



Enantioselective synthesis of vicinal (R,R)-diols by yeast butanediol dehydrogenase

Eduard Calam, Eva González-Roca, M Rosario Fernández, Sylvie Dequin, Xavier Parés, Albert Virgili, Josep A Biosca

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1 **Enantioselective synthesis of vicinal (*R,R*)-diols by yeast butanediol**
2 **dehydrogenase.**

3 Running title: Biotechnological applications of yeast Bdh1p.

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7 Eduard Calam, Eva González-Roca¹, M. Rosario Fernández, Sylvie Dequin²,
8 Xavier Parés, Albert Virgili³ and Josep A. Biosca*

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10
11 Department of Biochemistry and Molecular Biology, Universitat Autònoma de
12 Barcelona, E-08193 Bellaterra (Barcelona). 1 Present address
13 Autoinflammatory Disease Unit Immunology Service (CDB-IDIBAPS) Hospital
14 Clinic (Barcelona) 2 Laboratoire de Microbiologie et Technologie des
15 Fermentations, INRA-IPV, F-34600 Montpellier, France and 3 Department of
16 Chemistry, Universitat Autònoma de Barcelona, E-08193 Bellaterra (Barcelona).

17
18
19 *Correspondence to: Josep A. Biosca, Dept. of Biochemistry and Molecular
20 Biology, Faculty of Biosciences, Universitat Autònoma de Barcelona, E-08193
21 Bellaterra (Barcelona) Spain

22
23 Telefax: 34-93-581 1264

24 Telephone: 34-93-581 3070

25 E-mail: josep.biosca@uab.es

26

Abstract.

Butanediol dehydrogenase (Bdh1p) from *Saccharomyces cerevisiae* belongs to the superfamily of the medium chain dehydrogenases/reductases and converts reversibly *R*-acetoin and *S*-acetoin to (2*R*,3*R*)-2,3-butanediol and *meso*-2,3-butanediol, respectively. It is specific for NAD(H) as a coenzyme and it is the main enzyme involved in the last metabolic step leading to (2*R*,3*R*)-2,3-butanediol in yeast. In this study, we have used the activity of Bdh1p, in different forms: purified enzyme, yeast extracts, permeabilized yeast cells, and as a fusion protein (with yeast formate dehydrogenase, Fdh1p) to transform several vicinal diketones to the corresponding diols. We have also developed a new variant of the "delitto perfetto" methodology to place *BDH1* under the control of the *GAL1* promoter, resulting in a yeast strain that overexpresses butanediol dehydrogenase and formate dehydrogenase activities in the presence of galactose and regenerates NADH in the presence of formate. While the use of purified Bdh1p allows the synthesis of enantiopure (2*R*,3*R*)-2,3-butanediol, (2*R*,3*R*)-2,3-pentanediol, (2*R*,3*R*)-2,3-hexanediol and (3*R*,4*R*)-3,4-hexanediol, the use of the engineered strain (as an extract or as permeabilized cells), yields mixtures of the diols. The production of pure diol stereoisomers has also been achieved by means of a chimeric fusion protein combining Fdh1p and Bdh1p. Finally, we have determined the selectivity of Bdh1p towards the oxidation/reduction of the hydroxyl/ketone groups from (2*R*,3*R*)-2,3-pentanediol/2,3-pentanedione and (2*R*,3*R*)-2,3-hexanediol/ 2,3-hexanedione. In conclusion, Bdh1p is an enzyme with biotechnological interest that can be used to synthesize chiral building blocks. A scheme of the favored pathway with the corresponding intermediates is proposed for the Bdh1p reaction.

Introduction

Biocatalytic transformations using pure enzymes or whole-cell microorganisms offer mild and environmentally benign reaction conditions as opposed to the chemical processes that use harsh conditions and produce residual metals. Several diols, as 1,3-propanediol, 1,2-propanediol, 2,3-butanediol and 1,4-butanediol, are considered platform chemicals because of their many applications in the industry, including the synthesis of special chemicals and their use as precursors of polymers (1). Thus, 2,3-butanediol (2,3-BD) has been used as an antifreeze agent (the stereoisomeric form (2*R*,3*R*)-2,3-BD has a freezing point of -60 °C) and as a precursor of several compounds, through reactions of dehydration (obtaining methyl ethyl ketone, that can be used as a fuel additive), dehydrogenation (giving flavoring compounds as acetoin and diacetyl), ketalization (yielding a potential gasoline blending agent) and esterification (giving precursors of drugs and plasticizers) (2-3, for recent reviews). Several microbial systems have been optimized (by genetic engineering and by optimization of the fermentation conditions) for the production of 2,3-BD, such as *Klebsiella pneumoniae* (4) *Klebsiella oxytoca* or *Paenibacillus polymyxa* (2) and *E.coli* (5). The manipulation of the carbon flux and cofactor regeneration in *Bacillus amyloliquefaciens*, has resulted in a high-titer of 2,3-butanediol from biodiesel-derived glycerol (6). Recent work has also established the potential of some acetogenic bacteria to produce 2,3-butanediol using CO and/or CO₂ plus H₂ (7). *Saccharomyces cerevisiae* has also been used recently as a producer of 2,3-BD through genetic engineering (8-9). Thus, a pyruvate decarboxylase yeast mutant was used as a starting strain, that was

77 evolved for rapid glucose consumption, such that the resulting strain achieved
78 96.2 g/L of 2,3-butanediol production in the fermentation medium (8).

79 The pure stereoisomers of 2,3-butanediol, (2S,3S)-, (2R,3R)- and
80 *meso*- are useful as auxiliaries and can serve as building blocks in the
81 asymmetric synthesis of chiral compounds with two stereogenic centers (10).
82 The three stereoisomeric forms can be produced by microbial fermentation.
83 Thus, (2R,3R)-2,3-BD can be obtained in high purity from sucrose
84 fermentations by *Paenibacillus polymyxa* (11) and by metabolic engineering of
85 *E. coli* and *S. cerevisiae*, through the introduction of biosynthetic pathways
86 composed of endogenous and foreign genes (12-13). (2S,3S)-2,3-BD has been
87 obtained recently by fed-batch bioconversion from diacetyl using an *E. coli*
88 strain expressing 2,3-BD dehydrogenase and glucose or formate
89 dehydrogenases as cofactor-regenerating systems (14). *meso*-2,3-BD has been
90 produced from a metabolically engineered *E.coli* from glucose fermentation
91 under low oxygen (15). Several classes of oxidoreductases, belonging to the
92 superfamilies of medium chain dehydrogenases/reductases (MDR), aldoketo
93 reductases and short-chain dehydrogenases/reductases have been used in
94 those organisms to convert diacetyl and/or acetoin to 2,3-BD (16-17). A recent
95 biotechnological use for Bdh1p from *Bacillus subtilis* was the production of (3R)-
96 acetoin and (3S)-acetoin with high enantiomeric excess, from (2R,3R)-2,3-
97 butanediol and *meso*-2,3-butanediol respectively (18). They used Bdh1p
98 together with an NAD⁺-regenerating system, formed by NADH oxidase from
99 *Lactobacillus brevis* to regenerate NAD⁺ from NADH by reducing O₂ to H₂O.

100 We have previously characterized Bdh1p as an MDR that can reversibly
101 convert *R*-acetoin and *S*-acetoin to (2R,3R)-2,3-butanediol and *meso*-2,3-

102 butanediol, respectively using NAD(H) as a coenzyme (19). We have also
103 shown that Bdh1p is the main enzyme involved in the last metabolic step
104 leading to (2*R*,3*R*)-2,3-butanediol in yeast (20). In the present work we have
105 used the activity of Bdh1p in different conditions: (I) purified enzyme, (II) yeast
106 extracts, (III) permeabilized yeast cells and, (IV) as a fusion protein with yeast
107 formate dehydrogenase (Fdh1p), to transform diacetyl, 2,3-pentanedione, 2,3-
108 hexanedione and 3,4-hexanedione to the corresponding diols. By using purified
109 Bdh1p (together with exogenous Fdh to regenerate NADH) we have obtained
110 enantiopure (2*R*,3*R*)-2,3-butanediol, (2*R*,3*R*)-2,3-pentanediol, (2*R*,3*R*)-2,3-
111 hexanediol and (3*R*,4*R*)-3,4-hexanediol. We have also engineered a yeast
112 strain (by developing a new variant of the “delitto perfetto” methodology (21)
113 that overexpresses Bdh and Fdh activities in the presence of galactose. The
114 use of this engineered strain allowed obtaining several diols from the
115 corresponding diketones in the presence of formate, without the need of a
116 coenzyme-regenerating system. Although the use of yeast extracts or
117 permeabilized cells from this strain did not allow the production of pure
118 stereoisomers, we could separate and identify the hydroxyketones and diols
119 arising from the reduction of the diketones. To allow the production of pure diol
120 stereoisomers and to avoid the need of a coenzyme regenerating system, we
121 have constructed a chimeric fusion protein combining Fdh1p and Bdh1p.
122 Finally, we propose a mechanism to explain the selectivity shown by Bdh1p
123 towards the oxidation/reduction of the hydroxyl/ketone groups from (2*R*,3*R*)-2,3-
124 pentanediol/2,3-pentanedione and (2*R*,3*R*)-2,3-hexanediol/ 2,3-hexanedione.

125 **Materials and Methods**

126

127 **Materials.**

128 Restriction enzymes and T4 DNA ligase were from Roche (Basel,
129 Switzerland). Vent and KOD polymerases were from New England Biolabs, Inc.
130 (Beverly, USA) and Merck (Nottingham, United Kingdom) respectively. DNA
131 oligomers were synthesized and purified by Sigma-Genosys (Haverhill, United
132 Kingdom). Chemicals were purchased from Fluka or, Sigma-Aldrich (Saint
133 Louis, USA). *S.cerevisiae* alcohol dehydrogenase and *Candida boidinii* formate
134 dehydrogenase were from Sigma-Aldrich.

135

136 **Synthesis and NMR analysis of 2,3-pentanediol stereoisomers.**

137 To a stirred solution of 2,3-pentanedione (0.50g, 5 mmols) in methanol
138 (40 ml) a suspension of sodium borohydride (0.180 g, 4.8 mmols) in methanol
139 (20 ml) was added during 60 min. After stirring for 3 hours, the solvent was
140 gently evaporated and the residue was treated with water (50 ml), neutralized
141 and extracted with chloroform (3x30 ml). The solvent was removed obtaining
142 0.47g (4.5 mmols, 90% yield) of 2,3-pentanediol. The residue was analyzed by
143 ¹H- and ¹³C-NMR. To prepare (2*R*,3*R*)-2,3-pentanediol, we prepared a reaction
144 mixture containing 48 mM 2,3-pentanedione, 1 mM NADH, 100 mM sodium
145 formate, 25 U of Fdh and 10 U of Bdh1p. The mixture was allowed to react for
146 23 hours and then was extracted with chloroform. The chloroform was
147 evaporated with a rotavapor and the oily extract was dissolved in deuterated
148 chloroform before being analyzed by ¹H-NMR.

149

150 **Yeast and bacterial strains.**

151 *Escherichia coli* XL-1 blue (Stratagene, La Jolla, USA) or DH5 α was
152 used for cloning experiments. The *S. cerevisiae* strains used in this study were
153 derived from the wild type FY834 α (*MAT α* , *his3 Δ 200*, *ura3-52*, *leu2 Δ 1*,
154 *lys2 Δ 202*, *trp1 Δ 63*) (22) and from the Adh⁻ strain WV36-405 (*MAT α* , *ura3-52*,
155 *trp1*, *Δ adh1*, *Δ adh2*, *Δ adh3*, *adh4::TRP1*) constructed by Dr. Wolfgang Vogel
156 (Neuherberg, Germany). Mutant strain EG2 (FY834 α *MAT α* *his3 Δ 200* *ura3-52*
157 *leu2 Δ 1* *lys2 Δ 202* *trp1 Δ 63* *TRP1::bdh1*) (19) was used to characterize additional
158 NADH dependent diacetyl reductases. Yeast strains deleted for *bdh1* (*Δ bdh1*)
159 and/or *ara1* (*Δ ara1*) were constructed by the one-step gene replacement
160 method (23) with a fragment containing the *kanMX4* and/or *natMX4* (24-25)
161 genes flanked by sequences identical to *BDH1* and/or *ARA1* (20).

162

163 **Plasmids, DNA manipulations, cloning techniques and transformation** 164 **methods.**

165 All DNA manipulations were performed under standard conditions as
166 described (26). *E. coli* plasmid DNA was obtained by using a commercial kit
167 provided by Sigma. The plasmids used to overexpress Bdh1p (pYES2-*BDH1*)
168 and Bdh1p-(His)₆ (pYES2-*BDH1*-6His) have already been described (19-20).
169 The formate dehydrogenase gene (*FDH1*) from *S. cerevisiae* was amplified
170 from yeast genomic DNA in a PCR with the oligos o-FDH-fw and o-FDH-rv
171 (Table 1). The band containing *FDH1* was excised from an agarose gel,
172 digested with *Bam*HI and *Eco*RI and cloned in the pYES2 vector digested with
173 the same enzymes. The construct pYES2-*FDH1* was sequenced to verify that
174 no mutations had been introduced during the PCR. Plasmids pYES2-*BDH1*-
175 6His and pYES2-*FDH1* (see above) were used as starting points to construct

176 the plasmid pYES2-*FDH1-BDH1*-6His, expressing the fusion protein containing
177 Fdh1p in its N-terminus and Bdh1-(His)₆p in its C-terminus linked by the
178 heptapeptide (ENLYFQG). The gene coding for the fusion protein was
179 constructed in two steps: firstly, we used the oligonucleotides o-FDH-fw and
180 FPint-Rv (Table 1) together with the plasmid pYES2-*FDH1* as a template, which
181 in a PCR resulted in a fragment containing *FDH1* fused to the sequence coding
182 for the peptide linker (see above). Secondly, the oligos FPint-Fw and bdh1-his
183 together with the plasmid pYES2-*BDH1*-6His as a template, were used to
184 construct by PCR a fragment that contained the sequence coding for Bdh1p-
185 (His)₆ fused to the aforementioned peptide linker. A mixture of these two
186 fragments, containing a complementary region (underlined in the oligos, see
187 Table 1), was used as template in a PCR with the external oligos o-FDH-fw and
188 bdh1-his to obtain the complete gene coding for the fusion protein Fdh1p-
189 Bdh1p(His)₆. The amplified fragment was excised from an agarose gel and
190 cloned in pYES2 digested with *Bam*HI and *Eco*RI. The correctness of the
191 construct was verified by sequencing.

