakteristika wurden für die *meso*-BDH aus *Serratia marcescens* CECT 977 berichtet <sup>27</sup>. Hingegen zeigt die BcBDH ein anderes Verhalten. Hier wird ausgehend von Diacetyl das (*R,R*)-2,3-Butandiol gebildet, ohne dass das Zwischenprodukt (*R*)-Acetoin detektierbar ist. Bei der Verwendung vom racemischen Acetoin ist das Enzym in der Lage, beide Enantiomere zu verwerten, mit der Bildung von (*R,R*)- und *meso*-2,3-Butandiol. Selbiges Verhalten wurde für die *R*-selektive, und der Superfamilie der MDR zugehörige, BDH aus *Paenibacillus polymyxa* ATCC 12321 berichtet <sup>108</sup>. Auch die Zink-abhängige, und MDR zugehörige, (*R,R*)-BDH aus *Saccharomyces cerevisiae* YAL060W zeigt den Umsatz von racemischem Acetoin zu (*R,R*)- und *meso*-2,3-Butandiol <sup>89</sup>. Eine größere Datenlage zu den Stereospezifitäten für die einzelnen Enantiomere der physiologischen Substrate wäre von Nöten um generell von einem Charakteristikum, das typisch für BDHs der MDR oder SDR Superfamilie ist, sprechen zu können.

Wird der Umsatz von diversen nicht physiologischen Substraten mit der BIBDH oder BcBDH verglichen so sind hier deutliche Unterschiede festzustellen (Tabelle 4). BIBDH reduziert dabei die jeweiligen Diketone primär zum jeweiligen  $\alpha$ -Hydroxyketon als Hauptprodukt. Ausgehend von dem Reaktionsmechanismus für Diacetyl sollte es sich hier um das (*S*)-konfigurierte  $\alpha$ -Hydroxyketon handeln. Dabei ist das Hauptprodukt immer das 2-Hydroxy-3-keton Isomer. Als Nebenprodukte können hier sowohl das andere Strukturisomer des Hydroxyketons als auch geringe Mengen des Diols beobachtet werden. Interessant ist, dass dies nun nicht mehr mit der *meso*-BDH aus *Serratia marcescens* CECT 977 korreliert. Hier wird vorwiegend das Diol ausgehend von den Substraten 2,3-Pentandion und 3,4-Hexandion gebildet. Die Umsetzung vom 2,3-Hexandion führt zwar primär zur Bildung des  $\alpha$ -Hydroxyketons, aber des anderen Strukturisomeren 3-Hydroxy-2-hexanon <sup>27</sup>. Die Reaktionsbedingungen in der Arbeit von Médicci *et al.* sind dabei mit den Reaktionsbedingungen in dieser Arbeit durchaus vergleichbar (10 mM Substrat; 2 U/ml BDH). Vergleicht man die Ergebnisse der Umsetzungen mit der BIBDH für aliphatische lineare Substrate mit den Umsetzungen der BcBDH, so kann man feststellen, dass nicht nur eine andere Stereoselektivität vorhanden ist, das Einfügen eines *R*- anstatt eines *S*-Stereozenentrums, sondern auch die primäre Bildung des 3-Hydroxy-2-ketons als Hauptprodukt erfolgt. Mit Verlängerung der aliphatischen Kette ist hier auch ein Trend zu erkennen, bei dem symmetrische Moleküle bis zum Diol reduziert werden und die Reduktion asymmetrischer Moleküle beim  $\alpha$ -Hydroxyketon Intermediat stehen bleibt. 2,3-Pentandiol ist das einzige Molekül, bei dem noch beide Produkte beobachtet werden können.

# Diskussion

Tabelle 4: Vergleich der biokatalytischen Charakteristika der BIBDH und BcBDH gegenüber bezüglich diversen vicinalen Diketonen und  $\alpha$ -Hydroxyketonen. Bei den Produkten, die mit einem Stern markiert sind (\*), handelt es sich um das jeweilige Hauptprodukt. Alle Biokatalysen wurden mit 1 U/ml BDH über 1 Stunde durchgeführt (beschrieben in Publikation 2 und 3).