192 The plasmids were introduced into the yeast strains by the lithium
193 acetate method (23), and the transformants were selected on SC-Ura medium
194 supplemented with 2% glucose or galactose (in the case of the Adh⁻ strains).

195

196 **Construction of a yeast strain overexpressing Bdh1p and Fdh1p by self-** 197 **cloning.**

198 A slight modification of the *delitto perfetto* technique (21) was
199 performed to place *BDH1* under the control of the *GAL1* promoter in its
200 chromosomal *locus*. The promoter and part of the *BDH1* coding region from the

201 yeast strain WV36-405 were deleted with a double marker (consisting of *URA3*
202 from *Kluyveromyces lactis* fused to *natMX4* from *Streptomyces noursei*)
203 obtained by PCR using the plasmids pUG72 and pAG25 (obtained from
204 Euroscarf) as templates. The oligonucleotides used to amplify the double
205 marker by PCR were Pbdh-ura-fw and Bdh-nat1-rv (Table 1). The linear
206 fragment obtained upon PCR (containing two flanking regions identical to the
207 -520→ -473 and +1→ +48 sequences from *BDH1* and its promoter) was used
208 to transform the yeast strain WV36-405 by the lithium acetate method, and the
209 transformants were selected on YPD agar medium (1% Bacto yeast extract, 2%
210 Bacto peptone, 2% glucose-containing 2% agar) supplemented with 100 µg/ml
211 clonNAT (Hans-Knöll Institute für Naturstoffforschung, Jena, Germany).
212 Several transformants that grew on clonNAT medium were checked by PCR to
213 verify that the double marker was in the proper place, thus a yeast strain WV36-
214 405 *URA3-NAT1::P_{bdh1}-bdh1* was constructed. One of the transformants was
215 grown in SC-Ura medium and transformed with a recombinogenic linear
216 fragment containing the *GAL1* promoter flanked by the -520→ -473 sequence
217 of the *BDH1* promoter and by the +1→+48 sequence from *BDH1*. This fragment
218 had been obtained in a PCR with the oligos: Pbdh-gal-Fw and Bdh-gal-bis-rv
219 (Table 1) by using pYES2-*BDH1* as a template. Upon transformation by the
220 lithium acetate method, the mixture was plated on a YPD plate overnight. The
221 plate was replica plated on a SC plate containing 100 µg/ml uracil and
222 supplemented with 1 mg/ml 5-FOA (27), the following day to allow for counter-
223 selection. The colonies that grew in this plate were replica-plated on a YPD
224 plate containing clonNAT, to discard spontaneous mutations of *URA3* that
225 allowed cells to grow on the 5-FOA plate. Several transformants growing on

FOA, but not on clonNAT plates, were checked by PCR with oligos o-gal1-fw and o-bdh1-rv (Table 1) and one of the transformants was verified by sequencing. The new strain (WV36-405 $P_{bdh1}::P_{GAL1}-BDH1$) was transformed with plasmid pYES2-*FDH1* and selected on SC-Ura plates, obtaining a yeast strain named DP-BDH1 overexpressing Bdh1p (because of the presence of its engineered chromosomal *GAL1* promoter controlling *BDH1*) and Fdh1p (under the control of the *GAL1* promoter contained in the pYES2 plasmid) on galactose. Crude extracts were prepared from this strain grown in SC-Ura medium plus 2% galactose until the beginning of the stationary phase. Permeabilized yeast DP-BDH1 cells were prepared by adding 0.1% (w/v) digitonin to the cell suspension, essentially as described (28).

Media and growth conditions.

E. coli was grown at 37°C in Luria-Bertani medium supplemented with 50 µg/ml of ampicillin to select for the desired plasmid constructs. The yeast strains derived from WV36-405 containing pYES2 derived plasmids were grown at 28 °C on a rotatory shaker (250 rpm) in SC-Ura supplemented with 2% galactose.

Bdh1p, Bdh1p(His)₆ and Fdh1p-Bdh1(His)₆ purification. Molecular mass determination.

Bdh1p, Bdh1p(His)₆ and Fdh1p-Bdh1(His)₆ were purified from the yeast strains WV36-405 $\Delta ara1$ [pYES2-*BDH1*], WV36-405 $\Delta ara1 \Delta bdh1$ [pYES2-*BDH1(His)6*] and WV36-405 $\Delta ara1 \Delta bdh1$ [pYES2-*FDH1-BDH1-6His*] respectively, as described (19-20). The last step of the purification protocol served to determine the Mr of the fusion protein, Fdh1p-Bdh1p(His)₆ by gel-

251 filtration chromatography. A Hi-Load (26/60) SuperDex 200 prep grade column
252 from GE Healthcare equilibrated with 50 mM Hepes, pH 7 and 150 mM NaCl
253 was used. The column was eluted at a flow rate of 1.75 mL/min with the
254 equilibration buffer.

255

256 **Enzyme activities, chemical transformations, and coenzyme regenerating**
257 **systems.**

258 Enzyme activities were determined spectrophotometrically by measuring
259 the change of absorbance at 340 nm and 25°C, corresponding to the oxidation
260 of NADH ($\epsilon_{340} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$). One unit (U) of activity corresponds to 1
261 μmol of NAD⁺ formed per minute. To determine the steady-state parameters,
262 the initial velocities were measured in duplicate at eight different substrate
263 concentrations and the k_{cat} and K_{M} for the different substrates (Table 2) were
264 determined by using the nonlinear regression program Grafit 5.0 (Erithacus
265 Software Ltd., Horley, U.K.). All reported values are expressed as the mean \pm
266 S.E. of at least three separate experiments. The specific activity of Bdh1p was
267 measured in 33 mM sodium phosphate buffer at pH 7, in the presence of 50
268 mM (*R/S*)-acetoin and 0.2 mM NADH. To ascertain the stereoselectivities of the
269 enzymes, reaction mixtures were prepared in 5 mL tubes with O-ring from Nirco
270 (Barberà del Vallés, Spain) with continuous agitation at room temperature. The
271 initial composition of the mixtures when using pure Bdh1p were 50 mM
272 diketone, 200 U of Bdh1p, 1 mM NADH in 33 mM sodium phosphate, pH 7
273 buffer and an NADH-regenerating system containing 100 mM sodium formate
274 and 4 U of formate dehydrogenase from *Candida boidinii* (29). When using
275 crude extracts, yeast strain DP-BDH1 (WV36-405 $P_{\text{GAL1}}\text{-BDH1}$ [pYES2-FDH1])

276 was grown in SC-Ura plus galactose until the beginning of the stationary phase
277 and the pelleted cells were disrupted with glass beads. The extracts, containing
278 150 U of Bdh1 and 3 U of Fdh activities, were incubated with 50 mM diketone
279 together with 1 mM NADH and 100 mM formate. Yeast cells, permeabilized with
280 0.1 % digitonin (28), were used to drive the reduction of 50 mM diketone, in the
281 presence of 2% galactose, 1 mM NADH, 100 mM formate and 33 mM sodium
282 phosphate pH 7. When using the fusion protein Fdh1p-Bdh1p(His)₆, the
283 reaction solution contained 39 U of Bdh and 0.3 U of Fdh, which were incubated
284 with 50 mM diketone together with 5 mM NAD⁺ and 100 mM formate as an
285 NADH-regenerating system. For the oxidation of the diols with pure Bdh1p, we
286 used an NAD⁺ regenerating system containing α -ketoglutarate, ammonium
287 chloride and glutamate dehydrogenase in 33 mM sodium phosphate buffer at
288 pH 7 (29).

289

290 **Western blots and in-gel assays of Bdh activity of yeast extracts.**

291 Bdh1p(His)₆ and Fdh1p-Bdh1(His)₆ expressed from WV36-405 Δ *ara1*
292 Δ *bdh1* [pYES2-*BDH1*-6His] and WV36-405 Δ *ara1BDH1*[pYES2-*FDH1*-*BDH1*-
293 6His] yeast strains were detected by Western blotting using a mouse
294 monoclonal anti-His₆ antibody following a previously described method (20).

295 Yeast protein extracts from strains expressing Bdh1p(His)₆ and Fdh1p-
296 Bdh1(His)₆ in different genetic backgrounds were loaded on precast (pH 3 to 9)
297 IEF gels from Bio-Rad (Criterion). Their Bdh activities were visualized on the
298 gels by incubating them with 100 mM (2R,3R)-2,3-butanediol, 2 mM NAD⁺,
299 together with 0.08 mg/ml of phenazine methosulfate, and 0.8 mg/ml of nitroblue
300 tetrazolium, essentially as described (20).

301 **Analytical methods.**

302 The reaction mixtures were extracted with chloroform, to recover the
303 hydroxyketones and diols, together with 1-hexanol, as an internal standard,
304 essentially as described (30). A slight modification was introduced to quantify *R*-
305 and *S*-acetoin and the different 2,3-butanediol stereoisomers obtained from the
306 overnight reaction mixtures. To 1-mL reaction mixture, 1-hexanol was added up
307 to 8 mM, together with 2.5 g of K₂CO₃, followed by two successive extractions
308 with 2 and 4 mL chloroform. Both extractions were necessary to attain linearity
309 in the recovered acetoin and 2,3-butanediols from standard additions to the
310 reaction mixtures. Alternatively, the analytes were extracted from the reaction
311 mixtures with two successive ethyl acetate extractions (19). The different
312 stereoisomers of the hydroxyketones and diols were resolved on a chiral
313 column (Supelco β-DEX™ 120, 30 m length, 0.25 mm inner diameter), coupled
314 to a Hewlett-Packard gas chromatograph equipped with a mass
315 spectrophotometer as a detector, under conditions previously described (19).
316 The identity of the products was verified by known standards (when available)
317 and by mass spectrometry. To confirm the resolution between (3*R*)-hydroxy-2-
318 pentanone and (2*R*)-hydroxy-3-pentanone, we also used an HP-5MS Agilent
319 column, 30 m length, 0.25 mm inner diameter, coupled to a Hewlett-Packard
320 gas chromatograph equipped with a mass spectrophotometer as a detector.
321 The following temperature program was used: isotherm at 45°C for 2 min and
322 three ramps 5°C/min up to 100°C, 15°C/min up to 150°C and 30 °C/min up to
323 275°C. The identity of the products was verified by mass spectrometry.

324

325 **Results**

326

327 **Kinetic parameters and stereoselectivity of Bdh1p.**

328 Table 2 gives the kinetic parameters determined for Bdh1p towards
329 acetoin, diacetyl, 2,3-pentanedione, 2,3-hexanedione and 3,4-hexanedione.
330 The specificity constant k_{cat}/K_M decreased from acetoin to 2,3-hexanedione and
331 then increased for 3,4-hexanedione. The reaction of purified Bdh1p with diacetyl
332 specifically produced (2*R*,3*R*)-2,3-butanediol, with a 95% yield, and a minor
333 amount of *R*-acetoin (Fig 1A). To determine the stereoisomeric purity of the
334 (2*R*,3*R*)-2,3-butanediol obtained, we added a known concentration of *meso*-2,3-
335 butanediol and (2*S*,3*S*)-2,3-butanediol to half of the reaction mixture obtained
336 upon the reaction of Bdh1p with diacetyl (and the NADH-regenerating system).
337 An equal volume of buffer was added to the other half of the reaction. By
338 comparing the areas of the known added concentrations of *meso*-2,3-butanediol
339 and (2*S*,3*S*)-2,3-butanediol (panel B in Figure 2) and the corresponding ones in
340 panel A, we estimated a stereoisomeric purity of (2*R*,3*R*)-2,3-butanediol
341 greater than 99.6% (Fig 2).

342 When using Bdh1p with 2,3-pentanedione, 2,3-hexanedione and 3,4-
343 hexanedione only one diol was obtained (Fig 1B, C, D), identified as 2,3-
344 pentanediol, 2,3-hexanediol and 3,4-hexanediol, respectively, by their mass
345 spectra. Since there are not commercial sources of pure stereoisomers of these
346 compounds that could be used as standards, we assigned the configuration of
347 the stereoisomer of 2,3-pentanediol produced by Bdh1p, by comparing the ¹H-
348 NMR spectrum obtained from 2,3-pentanediol obtained chemically with the one
349 from the diol obtained with pure Bdh1p (Supplemental Fig S1).

350 The ^1H and ^{13}C -NMR spectra of chemically prepared 2,3-pentanediol
351 showed the presence of two diastereoisomers, (2*RS*,3*RS*) and (2*RS*,3*SR*) in
352 their racemic form, showing a good agreement with NMR data described
353 before (31). Thus, the pair (2*R*,3*S*) plus (2*S*,3*R*) [abbreviated (2*RS*,3*SR*)]
354 showed the chemical shifts at $\delta=3.81\text{ppm}$ (for the H-2), $\delta=3.55$ (for the H-3),
355 $\delta=1.15$ (for the H-1) and $\delta=1.00$ (for the H-5) (supplemental Fig 1, panel B). In
356 its turn, the pair (2*R*,3*R*) plus (2*S*,3*S*) [abbreviated (2*RS*,3*RS*)] showed the
357 chemical shifts at $\delta=3.61\text{ppm}$ (for the H-2), $\delta=3.27$ (for the H-3), $\delta=1.20$ (for the
358 H-1) and $\delta=1.00$ (for the H-5) (supplemental Fig S1, panelB). From the areas of
359 the corresponding signals, the two isomers were obtained in a ratio of 2.14
360 being the isomers (2*RS*, 3*SR*) the major compounds (Supplemental Fig S1,
361 panel B). When the same mixture of 2,3-pentanediols was loaded in a GC
362 equipped with a chiral column, the four stereoisomers were resolved, being
363 those eluting at $t_R=14.86$ and 15.51 minutes less abundant than the other two
364 (supplemental Figure S2). In consequence, these minority stereoisomers should
365 correspond to the pair (2*RS*, 3*RS*).

366 Moreover, when we prepared 2,3-pentanediol by the action of Bdh1p
367 and the NADH-regenerating system, only one 2,3-pentanediol stereoisomer
368 was obtained, eluting at 15.56 minutes (supplemental Figure S3) and with a
369 NMR spectrum coincident with the one from the pair (2*RS*, 3*RS*). The chemical
370 shifts for this compound were at $\delta=3.58\text{ppm}$ (for the H-2), $\delta=3.24$ (for the H-3),
371 $\delta=1.17$ (for the H-1) and $\delta=0.98$ (for the H-5) (supplemental Fig S1, panel A).
372 Since the NMR signals obtained for this compound are coincident with the ones
373 from the pair (2*RS*, 3*RS*), this stereoisomer should be (2*R*,3*R*)-2,3-pentanediol
374 or (2*S*,3*S*)-2,3-pentanediol. Given the stereoselectivity displayed by Bdh1p

375 towards diacetyl, we assign the peak eluting at 15.51 minutes to (2*R*,3*R*)-2,3-
376 pentanediol (also see Discussion) and automatically the stereoisomer eluting at
377 t_R =14.86 to (2*S*,3*S*)-2,3-pentanediol (supplemental Figure S2).

378

379 **Search for other yeast NADH-dependent diacetyl reductases.**

380 A protein extract of a yeast strain deleted for *bdh1*, namely EG2
381 (FY834α *TRP1::bdh1*) (19) grown in YPglucose displayed a diacetyl reductase
382 activity of 0.73 U/mg (measured in 50 mM diacetyl and 0.2 mM NADH). The
383 extract was loaded in an IEF gel and the diacetyl reductase activities were
384 visualized by activity staining with diacetyl and NADH (results not shown). A
385 clear band with a pI lower than the pI of Bdh1p (Bdh1p was not visible in this gel
386 since *BDH1* was disrupted in the strain EG2) indicated that another enzyme (or
387 enzymes) was able to reduce diacetyl in an NADH- dependent reaction.
388 Therefore, we developed a protocol to purify this diacetyl reductase activity from
389 the yeast strain EG2. The protocol consisted of a DEAE-Sepharose, followed by
390 a hydroxyapatite column and by a Cibachron Blue 3GA chromatography. We
391 obtained a homogeneous protein with an estimated Mr of approximately 42,000
392 (by SDS-PAGE), which upon digestion with trypsin was analyzed by MALDI-
393 TOF MS (conditions described in González et al., 2010). The protein was
394 identified as Adh2p, and thus we analyzed by GC-MS the products obtained in
395 the reduction of diacetyl in the presence of Adh2p, which resulted in *S*-acetoin.
396 Given the similarity between Adh2p, Adh1p and Adh3p, we cloned and over-
397 expressed Adh1p and Adh3p (by means of the galactose inducible pYES2
398 vector) in the WV36-405 strain, finding that both displayed diacetyl reductase
399 activity, also yielding *S*-acetoin. The supplemental Figure S4 shows that

400 diacetyl treated with yeast Adh yielded S-acetoin. When the filtrate obtained
401 upon this reduction, was treated with Bdh1p, meso-2,3-butanediol was
402 obtained (supplemental Figure S5). Moreover, when 2,3-pentanedione was
403 treated with yeast Adh yielded (2S)-hydroxy-3-pentanone (supplemental Figure
404 S6). When the filtrate obtained upon this reduction, was treated with Bdh1p,
405 (2S,3R)-2,3-pentanediol was obtained (supplemental Figure S7). The residual
406 amount of 2,3-pentanedione from the filtrate yielded (3R)-hydroxy-2-pentanone
407 and (2R,3R)-2,3-pentanediol (supplemental Figure S7). Thus, since Bdh1p is
408 enantioselective yielding (R)-acetoin from diacetyl and, (2R,3R)-2,3-butanediol
409 from (R)-acetoin, we decided to use the Adh-deficient strain WV36-405 when
410 working with extracts and permeabilized cells to minimize the production of S-
411 acetoin, that would yield meso-2,3-butanediol upon the action of Bdh1p.

412

413 **Diols and hydroxyketones obtained from vicinal diketones by yeast**
414 **extracts and permeabilized cells.**

415 Although purified Bdh1p yields enantiopure vicinal diols upon the
416 reduction of the corresponding diketones, an NADH-regenerating system was
417 necessary to displace the reaction towards the diols. To avoid the need of an
418 external source of FDH activity to regenerate NADH, we constructed a yeast
419 strain that overexpressed Bdh1p and yeast formate dehydrogenase (Fdh1p),
420 such that it would not be necessary to purify Bdh1p, nor the addition of
421 exogenous FDH. As a first step to construct such a strain, we used a
422 modification of the “*delito perfetto*” technique (21) developed in our laboratory to
423 change the chromosomal promoter of *BDH1* by the *GAL1-10* promoter. Then, a
424 multicopy inducible plasmid with cloned *FDH1* under the control of the *GAL1*

425 promoter was used to transform the previous yeast strain, such that the
426 presence of galactose in the growth medium induced the Bdh and Fdh
427 activities. The resulting yeast strain was named DP-BDH1 (WV36-405 P_{GAL1} -
428 *BDH1* [pYES2-*FDH1*]). To ascertain the overexpression of Bdh and Fdh
429 activities in the strain DP-BDH1, we grew it and its parental control strain
430 (WV36-405 P_{BDH1} -*BDH1* [pYES2]) in galactose containing medium. At the end
431 of their logarithmic phases, we obtained the yeast extracts by disrupting the
432 cells with glass beads and determined their Bdh and Fdh activities. While the
433 Bdh and Fdh specific activities from the control strain, WV36-405 P_{BDH1} -
434 *BDH1*[pYES2], were 0.26 and less than 0.001 U/mg, respectively (measured at
435 5 mM NAD^+ and 100 mM (2*R*,3*R*)-butanediol or 100 mM formate), the
436 corresponding ones from the strain DP-BDH1 were 1.22 and 0.13 U/mg,
437 respectively. The yeast strain DP-BDH1 was permeabilized with 0.1% digitonin,
438 following the technique of Cordeiro and Freire (1995) and incubated with the
439 different diketones and galactose containing medium. When using the protein
440 extracts or the digitonin permeabilized yeast cells DP-BDH1, grown in
441 galactose, on the different diketones, the products obtained were a mixture of
442 all possible stereoisomers (Figs. 3 and 4). Thus, (2*R*,3*R*)-2,3-butanediol, *meso*-
443 2,3-butanediol and traces of (2*S*,3*S*)-2,3-butanediol were the products upon the
444 reduction of diacetyl with permeabilized cells with a yield of 65% (adding the 3
445 butanediol stereoisomers) (Fig. 4). Furthermore, the additional enzymatic
446 activities present in the yeast extracts and permeabilized cells yielded several
447 diol stereoisomers from 2,3-pentanedione, 2,3-hexanedione and 3,4-
448 hexanedione (Figs. 3 and 4). For instance, the four stereoisomers of 2,3-

449 hexanediol could be detected from their mass spectra, upon the reaction of 2,3-
450 hexanedione with DP-BDH1 extracts or permeabilized cells (Figs. 3C and 4C).

451

452 **Identification of the stereoisomeric forms of the hydroxyketones and**
453 **vicinal diols.**

454 In the case of the acetoin and 2,3-butanediol stereoisomers obtained
455 upon reduction of diacetyl (Figs. 3A and 4A), the assignment of the
456 stereoisomeric forms was made by comparison with the retention times of the
457 pure (2*R*,3*R*)-2,3-butanediol, *meso*-2,3-butanediol and (2*S*,3*S*)-2,3-butanediol
458 and also after the oxidation of pure (2*R*,3*R*)-2,3-butanediol to *R*-acetoin (19)
459 and of *meso*-2,3-butanediol to *S*-acetoin. In the case of 2,3-pentanedione
460 (Figure 4B), the MS of the peaks that eluted at 7.06 and 7.77 min (*m/z* of 59, 43
461 and 31, in decreasing % abundance) identified them as 3-hydroxy-2-
462 pentanones (32), while the peak eluting at 7.64 min (*m/z* of 45, 57 and 43, in
463 decreasing % abundance) was identified as 2-hydroxy-3-pentanone (32). The
464 stereoisomeric form eluting at *t_R*=7.06 min was assigned to (3*R*)-hydroxy-2-
465 pentanone since its retention time was the same as the product obtained with
466 pure Bdh1p and 2,3-pentanedione (after one hour of reaction only). Thus, the 3-
467 hydroxy-2-pentanone eluting at 7.77 min must be (3*S*)-hydroxy-2-pentanone.
468 The 2-hydroxy-3-pentanone eluting at 7.64 min was assigned to (2*S*)-hydroxy-
469 3-pentanone, since this retention time was the same as the product obtained
470 upon reduction of 2,3-pentanedione with pure Ara1p (33). Further evidences for
471 the assignments of the hydroxyketones were obtained from the oxidations of
472 (2*R*,3*R*)-2,3-pentanedione and (2*R*,3*R*)-2,3-hexanediol with pure Bdh1p (see
473 below).

474 The 2,3-pentanediol peaks observed upon reduction of 2,3-
475 pentanedione with yeast extracts or permeabilized cells overexpressing Bdh1p
476 and Fdh1p (Figures 3B and 4B) were assigned according to the signal obtained
477 with pure Bdh1p on 2,3-pentanedione (see above), for (2*R*,3*R*)-2,3-pentanediol
478 (eluting at t_R =16.87 min, Fig 4, panel B). The identities of the stereoisomers
479 (2*R*,3*S*)-2,3-pentanediol and (2*S*,3*R*)-2,3-pentanediol, were established by
480 treating 2,3-pentanedione with Ara1p followed by Bdh1p: by our previous work
481 (33), we knew that (2*S*)-hydroxy-3-pentanone should be obtained from Ara1p
482 and (2*S*,3*R*)-2,3-pentanediol after treating this hydroxyketone with Bdh1p. This
483 compound corresponds to the peak eluting at t_R =18.19 minutes (Fig 4, panel B),
484 so finally the peak eluting at t_R =18.07 minutes should be (2*R*,3*S*)-2,3-
485 pentanediol (Fig 4, panel B). No (2*S*,3*S*)-2,3-pentanediol is observed in these
486 figures.

487 In regard to the 2,3-pentanediol stereoisomers obtained chemically
488 (supplemental Figure S2), the identities of the different stereoisomers are
489 marked in the figure. The elution times are different from the ones obtained in
490 the reduction of 2,3-pentanedione by yeast extracts (and permeabilized cells)
491 because the reaction mixtures were extracted with ethyl acetate and were run at
492 different days.

493 The same experimental strategy and reasoning was followed to assign
494 the hydroxyketones and 2,3-hexanediols derived from 2,3-hexanedione (Fig.
495 4C). First, the four peaks eluting from t_R =20 to 21 min, were assigned to 2,3-
496 hexanediol by their MS (with m/z of 55, 73, 45 and 43, in decreasing %
497 abundance), characteristic of 2,3-hexanediols (34). Second, the treatment of
498 2,3-hexanedione with pure Bdh1p allowed us to assign (2*R*,3*R*)-2,3-hexanediol

499 to the $t_R=20.25$ minutes peak, and the treatment of 2,3-hexanedione with Ara1p
500 followed by Bdh1p, allowed to assign (2*S*,3*R*)-2,3-hexanediol to the 20.74 min
501 peak (Fig. 4C). The remaining stereoisomers were assigned as in the case of
502 the reduction of 2,3-pentanedione. In fact, the same order of elution of the 2,3-
503 hexanediols stereoisomers was also obtained by Schröder et al. (1994),
504 working with a β -cyclodextrin chiral column (34).

505 In the case of the diols produced upon the reduction of 3,4-hexanedione (Fig.
506 4D), the assignation of the stereoisomeric forms was made as in the case of
507 diacetyl reduction.

508 **Properties of the fusion protein Fdh1p-Bdh1p(His)₆.**

509 The fusion protein Fdh1p-Bdh1p(His)₆ was purified from the yeast
510 strain WV36-405 Δ *ara1* Δ *bdh1* [pYES2-*FDH1-BDH1*-6His], by means of a Ni-
511 Sepharose column, followed by gel-filtration on a Hi-Load SuperDex 200 that
512 also served to determine its Mr. The estimated Mr for Fdh1p-Bdh1p(His)₆ and
513 Bdh1p(His)₆ were 133,000 and 70,500, respectively (Fig. 5C). Given the Mr of
514 Fdh1p (Mr=41,700) and Bdh1p (Mr=41,500) monomers, the active form of the
515 fusion protein is a dimer of two Fdh1-Bdh1(His)₆p polypeptides. The fusion
516 protein is rather unstable as it can be deduced from the Western blot analysis
517 (Fig. 5A, lane 2). A main band of an apparent Mr close to 95,000 (consistent
518 with the Mr of a Fdh1p-Bdh1p(His)₆ polypeptide) and several bands
519 (presumably degradation products) of lower Mr are visible in the blot. Under
520 these conditions Bdh1p(His)₆ is stable as only one band of the correct Mr is
521 visible in the blot (Fig. 5A, lanes 3 and 4). Furthermore, the presence of Fdh1p
522 in the fusion protein decreases the pI of Bdh1p(His)₆p (compare lanes 3 and 4
523 with lane 2 in Fig. 5B). Thus, the activity bands seen in the gel (Fig. 5B, lane 2)

confirm that Fdh1p-Bdh1p(His)₆ had a lower pI than Bdh1p(His)₆ in two genetic backgrounds, with endogenous Bdh1p (Fig 5B, lane 3) and without endogenous Bdh1p (Fig. 5B, lane 4). Moreover, we determined the kinetic constants of Fdh1p-Bdh1p(His)₆ towards (*R/S*)-acetoin as $K_M=2.2$ mM (determined at 0.2 mM NADH) and $k_{cat}=31s^{-1}$. From the specific activity of Fdh1p-Bdh1p(His)₆ towards formate (at 5 mM NAD⁺), an apparent k_{cat} of $0.25 s^{-1}$ was estimated at 25 °C. Thus, the k_{cat} of the fusion protein for acetoin was approximately 50 times lower than the k_{cat} of Bdh1p (Table II), while the K_M values were similar. The k_{cat} for formate was 24 times lower than the published value obtained at 30°C (35).