Substrat	BcBDH		BIBDH	
	$\alpha$ -Hydroxyketon	Vicinales Diol	$\alpha$ -Hydroxyketon	Vicinales Diol
	-		-	
	-			-
			 	-
	 	-	 	-
	-			
	  	-	 	
		-	  	
	 	-	 	-
	-		-	-
	-		-	-
	-	-	-	
	-	-	-	



Die Umsetzung von 1-Phenyl-1,2-propandion und den daraus resultierenden Hydroxyketon-isomeren 1-Hydroxy-1-phenylpropanon (PAC) und 2-Hydroxy-1-phenylpropanon (HPP) erlaubt weitere Einsichten in die Substratbindung. So präferiert BIBDH die Ketogruppe, die in der Nähe des kleineren Rests des Moleküls liegt. BcBDH präferiert grundsätzlich die Ketogruppe, die am größeren Rest des Moleküls liegt. Bei der Reduktion des 1-Phenyl-1,2-propandions werden von beiden Enzymen  $\alpha$ -Hydroxyketone als Produkte gebildet, die aber weiterhin nicht mehr als Substrate angenommen werden. Dies deutet vermutlich auf sterische Hindernisse hin, die eine Umorientierung des Reaktionsintermediats verhindern. Deutlich wird dies auch bei der Reduktion der Hydroxyketone PAC und HPP. Hier wird jeweils das gegenteilige Stereoisomer angenommen. In einer Arbeit von Kurina-Sanz *et al.* werden für die Alkoholdehydrogenasen aus *Lactobacillus brevis* (LBADH), *Rhodococcus ruber* (ADH-A) und aus *Thermoanaerobacter sp.* (ADH-T) mögliche Substratbindungsmodelle basierend auf unterschiedlich großen Bindetaschen für die jeweiligen Seitenkettenreste des Substrats dargestellt (Abb. 17). Die LBADH ist in der Lage, ausgehend vom 2,3-Pentandion das *syn*-Diol zu bilden, aber das 2,3-Hexandion und 2,3-Heptandion wird zum *anti*-Diol umgesetzt, jedoch mit einem Umsatz kleiner 10% <sup>109</sup>. Ein ähnliches Modell würde die Reaktionsprodukte der BcBDH erklären. So ist ein Drehen des Moleküls erforderlich, was bei symmetrischen Molekülen wie dem 3,4-Hexandion oder Diacetyl möglich ist, aufgrund der auf beiden Seiten kurzen Methyl- bzw. Ethylreste. Hier kann die Bildung eines Diols beobachtet werden. Bei asymmetrischen Molekülen mit Propyl- oder Butylresten ist dies offenbar nicht möglich. Das würde für die BcBDH auch für ein Modell sprechen, das eine kleine und eine große Substratbindetasche beinhaltet. Da jedoch bei asymmetrischen Molekülen keine Diolbildung beobachtet wurde, scheint die Diskriminierung der Reste hier größer zu sein. Auch für die BIBDH ist dieses Modell denkbar. So wäre für die Generierung von *meso*-Diolen ein Spiegeln des Substrats aber kein gänzlich Drehen erforderlich. Dies geht auch mit den Umsetzungen der HPP und PAC Substrate einher. Jedoch ist es hier von Nöten die absolute Stereokonfiguration aller gebildeten Diole zu ermitteln, um dieses Modell für die BDHs zu untermauern.