534

535 **Hydroxyketones and diols produced by the fusion protein Fdh1p-** 536 **Bdh1p(His)₆.**

537 The purified fusion protein Fdh1p-Bdh1p(His)₆ yielded a single form of
538 diol, namely (2*R*,3*R*)-2,3-butanediol, upon diacetyl reduction (Fig. 6A) with a
539 conversion of 31%. Moreover, when starting with either 2,3-pentanedione, 2,3-
540 hexanedione or 3,4-hexanedione a single stereoisomeric form of diol was
541 obtained also (Figs. 6B, C and D). Since these diols have the same retention
542 times than the diols obtained with pure Bdh1p, they have been tentatively
543 assigned as the corresponding (*R,R*) stereoisomers. The reduction of the
544 diketones by Fdh1p-Bdh1p(His)₆ yielded also hydroxyketones. In the case of
545 diacetyl, the hydroxyketone was identified as *R*-acetoin, while in the case of 2,3-
546 pentanedione and 2,3-hexanedione, they were identified by their mass spectra
547 as 3-hydroxy-2-pentanone and 3-hydroxy-2-hexanone, respectively. We
548 assigned them the *R* configuration (Fig. 6) because we assume that, for these

549 compounds, the fusion protein displays the same enantioselectivity shown
550 towards diacetyl.

551

552 **Oxidation of the vicinal diols with Bdh1p.**

553 We first studied the products obtained in the oxidation reaction of (2*R*,
554 3*R*)-2,3-butanediol by Bdh1p in the presence of NAD⁺ and an NAD⁺-
555 regenerating system, consisting of α -ketoglutarate, ammonium chloride and
556 glutamate dehydrogenase. We obtained *R*-acetoin as expected from the
557 oxidation of (2*R*,3*R*)-2,3-butanediol (results not shown). Since (2*R*,3*R*)-2,3-
558 pentanediol, (2*R*,3*R*)-2,3-hexanediol and (3*R*,4*R*)-3,4-hexanediol are not
559 commercially available, they were obtained *in situ* from the reaction products
560 upon reduction of 2,3-pentanedione, 2,3-hexanedione and 3,4-hexanedione by
561 Bdh1p (presumably the (*R,R*)-stereoisomers, see above). When using (2*R*,3*R*)-
562 2,3-pentanediol and NAD⁺ with Bdh1p, together with the NAD⁺-regenerating
563 system, one peak was obtained, with a MS pattern compatible with 2-hydroxy-3-
564 pentanone (Fig. 7C). We also analyzed the reaction products obtained upon the
565 reduction of 2,3-pentanedione with Bdh1p (after one-hour reaction) and
566 observed a peak, identified as 3-hydroxy-2-pentanone (Fig. 7A). The insets
567 from Figs. 7A and 7C show only the most abundant and selective ions for (3)-
568 hydroxy-2-pentanone (*m/z* of 59, black) and (2)-hydroxy-3-pentanone (*m/z* of
569 45, red) for clarity. Both compounds should have the *R*-configuration and
570 showed close retention times on the chiral column. We obtained an additional
571 proof of their identities by loading them (as a chloroform extract) on an HP-5MS
572 column, which resolved two peaks corresponding to (3*R*)-hydroxy-2-pentanone
573 and (2*R*)-hydroxy-3-pentanone (results not shown). Furthermore, a mixture of

the four stereoisomeric forms of 2,3-pentanediol was oxidized with Bdh1p and, the most reactive species were (2*R*,3*R*)-2,3-pentanediol, yielding essentially (2*R*)-hydroxy-3-pentanone (t_R = 6.36 min), but also a minor amount of (3*R*)-hydroxy-2-pentanone (t_R = 6.31 min) and, (2*S*,3*R*)-2,3-pentanediol that yielded (2*S*)-hydroxy-3-pentanone (t_R = 6.76 min). A minor amount of (3*S*)-hydroxy-2-pentanone (t_R = 6.91 min) was obtained from the oxidation of (2*R*,3*S*)-2,3-pentanediol by Bdh1p (supplemental Figure S8). We did a parallel study with (2*R*,3*R*)-2,3-hexanediol with similar results. Thus, the reduction of 2,3-hexanedione yielded (3*R*)-hydroxy-2-hexanone (Fig. 8A and C), while the oxidation of (2*R*,3*R*)-2,3-hexanediol yielded (2*R*)-hydroxy-3-hexanone (Fig. 8B and D). Fig 8C and D show only the most abundant and selective ions for 3-hydroxy-2-hexanone (m/z of 55, black), and 2-hydroxy-3-hexanone (m/z of 45, red). However, since the reduction of 2,3-hexanedione was not complete (Fig. 8A), some (3*R*)-hydroxy-2-hexanone was carried over together with (2*R*,3*R*)-2,3-hexanediol, which is visible in the chromatogram (Fig. 8B). With all these data on the oxidation/reduction of vicinal diols/diketones with Bdh1p, we propose as a mechanism that the more reactive carbon upon oxidation (of the diol) and reduction (of the diketone) would be that corresponding to the most internal hydroxyl (or keto) group (Fig. 9). Furthermore, in order to resolve all the stereoisomeric forms of 2-hydroxypentanone and 3-hydroxypentanone (as well as the corresponding hydroxyhexanones), we induced the racemization and isomerization through enolate species of the hydroxyketones by adding NaOH to the products obtained upon oxidation of (2*R*,3*R*)-2,3-pentanediol and (2*R*,3*R*)-2,3-hexanediol (or reduction of the diketones). Figure 10 shows that the chiral column successfully resolved all the hydroxyketone stereoisomers.

599

Discussion

600 The main aims of the present work were to develop a system able to
601 produce (*R,R*) diols from vicinal diketones, to determine the stereoselectivity of
602 Bdh1p towards the reduction of the two carbonyl groups, to determine the
603 kinetic constants of the reactions and to elucidate its preference towards the
604 keto groups of the vicinal diketones. In the first part of the study, we showed
605 that pure Bdh1p reduced both carbonyl groups from diacetyl to
606 stereoisomerically pure (*2R,3R*)-2,3-butanediol (Fig. 2A and B). Furthermore,
607 when using 2,3-pentanedione, 2,3-hexanedione and 3,4-hexanedione with pure
608 Bdh1p, only one stereoisomeric form of the corresponding diols was identified in
609 the reaction mixtures. Given the stereoselectivity displayed by Bdh1p towards
610 diacetyl and, since there are not commercial sources of those pure
611 stereoisomers, we tentatively assigned them to the corresponding (*R, R*)
612 stereoisomers. We performed, however, NMR and GC experiments to assign
613 the configuration of the 2,3-pentenediol stereoisomer produced by Bdh1p from
614 2,3-pentanedione. There are several reasons to conclude that the stereoisomer
615 is (*2R,3R*)-2,3-pentenediol. Thus, the NMR experiments on the four 2,3-
616 pentenediol stereoisomers obtained chemically show the signals from the pair
617 (*2R,3S*) plus (*2S,3R*) and the pair (*2R,3R*) plus (*2S,3S*). The NMR signals show
618 that the pair (*2R,3R*) plus (*2S,3S*) is in a ratio twice as low as the pair (*2R,3S*)
619 plus (*2S,3R*). Since the same mixture loaded in a GC show two peaks in a ratio
620 twice as low as the two other peaks, those peaks correspond to the pair
621 (*2R,3R*) plus (*2S,3S*). Thus, the results from the NMR data alone do not discard
622 a mixture of (*2R,3R*)-2,3-pentenediol and its enantiomer (*2S,3S*)-2,3-
623 pentenediol, on the reduction of 2,3-pentanedione by Bdh1p (supplemental

624 Figure S1). However, only one stereoisomer was obtained, when the reaction
625 mixture was loaded in the chiral column (supplemental Figure S2).
626 Consequently, the product can be (2*R*,3*R*)-2,3-pentanediol or (2*S*,3*S*)-2,3-
627 pentanediol. Given the stereospecificity shown by Bdh1p towards the
628 production of (2*R*,3*R*)-2,3-butanediol from diacetyl, the production of pure
629 (2*S*,3*S*)-2,3-pentanediol from 2,3-pentanedione, without any production of the
630 (2*R*,3*S*)-2,3-pentanediol nor (2*S*,3*R*)-2,3-pentanediol seem very unlikely.

631 The steady-state kinetic studies performed with pure Bdh1p and
632 several vicinal diketones show that the specificity constant k_{cat}/K_M decreased
633 from diacetyl to 2,3-hexanedione and then increased for 3,4-hexanedione
634 (Table 2). Bdh1p displayed the highest specificity constants towards the
635 substrates known to be transformed by Bdh1p in the *Saccharomyces cerevisiae*
636 metabolism: i.e. acetoin, diacetyl and 2,3-pentanedione.

637 Although the use of pure Bdh1p yielded only one stereoisomeric form
638 of the diols, it was necessary to use formate and exogenous formate
639 dehydrogenase to regenerate NADH. To avoid this drawback, we explored the
640 possibility of using a yeast strain derived from FY834 (22) which could
641 overexpress their endogenous *BDH1* and *FDH1*. To choose a convenient
642 genetic background, we tested first whether there were any diacetyl reductases
643 other than Bdh1p in the yeast extracts derived from the wild type strain FY834.
644 We detected Adh2p with an NADH-dependent reductase activity that yielded S-
645 acetoin from diacetyl. Besides, we showed that Adh1p and Adh3p could also
646 reduce diacetyl to S-acetoin, in NADH-dependent reactions. Furthermore, with a
647 commercial preparation of yeast Adh, we obtained also S-acetoin from diacetyl
648 (supplemental Figure S4). Consequently, we decided to work with a yeast strain

649 with a genetic background $\Delta adh1$, $\Delta adh2$, $\Delta adh3$ (as WV36-405), to avoid the
650 production of *meso*-2,3-butanediol, that would be obtained from *S*-acetoin by
651 Bdh1p (19) (supplemental Figure S5). We developed a modified *delitto perfetto*
652 technique (21), to overexpress Bdh1p and Fdh1p in the strain WV36-405. The
653 counter-selection marker used, *URA3* from *Kluyveromices lactis*, being different
654 in sequence to *URA3* from *S.cerevisiae*, minimized the homologous
655 recombination events to the endogenous *URA3* locus, while the selection
656 marker *NAT1* yields less spontaneous mutants than the *kanMX4* marker (25).
657 Thus, by placing the *GAL1-10* promoter controlling *BDH1* and transforming the
658 resultant strain with a multicopy galactose-inducible plasmid carrying *FDH1*, we
659 obtained a modified yeast strain (named DP-BDH1) that overexpressed Bdh1p
660 and Fdh1p in the presence of galactose. We measured a 4.5-fold increase in
661 Bdh specific activity towards (2*R*,3*R*)-2,3-BD and more than 100 fold increase in
662 Fdh specific activity towards formate in the corresponding protein extracts, in
663 comparison to the values of the parental strain, transformed with an empty
664 plasmid. We used this engineered strain in the form of protein extracts and
665 permeabilized cells to drive the reductions of diketones without exogenously
666 added Fdh. Although the diketones were reduced, the obtained diols were a
667 mixture of different stereoisomers. Thus, with diacetyl, *meso*-2,3-butanediol and
668 (2*R*,3*R*)-2,3-butanediol were identified as products by using protein extracts or
669 permeabilized cells (Figs. 3 and 4). A mixture of different stereoisomeric forms
670 of 2,3-pentanediol, 2,3-hexanediol and 3,4-hexanediol were obtained in the
671 reductions of 2,3-pentanedione, 2,3-hexanedione and 3,4-hexanedione,
672 respectively (Figs. 3 and 4). Although it was advantageous to use DP-BDH1
673 with no need of coenzyme regenerating processes, the diastereoisomeric

674 composition of the products precludes its use to obtain pure (*R,R*)-diols.
675 Mixtures of stereoisomeric alcohols are formed because of the presence of
676 other enzymes that could convert the same substrates but with different
677 stereoselectivities. Thus, the activities of other diketone reductases (NADH- and
678 also NADPH-dependent in the permeabilized cells) in yeast would be
679 responsible for the production of *S*-hydroxyketones and the different
680 stereoisomeric forms of the diols (20, 32). In fact, the reduction of diacetyl and
681 2,3-pentanedione for only one hour, allowed the observation of *S*-acetoin, (2*S*)-
682 hydroxy-3-pentanone and (3*S*)-hydroxy-2-pentanone (Fig. 4A and 4B). Then,
683 Bdh1p and/or other reductases would reduce these mixtures of hydroxyketones
684 to the corresponding mixture of diols. A mixture of stereoisomeric alcohols
685 derived from the reduction of α - and β -ketoesters was already observed working
686 with yeast cells (17).