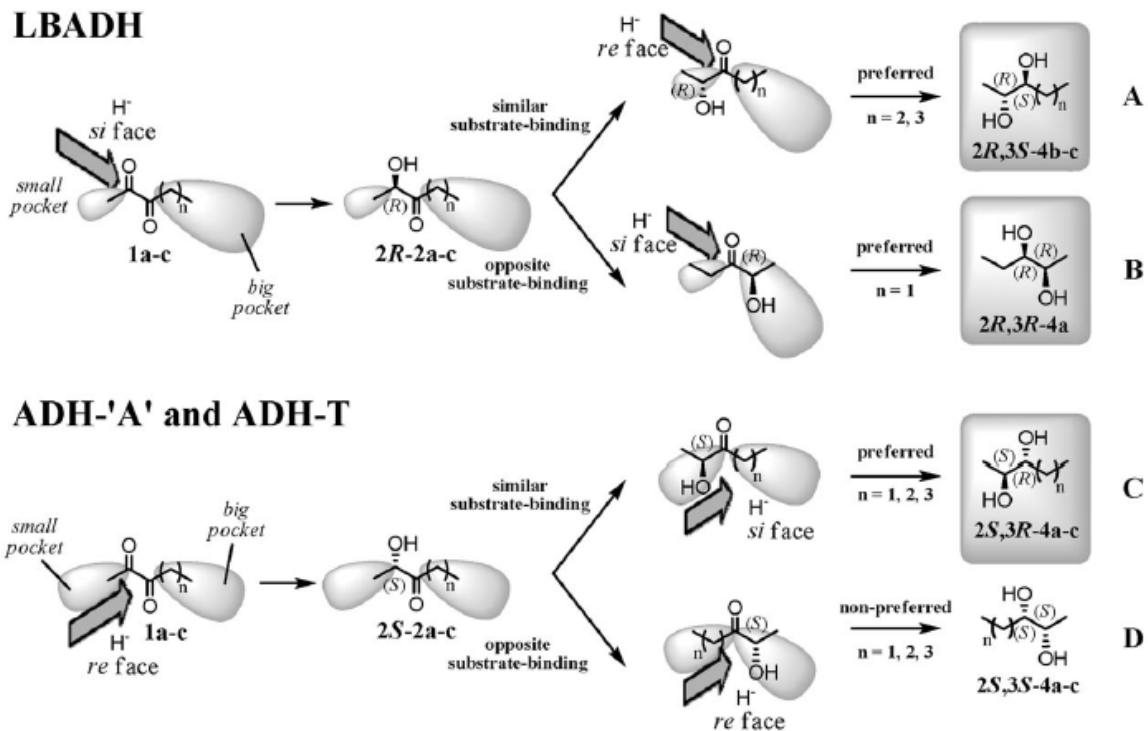


Abbildung 17: Modell für mögliche Substratbindemodi von vicinalen Diketonen (1a-c) und  $\alpha$ -Hydroxyketonen (2a-c) für die Alkoholdehydrogenasen aus *Lactobacillus brevis* (LBADH; Bindemodi A und B), *Rhodococcus ruber* (ADH-A; Bindemodi C und D) und aus *Thermoanaerobacter sp.* (ADH-T; Bindemodi C und D). Entnommen aus Kurina-Sanz *et al* <sup>109</sup>.

Insgesamt kann festgestellt werden, dass die BcBDH unter den gewählten Bedingungen diverse Diketone selektiver reduziert als die BIBDH. Gerade bei Molekülen mit längeren aliphatischen Ketten, wie 5-Methyl-2,3-hexandion und 2,3-Heptandion, zeigt die BcBDH ausgesprochen hohe Selektivitäten für die Bildung eines Strukturisomers des  $\alpha$ -Hydroxyketon (Tabelle 5).

Tabelle 5: Vergleich der Stereo- bzw. Regioselektivität der BcBDH und BIBDH für einige Diketone. Beschrieben in den Publikationen 1 und 2.

Substrat	BIBDH <i>ee/de</i> -Wert [%]	BcBDH <i>ee/de</i> -Wert [%]
5-Methyl-2,3-hexandion	71	97
2,3-Hexandion	72	89
2,3-Heptandion	-	99
1-Phenyl-1,2-propandion	76	86

Die Reduktion des Diketons 2,3-Heptandion mit der BIBDH verläuft hingegen unspezifisch mit der Bildung mehrere Hydroxyketon Intermediate und des Diols, sodass hier kein *ee/de* Wert angegeben werden kann. Verglichen mit der *meso*-BDH aus *Serratia marcescens* CECT 977

zeigt diese eine deutlich höhere Selektivität für die verwendeten Diketone als die hier untersuchte *meso*-BDH aus *Bacillus licheniformis*. So ist die *Serratia marcescens* CECT 977 BDH in der Lage, 2,3-Hexandion zu (*S*)-3-Hydroxy-2-hexanon mit einem *ee*-Wert von 99% zu reduzieren. 2,3-Pentandion konnte zum korrespondierenden Diol überführt werden mit einem *ee*-Wert von 96%<sup>27</sup>.