687 To avoid these interfering activities, and the use of exogenous Fdh, we
688 constructed a bifunctional fusion protein containing Fdh and Bdh activities with
689 a His-tag at its C-terminus Fdh1p-Bdh1p(His)₆. By using this protein, pure
690 (2*R*,3*R*)-2,3-butanediol, (2*R*,3*R*)-2,3-pentanediol, (2*R*,3*R*)-2,3-hexanediol and
691 (3*R*,4*R*)-3,4-hexanediol (Fig. 6) were obtained, but with lower yield (31% with
692 (2*R*,3*R*)-2,3-butanediol) than when using pure Bdh1p and exogenous formate
693 dehydrogenase (95% conversion of diacetyl into (2*R*,3*R*)-2,3-butanediol). The
694 fusion Fdh1p-Bdh1p(His)₆ behaved as a dimer in a gel-filtration column (Fig.
695 5C), consistent with the fact that Bdh1p is a dimer (19) and the orthologous
696 formate dehydrogenase from *Candida boidinii* is also a dimer (36). The stability
697 of the fusion protein was checked by Western-blot and zymogram analyses
698 (Fig. 5A and B), showing that the protein suffered proteolysis but maintained

699 Bdh activity on the zymogram. The use of this fusion protein allowed the
700 production of pure (*R,R*)-diols from vicinal diketones, without the need of adding
701 an external cofactor-regenerating system, but with a low yield. In fact the k_{cat}
702 values of the Bdh and Fdh activities were 50 and 24 times lower, respectively,
703 for the fusion protein than for the individual enzymes. However, this strategy
704 allowed the rapid purification of the fusion protein and the production of
705 enantiopure (*R,R*)-diols. However, further work needs to be done, to increase
706 the stability of the fusion protein and the expression level and/or the turnover
707 rate of Fdh1p. A similar approach for cofactor regeneration was used recently
708 by Hölsch and Weuster-Botz (2010) to convert a prochiral ketone to (*S*)-1-
709 (pentafluorophenyl)-ethanol. They used a bifunctional protein composed of an
710 NADP⁺ dependent mutant of formate dehydrogenase linked to a NADPH-
711 reductase, in the whole-cell reduction of pentafluoroacetophenone. In their
712 case, however, the activity of the oxidoreductase was almost not affected by the
713 fusion.

714 A different genetic engineering approach was taken by Kim et al. (8)
715 and by Lian and coworkers (13) that constructed pyruvate decarboxylase
716 mutant yeasts strains as starting point to develop high titer producers of 2,3-
717 butanediol. Thus, more than 96.2 g/L of 2,3-butanediol by fed-batch
718 fermentation with glucose (8) and more than 100 g/L of (*2R,3R*)-2,3-BD, from
719 glucose and galactose were obtained (13). However, those engineered strains
720 did not produce (*2R,3R*)-2,3-pentanediol, (*2R,3R*)-2,3-hexanediol nor (*3R,4R*)-
721 3,4-hexanediol.

722 We also studied the specificity of Bdh1p towards the two hydroxy
723 groups of (*2R,3R*)-2,3-pentanediol and (*2R,3R*)-2,3-hexanediol upon oxidation

724 with NAD^+ , as well as for the reverse reaction, the reduction of 2,3-
725 pentanedione and 2,3-hexanedione with NADH. The reduction of 2,3-
726 pentanedione after one hour of reaction yielded (3*R*)-hydroxy-2-pentanone as
727 intermediate (Fig. 7A). When the reduction reaction was complete (Fig. 7B), the
728 selectivity was studied in the oxidation direction, yielding (2*R*)-hydroxy-3-
729 pentanone. Furthermore, we prepared the four 2,3-pentanediol stereoisomers
730 by borohydride reduction of 2,3-pentanedione finding that the most abundant
731 species (from their NMR signals) were the enantiomeric pair (2*R*,3*S*)-2,3-
732 pentanediol and (2*S*,3*R*)-2,3-pentanediol (supplemental Figure S1), that when
733 loaded into the chiral column yielded the two more abundant peaks with longer
734 retention times (supplemental Figure S2). To characterize the configurations of
735 these two stereoisomers, we reduced 2,3-pentanedione with Ara1p (the aldo-
736 keto reductase AKR31C) that produced (2*S*)-hydroxy-3-pentanone (our work,
737 reference 33). The reduction of this hydroxyketone with Bdh1p gave a single
738 peak, that given the stereoselectivity of Bdh1p should be (2*S*,3*R*)-2,3-
739 pentanediol, with a retention time of $t_R=16.87$ minutes (results not shown).
740 Consequently, we could assign the remaining peak at $t_R=16.63$ minutes to
741 (2*R*,3*S*)-2,3-pentanediol. The identity of the peaks eluting at $t_R=14.86$ and
742 $t_R=15.51$ was assigned from the reduction of 2,3-pentanedione with Bdh1p,
743 obtaining only one stereoisomer that corresponds to the one eluting at $t_R=15.51$
744 minutes (supplemental Figure S3). After our previous discussion (see above),
745 we can safely assume that this stereoisomer should be (2*R*, 3*R*)-2,3-
746 pentanediol and the one eluting at $t_R=14.86$, should then be (2*S*,3*S*)-2,3-
747 pentanediol. Upon oxidation of the mixture of the four 2,3-pentanediol
748 stereoisomers, it can be observed that the most reactive species were (2*R*,3*R*)-

749 2,3-pentanediol and (2*S*,3*R*)-2,3-pentanediol (supplemental Figure S8) yielding
750 (2*R*)-hydroxy-3-pentanone and (2*S*)-hydroxy-3-pentanone, indicating the
751 preference towards the oxidation of the most internal carbon with *R*
752 configuration.

753 A consistent result with these observations was obtained when
754 studying the oxidation/reduction of (2*R*,3*R*)-2,3-hexanediol/2,3-hexanedione by
755 Bdh1p. The enzyme showed preference for the functional group bound to the
756 most internal carbon, namely C-3, in the oxidation and reduction reactions (Fig.
757 8). Thus, 3-hydroxy-2-hexanone was identified in the reduction direction and 2-
758 hydroxy-3-hexanone in the oxidation direction by its mass spectra. With the
759 characterization of all these intermediates and products, we propose the
760 mechanism shown in Fig. 9, which indicates that the most internal carbon (C-3
761 for 2,3-pentanedione and hexanedione) is the most reactive, since it is the one
762 that predominantly reacts in both directions. Although we did not detect (2*R*)-
763 hydroxy-3-pentanone in the reduction direction of 2,3-pentanedione by Bdh1p
764 (Fig.7A), it would be difficult to discard it completely (Fig. 9 discontinuous line).
765 For the same reason, we draw a discontinuous line in the oxidation of (2*R*,3*R*)-
766 2,3-pentanediol to (3*R*)-hydroxy-2-pentanone, that is much less important than
767 the oxidation of (2*R*,3*R*)-2,3-pentanediol to (2*R*)-hydroxy-3-pentanone
768 (supplemental Figure S8). Since no 3D structure of any (2*R*,3*R*)-2,3-butanediol
769 dehydrogenase has been published yet, it would be speculative to give a
770 structural reason for this preference. A recent article (38) reports the
771 crystallization of the orthologous (2*R*,3*R*)-2,3-butanediol dehydrogenase from
772 *Bacillus coagulans*, therefore we will be able to discuss more deeply the

773 differential reactivity of both stereogenic centers, once the 3D structure of the
774 enzyme is solved.

775 Finally, we showed the resolution power of the β -cyclodextrin column
776 that was able to resolve the four hydroxyketones derived from 2,3-pentanedione
777 and the four derived from the 2,3-hexanedione (Fig 10). Although *R*-3-hydroxy-
778 2-pentanone and *R*-2-hydroxy-3-pentanone are not completely resolved with
779 the chiral column under the conditions used, we obtained a better resolution
780 and confirmed their identities by the use of an HP-5MS column (results not
781 shown).

782

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792

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930 *Bacillus coagulans* 2-6.
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932
933

934 **Table I. List of oligonucleotide primers used.**

935 The restriction sites and self-complementary regions of the primers FPint-Fw
936 and FPint-Rv are underlined.

| Function | Name | Sequence |
|--|----------------|---|
| Cloning <i>FDH1</i> and <i>BDH1</i> -6His in vector pYES2 | o-FDH-fw | 5' CGC <u>GGA TCC AAT</u> ATG TCG AAG GGA AAG G 3' |
| Cloning <i>FDH1</i> in vector pYES2 | o-FDH-rv | 5' CCG <u>GAA TTC TTA</u> TTT CTT CTG TCC ATA AGC TCT GG 3' |
| Construction of the template for FDH1-BDH1-6His | FPint-Rv | 5' <u>CAA AGC TCT CAT ACC TTG</u> <u>AAA ATA CAA ATT TTC TTT</u> <u>CTT CTG</u> TCC ATA AGC TCT GG 3' |
| Construction of the template for FDH1-BDH1-6His | FPint-Fw | 5' <u>CAG AAG AAA GAA AAT TTG</u> <u>TAT TTT CAA GGT ATG AGA</u> <u>GCT TTG</u> GCA TAT TTC AAG AAG 3' |
| Cloning FDH1-BDH1-6His in vector pYES2 | bdh1-his | 5' GCG <u>GAA TTC TTA</u> ATG ATG ATG ATG ATG ATG CTT CAT TTC ACC GTG ATT GTT AGG 3' |
| Strain generation WV36-405 <i>URA3-NAT1::p_{bdh1}-bdh1</i> | Pbdh-ura-fw | 5' GAG CAG TCG GAA AGA TCA AGA AAG ACT ACG AGA ATC AAT AAA CGA GGC CAA TAC AAC AGA TCA CGT G 3' |
| Strain generation WV36-405 <i>URA3-NAT1::p_{bdh1}-bdh1</i> | Bdh-nat1-rv | 5' ATT AGT GAA GTG AAT ATC ACC CTT CTT GAA ATA TGC CAA AGC TCT CAT TCG ACA CTG GAT GGC GGC G 3' |
| Strain generation WV36-405 <i>P_{GAL1}-BDH1</i> | Pbdh-gal-Fw | 5'GAG CAG TCG GAA AGA TCA AGA AAG ACT ACG AGA ATC AAT AAA CGA GGC ACG GAT TAG AAG CCG CCG AGC 3' |
| Strain generation WV36-405 <i>P_{GAL1}-BDH1</i> | Bdh-gal-bis-rv | 5' ATT AGT GAA GTG AAT ATC ACC CTT CTT GAA ATA TGC CAA AGC TCT CAT TAT CCG GGG TTT TTT CTC CTT GAC G 3' |
| Verification of the construct <i>P_{GAL1}-BDH1</i> | o-gal1-fw | 5' CCG ACG GAA GAC TCT CCT CCG 3' |
| Verification of the construct <i>P_{GAL1}-BDH1</i> | o-bdh1-rv | 5' GGT CCG CAC AAC TGC TGG CAG C 3' |

937

938 **Table II.**
939 **Steady-state kinetic constants of yeast Bdh1p towards acetoin and**
940 **several vicinal diketones.**

941
942 Acetoin and diketone reduction activities were measured at 25°C in 33 mM
943 sodium phosphate pH 7.0 with 0.2 mM NADH.

944
945

| | k_{cat} (s^{-1}) | K_{M} (mM) | $k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \cdot \text{M}^{-1}$) |
|------------------------|--------------------------------------|---------------------|---|
| (<i>R/S</i>)-acetoin | 1,625±83 | 3±0.6 | $5.4 \times 10^5 \pm 1.3 \times 10^5$ |
| diacetyl | 1,735±165 | 60±2 | $2.9 \times 10^4 \pm 0.4 \times 10^4$ |
| 2,3-pentanedione | 826±83 | 33±7 | $2.5 \times 10^4 \pm 0.8 \times 10^4$ |
| 2,3-hexanedione | 55±8 | 6±3 | $9 \times 10^3 \pm 6 \times 10^3$ |
| 3,4-hexanedione | 22±1 | 0.7±0.2 | $3 \times 10^4 \pm 1 \times 10^4$ |

946

FIGURE LEGENDS**Figure 1. Production of diols by purified Bdh1p.**

Gas chromatograms obtained after extraction with chloroform of the products obtained from a reaction mixture containing 50 mM diketone, 1 mM NADH, 100 mM sodium formate and 4 U of FDH in 33 mM sodium phosphate, pH 7, after the addition of 200 U of pure Bdh1p, together with 1-hexanol as an internal standard. Total reaction time was 20 h.

Figure 2. Stereoisomer purity of the (2R,3R)-2,3-butanediol obtained with pure Bdh1p and diacetyl.

Panels A and B correspond to the enlarged region of 2,3-butanediols stereoisomers, before (panel A) and after (panel B) adding external (2S,3S)-2,3-butanediol and (*meso*)-2,3-butanediol. (A) Enlarged chromatogram corresponding to a reaction with diacetyl, Bdh1p and an NADH-regenerating system where the concentration of the reaction product (2R,3R)-2,3-butanediol was 13.7 mM.

(B) Enlarged chromatogram containing the reaction mixture from panel A, to which (2S,3S)-2,3-butanediol and (*meso*)-2,3-butanediol were added as controls. The final concentration of (2R,3R)-2,3-butanediol was 12.9 mM, while the concentrations of (2S,3S)-2,3-butanediol and (*meso*)-2,3-butanediol were 0.1 mM each.

Figure 3. Production of diols by yeast extracts overexpressing Bdh1p and Fdh1p.

971 Gas chromatograms obtained after extraction with chloroform of the products
972 obtained from a reaction mixture containing 50 mM diketone, 1 mM NADH, 100
973 mM sodium formate in 33 mM sodium phosphate, pH 7, 20 h after the addition
974 of an extract of yeast strain DP-BDH1 containing 3 U of Fdh and 150 U of Bdh,
975 together with 1-hexanol as an internal standard.

976

977 **Figure 4. Production of diols by permeabilized yeast cells overexpressing**
978 **Bdh1p and Fdh1p.**

979 Gas chromatograms obtained after extraction with chloroform of the products
980 obtained from a reaction mixture containing 2% galactose, 50 mM diketone, 5
981 mM NAD⁺, 100 mM sodium formate in 33 mM sodium phosphate, pH 7, after
982 the addition of permeabilized DP-BDH1 yeast cells, together with 1-hexanol as
983 an internal standard. Total reaction time was one hour for diacetyl and 2,3-
984 pentanedione and 20 h for 2,3-hexanedione and 3,4-hexanedione.