### 3.3.2 Einsatz der BcBDH in einem potentiometrischen Biosensor zur Messung von Acetoin und Diacetyl in Fermentationsprozessen

Im Rahmen eines Kooperationsprojekts erfolgte die Mitarbeit an der Entwicklung eines Biosensors zur Konzentrationsmessung der Überschussmetabolite Acetoin und Diacetyl in Fermentationsprozessen. Dies ist von besonderem Interesse, da viele Bakterien und Hefen Überschussmetabolite in fermentativen Prozessen produzieren, wenn eine hohe Glukosekonzentration gegeben ist. In dem Fall sind die Mikroorganismen in der Lage ausreichend Energie in Form von ATP über die Glykolyse zu generieren. Eine vollständige Oxidation der Glukose über den Tricarbonsäure-Zyklus findet nicht statt<sup>30</sup>. Als Überschussmetabolite dienen oft Acetat oder Lactat. Aber auch andere Metabolite, wie zum Beispiel die aerobe Bildung von Citronensäure bei *Aspergillus niger*<sup>110</sup> oder die aerobe Bildung von Ethanol durch einige Hefen (Crabtree-Effekt)<sup>111</sup> können auf einen Überflussmetabolismus zurückgeführt werden. Hingegen bilden Mikroorganismen vor allem aus der Klasse der Enterobakterien und Bacilli besonders häufig Acetoin und 2,3-Butandiole als Überflussmetabolite<sup>31</sup>. Viele Stämme aus der Gattung *Bacillus*, wie *B. subtilis*, *B. licheniformis* und *B. amyloliquefaciens* sind auch wichtige industriell genutzte Produktionsorganismen. Eine Methode zur zeitnahen Messung dieser Überflussmetabolite könnte zur verbesserten Prozessoptimierung von Fermentationen führen. Auch bei der Bier- und Weinherstellung ist die Messung von Acetoin und Diacetyl von besonderem Interesse. Diese Moleküle werden als Überflussmetabolite im Kohlenhydratmetabolismus der Hefe gebildet<sup>29</sup>. Aufgrund ihres butterähnlichen Geschmacks ist deren Konzentration ein wichtiges Qualitätsmerkmal. So dient die Acetoinkonzentration bei der Bierherstellung als wichtiger Parameter zur Bestimmung des Reifungsgrades während der Lagerung<sup>112</sup>. Bisherige Methoden zur Bestimmung des Acetoingehalts sind oft kolorimetrische Tests wie der Voges-Proskauer Test oder die Bestimmung erfolgt mittels Gaschromatographie<sup>113</sup>.

Der in diesem Kooperationsprojekt entwickelte Biosensor (Promotion Dr. Molinnus, AG Prof. Schöning) besteht aus Schichten aus Al, p-Si, SiO<sub>2</sub> und der pH sensitiven Ta<sub>2</sub>O<sub>5</sub> Schicht (Publikation 4; Abbildung 18). Auf deren Oberfläche wird die BcBDH mittels Querverknüpfung immobilisiert.

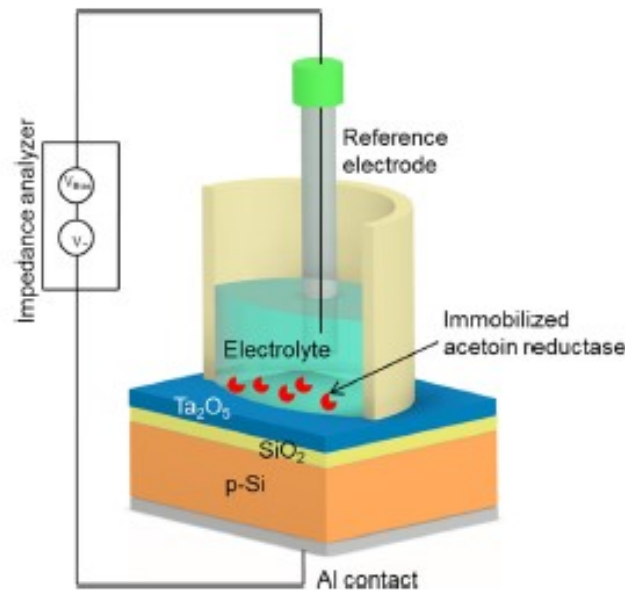


Abbildung 18: Schematischer Aufbau des Biosensors bestehend aus Al-p-Si-SiO<sub>2</sub>-Ta<sub>2</sub>O<sub>5</sub> Schichten mit immobilisierter BcBDH auf der Ta<sub>2</sub>O<sub>5</sub> Oberfläche ( entnommen aus Publikation 4) <sup>79</sup>.

Durch die von der BcBDH katalysierten Reduktion von Acetoin oder Diacetyl zum jeweiligen Diol, wird die Hydridionenkonzentration im Elektrolyten erniedrigt. Dies führt zu einer Änderung des pH-Wertes des Elektrolyts und damit verbundenen Modulation des Flachbandpotentials der Sensorstruktur und daraus resultierenden Änderung der Kapazitätsspannung (Publikation 4). Die Funktionsweise des Biosensors konnte durch die Zugabe von 30 – 90 µM Acetoin zum Elektrolyten demonstriert werden (Publikation 4; Abbildung 19). Dabei ist eine Zunahme des Sensorsignals mit zunehmender Acetoinkonzentration zu verzeichnen. Dies konnte auch für reale Weinproben (1:50 Mischung mit Puffer) gezeigt werden <sup>79</sup>.

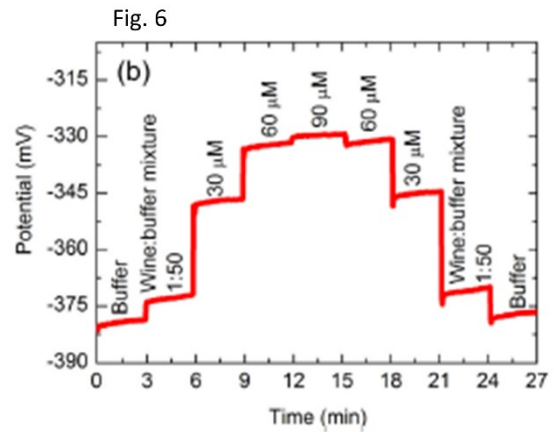
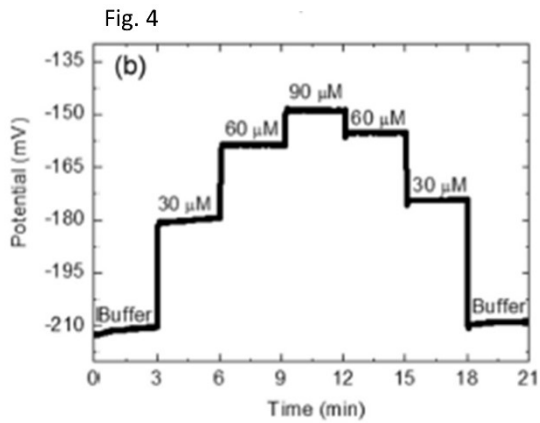


Abbildung 19: Konstante Kapazitätsmessung des Biosensors in Abhängigkeit verschiedener Acetoinkonzentrationen. Messungen erfolgten durch Zugabe von 30  $\mu$ M – 90 mM Acetoin in TRIS-HCl Puffer pH 7.1 + 150 mM NaCl (Fig.4 (b)) oder durch Zugabe von 30  $\mu$ M – 90 mM Acetoin in eine Weinprobe 1:50 verdünnt mit Puffer (TRIS-HCl Puffer pH 7.1 + 150 mM NaCl; Fig. 6 (b)). Entnommen aus Publikation 4.