985

986 **Figure 5. Determination of the molecular properties of the fusion protein**
987 **Fdh1p-Bdh1p(His)₆.**

988 (A) Western blot analysis of yeast protein extracts developed with an anti-His
989 antibody, Lane 1, Mr standards; lane 2, 15 µg from strain WV36-405Δ*ara1* Δ
990 *bdh1* [pYES2-*FDH1-BDH1*-6His] extract; lane 3, 17 µg from strain WV36-
991 405Δ*ara1BDH1* [pYES2-*BDH1*-6His] extract; lane 4, 21 µg from strain WV36-
992 405Δ*ara1* Δ*bdh1* [pYES2-*BDH1*-6His] extract. (B), IEF gel (pH 3 to 9) stained
993 with 100 mM (2R, 3R)-2,3-butanediol. *Lane 1*, pl standards; *lane 2*, 30 mU of
994 butanediol dehydrogenase activity, from strain WV36-405 Δ*ara1* Δ*bdh1*
995 [pYES2-*FDH1-BDH1*-6His] extract; *lane 3*, 7 mU of Bdh from a yeast strain

996 WV36-405 $\Delta ara1 BDH1$ [pYES2-*BDH1*-6His] extract; *lane 4*, 6 mU of Bdh from
997 strain WV36-405 $\Delta ara1 \Delta bdh1$ [pYES2-*BDH1*-6His] extract. (C), size exclusion
998 chromatography of purified Bdh1p(His)₆ and fusion protein Fdh1p-Bdh1p(His)₆
999 on a SuperDex 200 prep grade column. *a*, β -amylase (200 kDa); *b*, yeast
1000 alcohol dehydrogenase (150 kDa); *c*, bovine serum albumin (66 kDa); *d*,
1001 carbonic anhydrase (29 kDa); *e*, cytochrome *c* (12.4 kDa).

1002

1003 **Figure 6. Stereoisomeric composition of the products obtained from**
1004 **reaction mixtures containing vicinal diketones and purified fusion protein**
1005 **Fdh1p-Bdh1p(His)₆.**

1006 Stereoisomer composition of the products obtained with a reaction mixture
1007 containing purified fusion protein Fdh1p-Bdh1p(His)₆, 37 U of Bdh activity and
1008 0.3 U of Fdh activity, 5 mM NAD⁺, 100 mM sodium formate, in 33 mM sodium
1009 phosphate buffer at pH 7, together with 50 mM of the compounds diacetyl (A),
1010 2,3-pentanedione (B), 2,3-hexanedione (C) and 3,4-hexanedione (D). Total
1011 reaction time was 20 h.

1012

1013 **Figure 7. Stereoisomeric composition of the products from the**
1014 **reduction/oxidation of 2,3-pentanedione/(2*R*,3*R*)-2,3-pentanediol with**
1015 **Bdh1p.**

1016 (A) Stereoisomer composition of the products obtained in a reaction mixture
1017 containing Bdh1p, 50 mM 2,3-pentanedione, 1 mM NADH and an NADH-
1018 regenerating system (100 mM sodium formate and 1 U of formate
1019 dehydrogenase) in 33 mM sodium phosphate, pH 7, after the addition of 40 U of
1020 pure Bdh1p, together with 1-hexanol as an internal standard, with a total

1021 reaction time of 1 h. The inset shows the relative abundances of the ions with
1022 an m/z of 59 (in black) and of 45 (in red), for the peak eluting at 6.1 min from the
1023 chiral column.

1024 (B) Stereoisomer composition of the products obtained in a reaction mixture
1025 containing Bdh1p, 50 mM 2,3-pentanedione, the same components of reaction
1026 solution as A, after the addition of 40 U of pure Bdh1p, together with 1-hexanol
1027 as an internal standard, with a total reaction time of 20 h.

1028 (C) Stereoisomer composition of the products obtained in a reaction mixture
1029 containing Bdh1p, (2*R*,3*R*)-2,3-pentanediol, 5 mM NAD⁺ and a NAD⁺-
1030 regenerating system (50 mM α -ketoglutarate, 100 mM ammonium chloride and
1031 6 U of glutamate dehydrogenase) in 33 mM sodium phosphate, pH 7, after the
1032 addition of 6 U of Bdh1p, together with 1-hexanol as an internal standard, with a
1033 total reaction time of 20 h. The inset shows the relative abundances of the ions
1034 with an m/z of 59 (in black) and of 45 (in red), for the peak eluting at 7.1 min
1035 from the chiral column. The differences in retention times were due to running
1036 some samples in different days.

1037

1038 **Figure 8. Stereoisomeric composition of the products from the**
1039 **reduction/oxidation of 2,3-hexanedione/(2*R*,3*R*)-2,3-hexanediol with**
1040 **Bdh1p.**

1041 (A) Stereoisomer composition of the products obtained in a reaction mixture
1042 containing Bdh1p, 2,3-hexanedione 50 mM, 1 mM NADH and an NADH-
1043 regenerating system (100 mM sodium formate and 3 U of formate
1044 dehydrogenase) in 33 mM sodium phosphate, pH 7, after the addition of 40 U of

1045 Bdh1p, together with 1-hexanol as an internal standard, with a total reaction
1046 time of 20 h.

1047 (B) Stereoisomer composition of the products obtained in a reaction mixture
1048 containing Bdh1p and (2*R*,3*R*)-2,3-hexanediol, 5 mM NAD⁺ and a NAD⁺-
1049 regenerating system (50 mM α -ketoglutarate, 100 mM ammonium chloride and
1050 6 U of glutamate dehydrogenase) in 33 mM sodium phosphate, pH 7, after the
1051 addition of 6 U of Bdh1p with 1-hexanol as an internal standard, with a total
1052 reaction time of 20 h.

1053 (C) Relative abundances of the ions with an *m/z* of 55 (in black) and of 45 (in
1054 red), for the peak eluting at 11 min from the chiral column (panel A).

1055 (D) Relative abundances of the ions with an *m/z* of 45 (in red) and of 55 (in
1056 black), for the peak eluting at 10.7 min from the chiral column (panel B).

1057

1058 **Figure 9. Reaction schemes for the oxidation and reduction of (2*R*,3*R*)-2,3-**
1059 **pentanediol and 2,3-pentanedione by Bdh1p.**

1060 The scheme accounts for the relative abundances of (*R*)-3-hydroxy-2-
1061 pentanone and (*R*)-2-hydroxy-3-pentanone in the reduction of 2,3-pentanedione
1062 and in the oxidation of (2*R*,3*R*)-2,3-pentanediol with the C-3 position being the
1063 most reactive center in both directions. The discontinuous lines correspond to
1064 putative pathways, that although not observed, it would be difficult to rule out
1065 completely.

1066

1067 **Figure 10. Separation of the four hydroxy-pentanones and four hydroxy-**
1068 **hexanones obtained upon racemization and isomerization of the products**

1069 **obtained from the oxidations of (2R,3R)-2,3-pentanediol and (2R,3R)-2,3-**
1070 **hexanediol by Bdh1p.**

1071 (A) 40 mM 2,3-pentanedione and 40 mM 2,3-hexanedione were separately
1072 reduced by 6 U of Bdh1p in the presence of 1 mM NADH, 100 mM sodium
1073 formate and 4 U of formate dehydrogenase from *Candida boidinii*, in 33 mM
1074 sodium phosphate buffer pH 7. After 20 h of reaction, the products were
1075 recovered by filtration on an Amicon column and the filtrates (containing the
1076 (2R,3R)-2,3-diols) were subjected to an overnight oxidation by 6 U of Bdh1p in
1077 the presence of 5 mM NAD⁺, 50 mM α -ketoglutarate, 100 mM ammonium
1078 chloride and 6 U of glutamate dehydrogenase. The final reaction products were
1079 made 1N in NaOH to induce racemization and isomerization and were loaded
1080 (after chloroform extraction) on a β -cyclodextrin gas chromatography column
1081 coupled to a mass spectrophotometer.

1082 (B) Relative abundances of the ions with an m/z of 59 (in black) and of 45 (in
1083 red), for the peaks eluting at 5.67, 5.72, 6.16 and 6.27 min from the chiral
1084 column, labeled 1, 2, 3 and 4.

1085 (C) Relative abundances of the ions with an m/z of 55 (in blue) and of 45 (in
1086 red), for the peaks eluting at 9.03, 9.21, 9.59 and 10.31 min from the chiral
1087 column, labeled 5, 6, 7 and 8.

1088

Figure 1

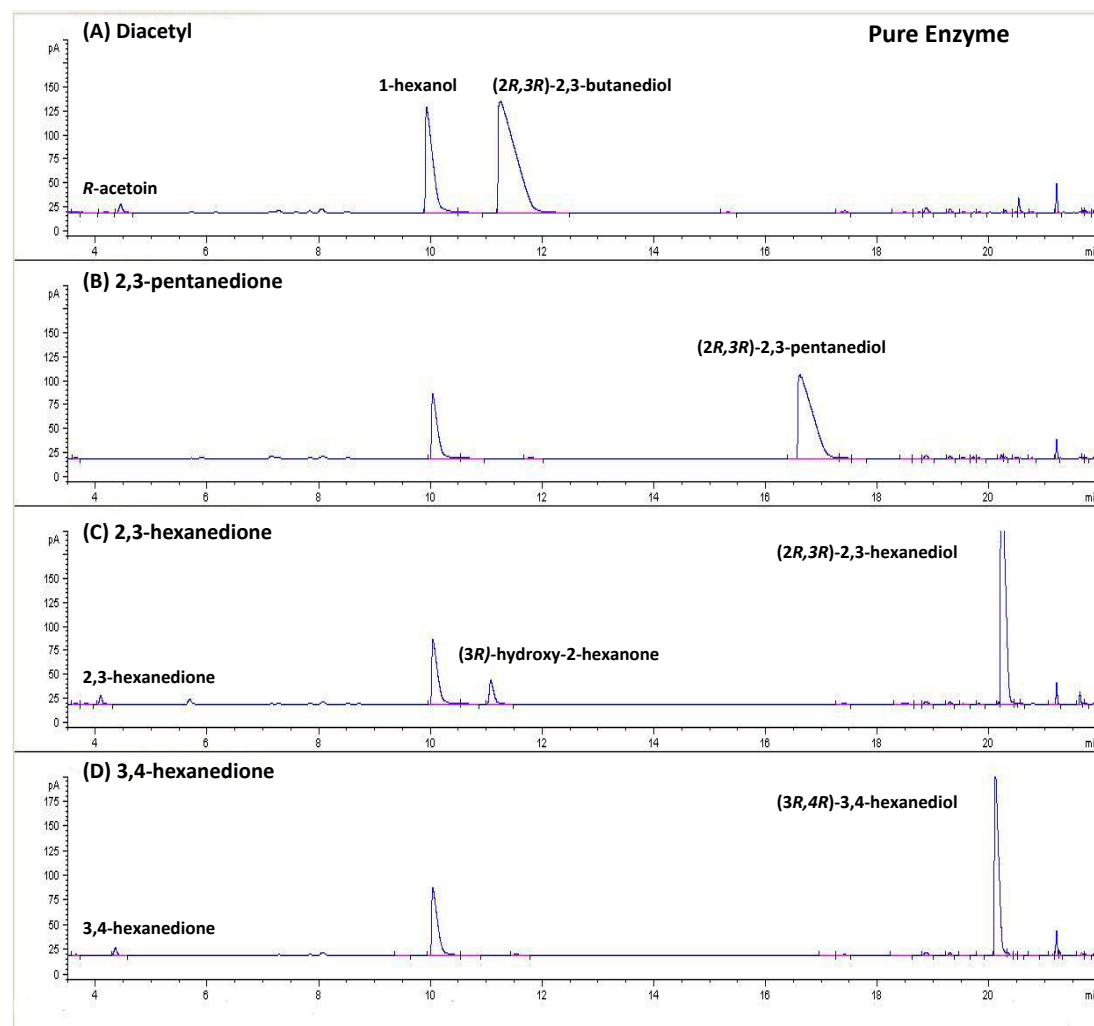
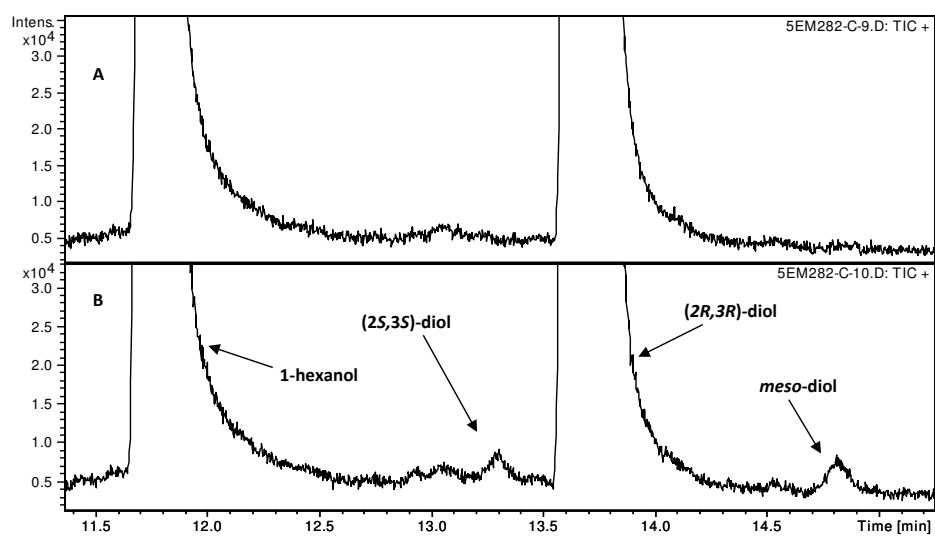


Figure 2



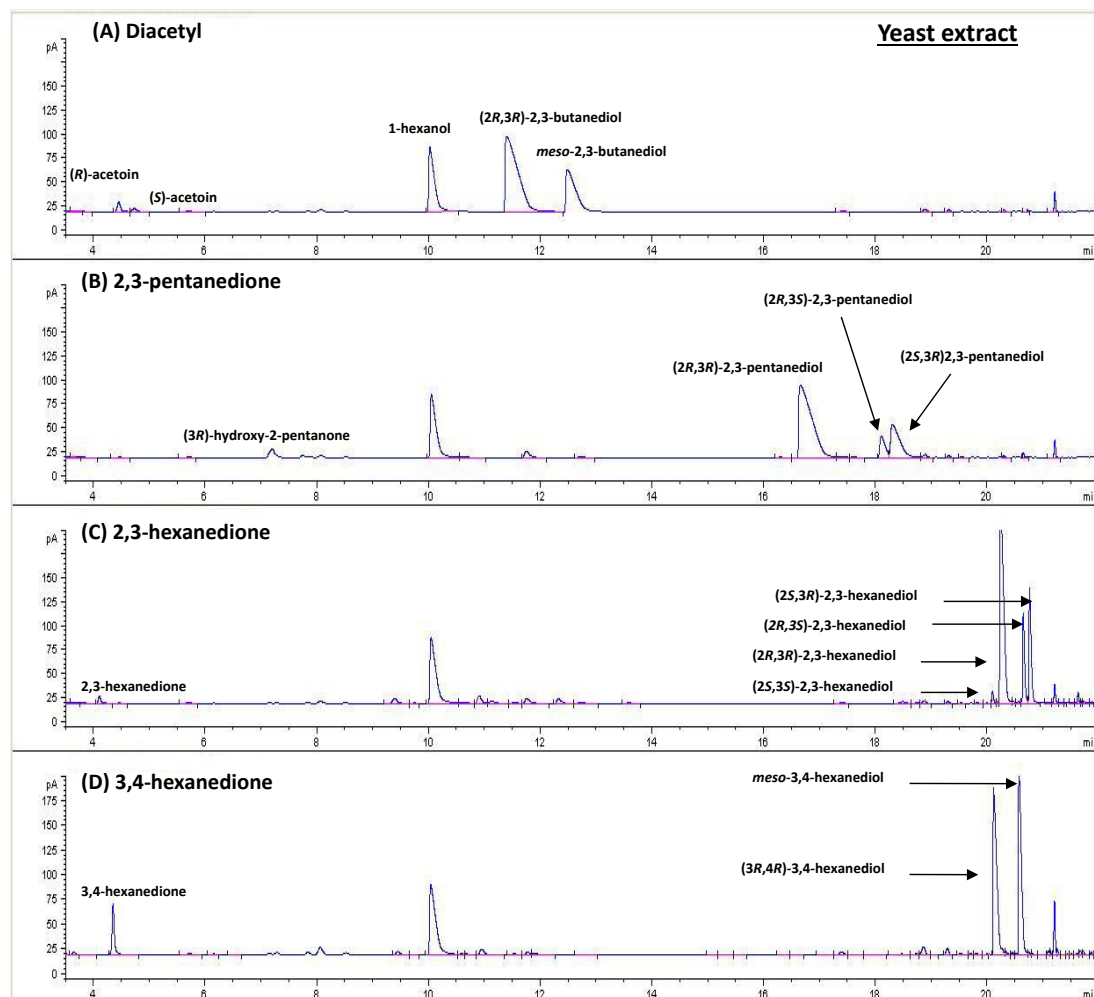


Figure 3

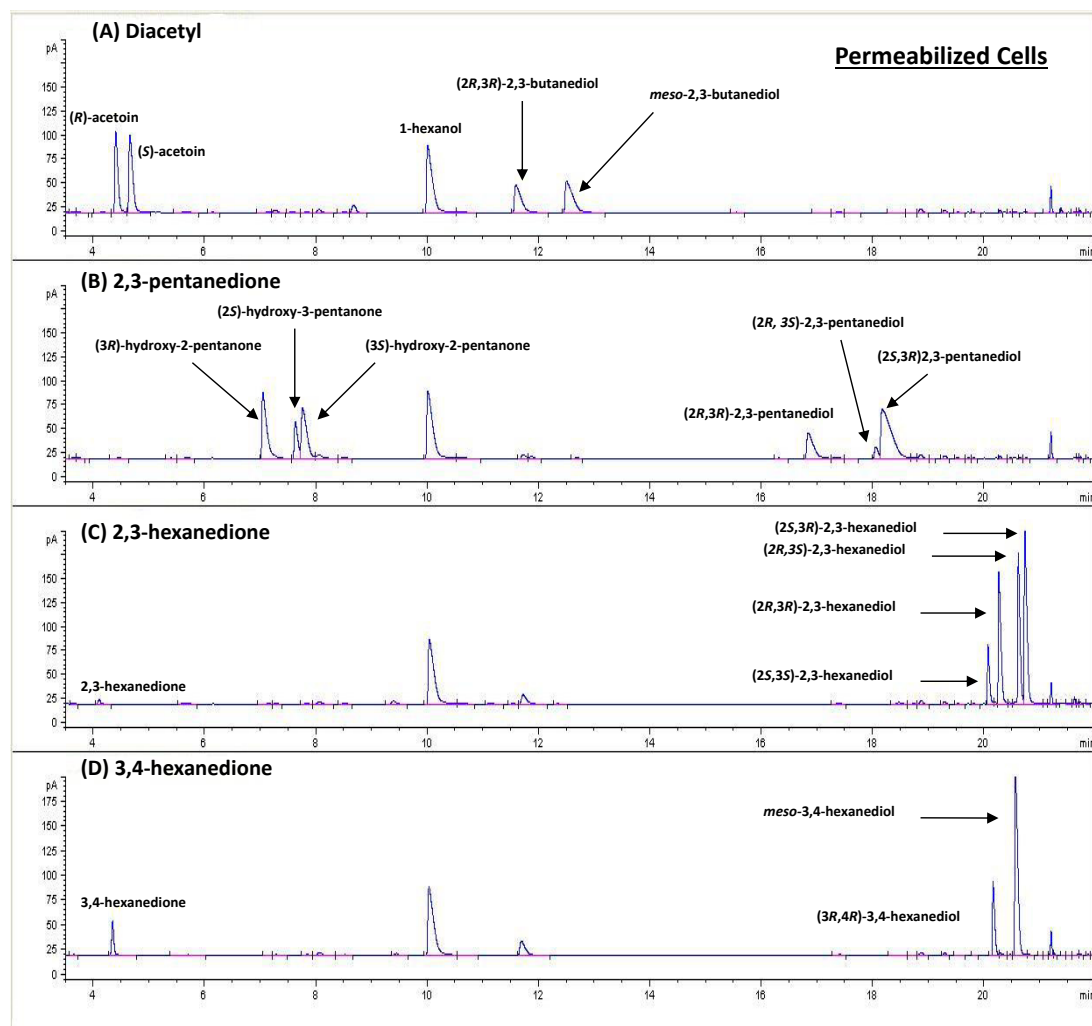


Figure 4

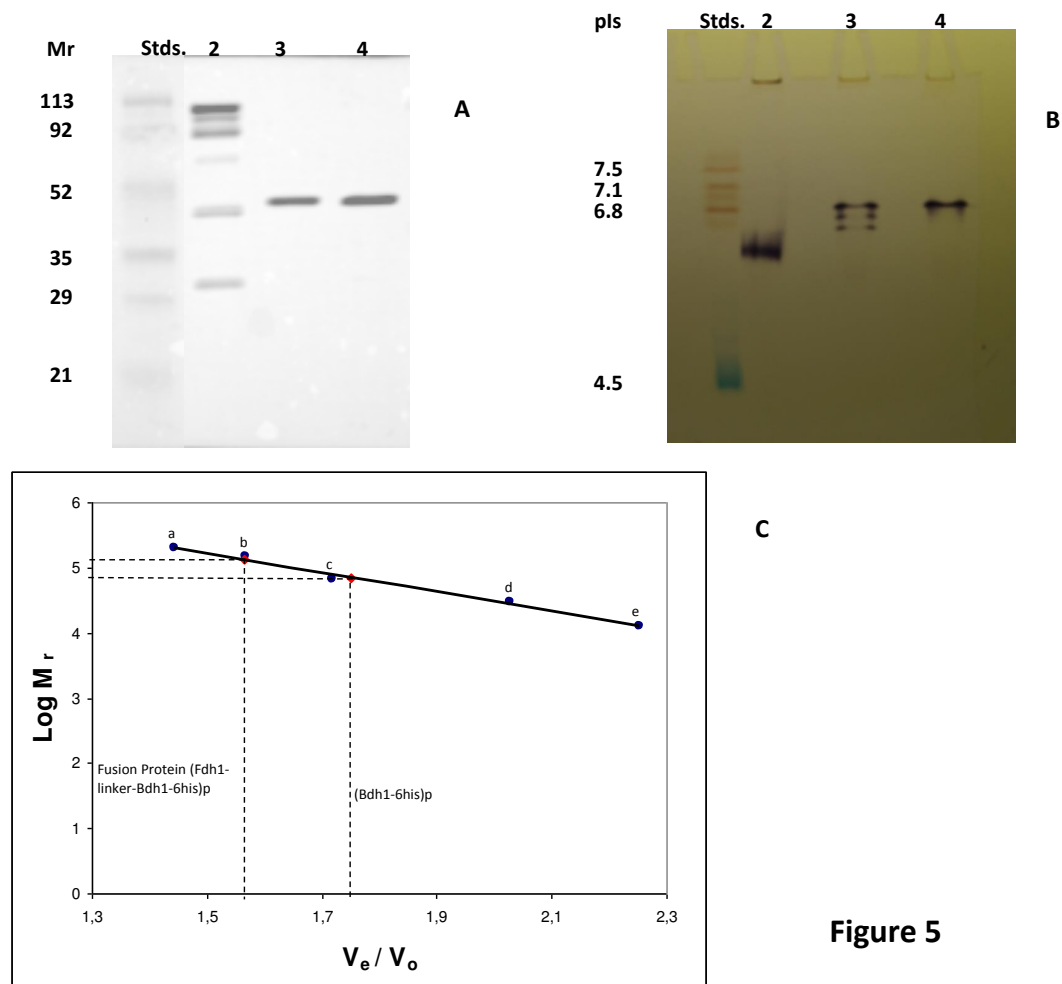


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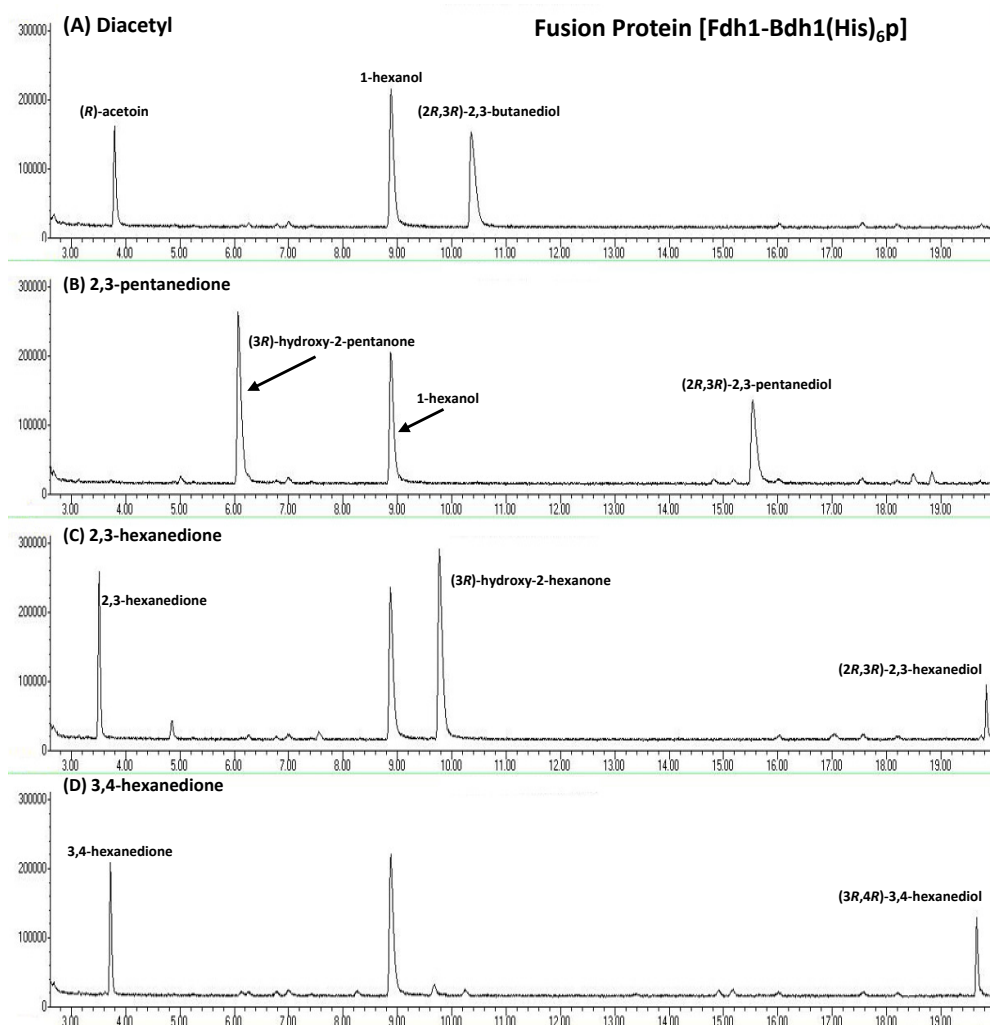
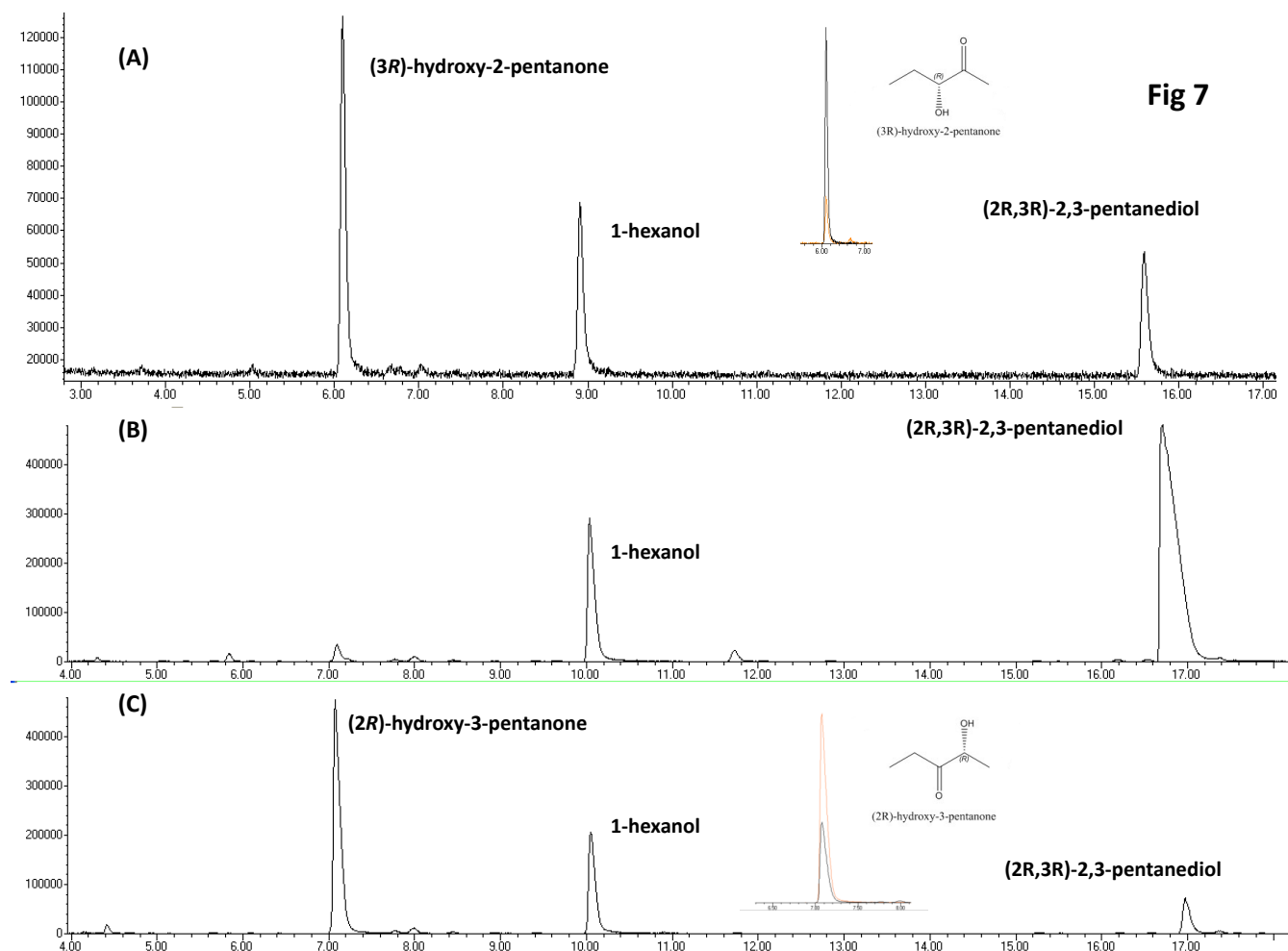