## 4 Zusammenfassung und Ausblick

Im Rahmen dieser Arbeit wurde das biokatalytische Potential dieser Enzymgruppe speziell für die Synthese von  $\alpha$ -Hydroxyketonen und vicinalen Diolen am Beispiel der Butandioldehydrogenasen aus *Bacillus clausii* DSM 8716<sup>T</sup> (BcBDH) und *Bacillus licheniformis* DSM 13<sup>T</sup> (BIBDH) verdeutlicht. Die BcBDH wurde in dieser Arbeit als bisher noch unbekanntes Enzym erstmals biochemisch charakterisiert und bezüglich ihres biokatalytischen Potentials untersucht. Im Rahmen dieser Arbeit wurde über die anfängliche biochemische Charakterisierung der BIBDH hinaus<sup>14</sup>, eine biokatalytische Charakterisierung des Enzyms durchgeführt.

Beide Enzyme gehören unterschiedlichen Superfamilien an (SDR und MDR) und sind somit strukturell deutlich verschieden. Jedoch weisen beide Enzyme ein ähnliches Substratspektrum auf. Es werden jedoch vorwiegend nur vicinale Diketone,  $\alpha$ -Hydroxyketone oder vicinale Diole als Substrate angenommen. Dabei zeigen diese Enzyme aber auch eine Akzeptanz für Moleküle mit längeren aliphatischen Ketten, wie z.B. das 2,3-Heptandion. Anders als bei Alkoholdehydrogenasen üblich, werden keine einfachen Ketone oder Alkohole als Substrate akzeptiert. Das Enzym BIBDH zeigt zudem geringe Aktivität für  $\alpha$ -Ketosäuren und  $\alpha$ -Ketoester. Hingegen zeigt die BcBDH ausgesprochen hohe Aktivitäten für Moleküle mit Phenylrest wie 1-Phenyl-1,2-propandion, 2-Hydroxy-1-phenylpropan-1-on und 1-Hydroxy-1-phenylpropan-2-on.

Es konnte dargestellt werden, dass sich beide BDHs für die enantioselektive Synthese von  $\alpha$ -Hydroxyketonen oder vicinale Diolen eignen, wobei sie unterschiedliche biokatalytische Charakteristika aufweisen. Während die *meso*-BIBDH vorwiegend das *R*-Enantiomer präferiert und ein *S*-konfiguriertes Stereozentrum einfügt, ist die BcBDH *R*-selektiv und zeigt eine deutliche Diskriminierung zwischen symmetrischen und asymmetrischen Molekülen, welche sich auf die Produktbildung auswirkt. Durch die Wahl des Substrats oder der Reaktionsbedingungen kann so die Synthese eines Produkts forciert werden. Generell hat sich aber in der vergleichenden Analyse herausgestellt, dass die BcBDH bei vielen Substraten die Reduktion deutlich selektiver katalysiert.

Als weiterführende Arbeiten wäre es nun interessant, diese Enzyme in präparativen Synthesen unterschiedlicher Maßstäbe einzusetzen. Erste Versuche hierzu wurden im Rahmen dieser

Arbeit bereits durchgeführt. Auch ist die Strukturaufklärung beider Enzyme ein attraktiver Ansatzpunkt, um genauere Einblicke in den Reaktionsmechanismus zu bekommen, sowie die unterschiedlichen Spezifitäten/Selektivitäten zu erklären und Ansatzpunkte für gerichtete Mutagenese, um die Synthesen im Hinblick einer noch höheren Stereoselektivität zu verbessern. Im Gegensatz zu den BDHs aus der Superfamilie der SDRs, sind bei den BDHs aus der Superfamilie der MDRs bis jetzt keine dreidimensionalen Strukturen bekannt. Daher wären hier kristallographische Studien besonders interessant.

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