Figure 6

Fig 7



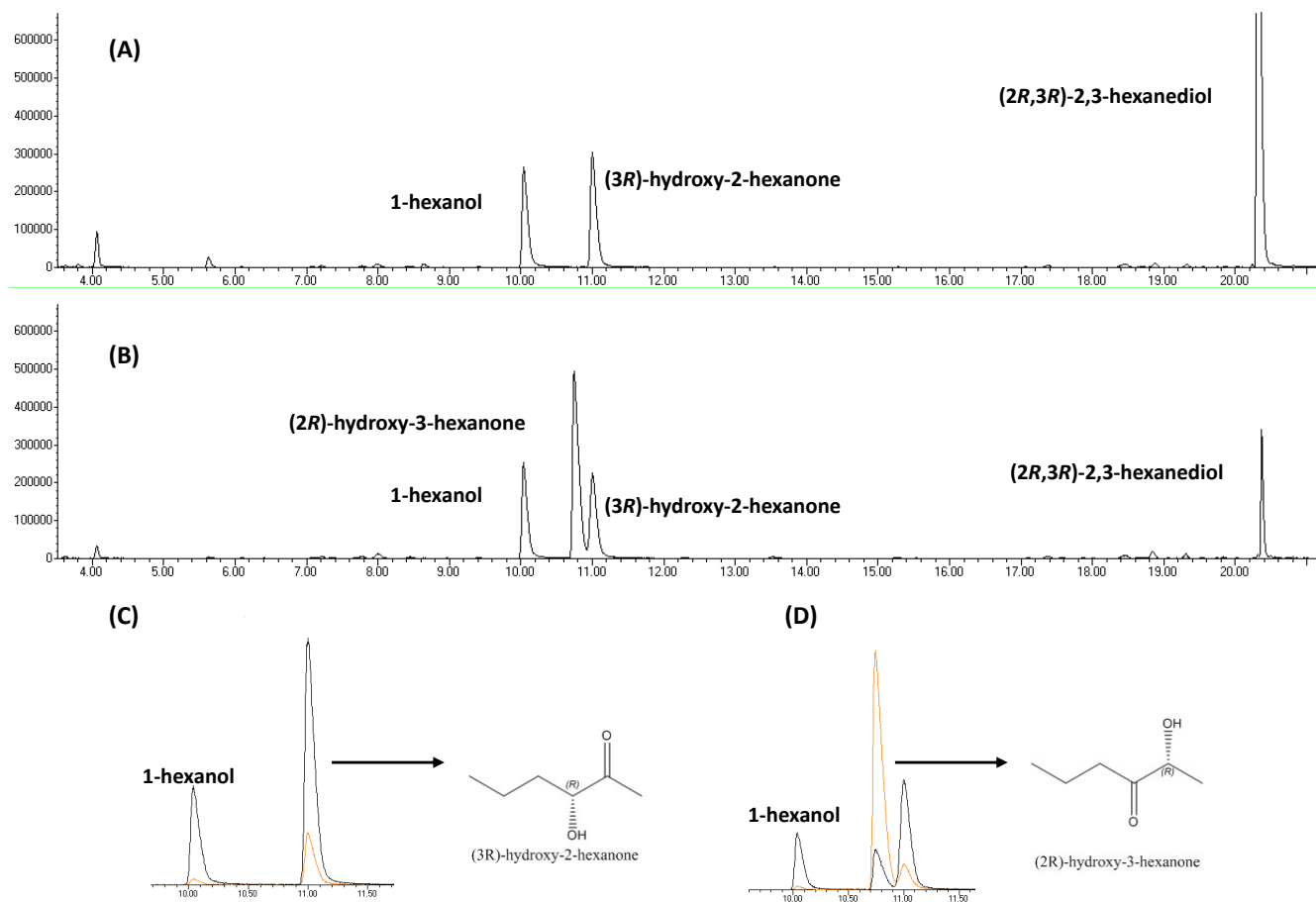


Figure 8

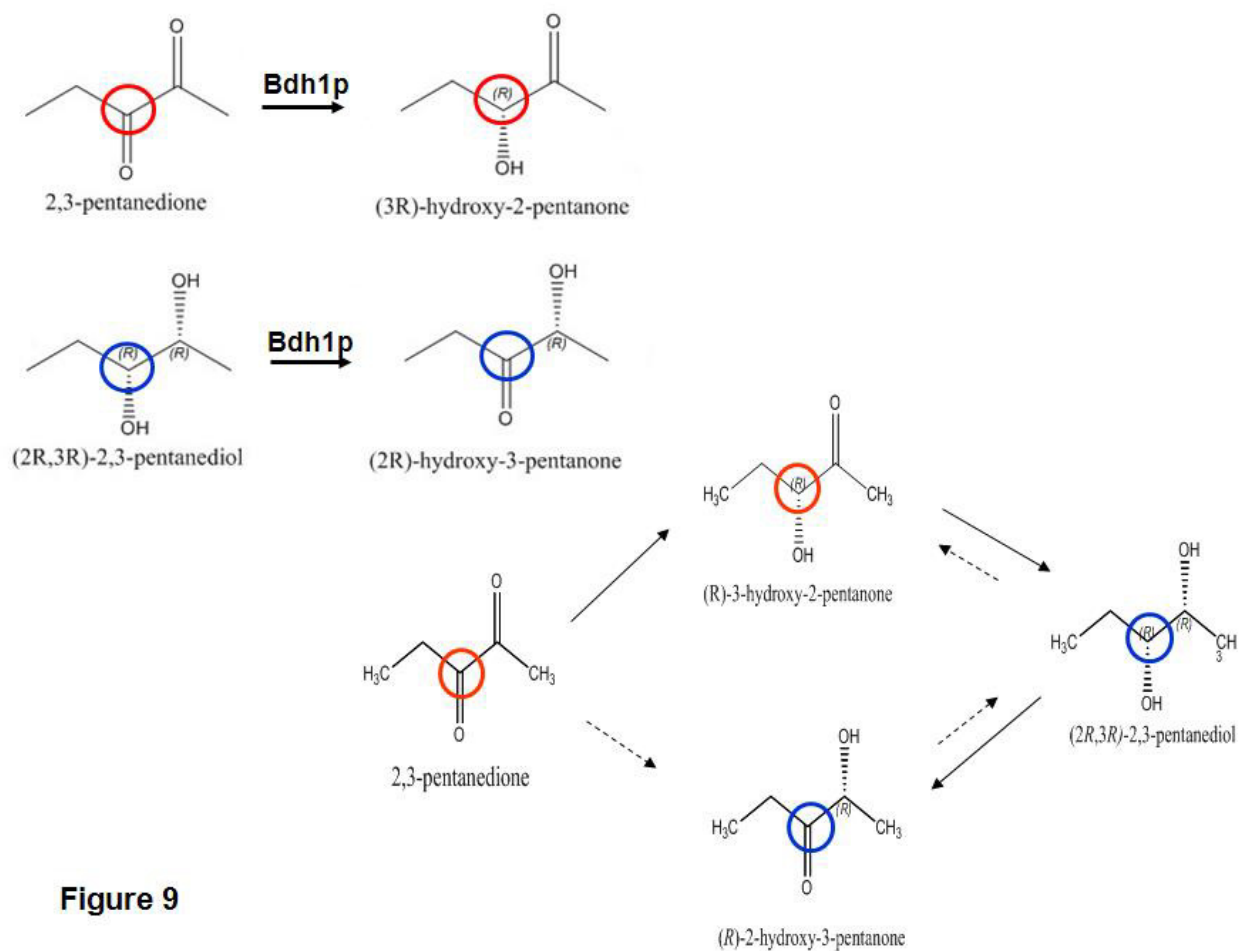


Figure 9

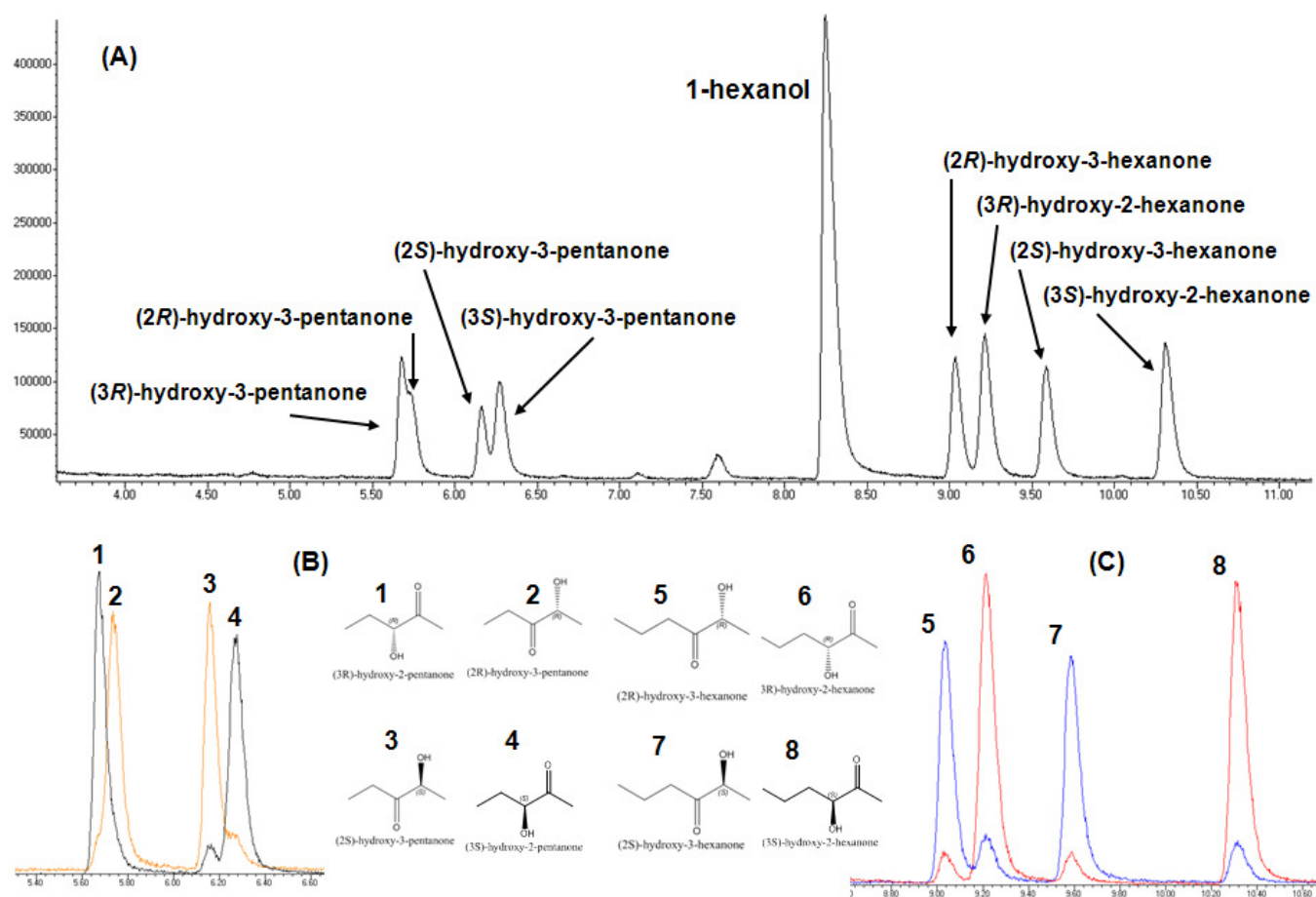


Figure 10