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New strategies improve the efficiency of the *baker's yeast* reduction of ketoesters: near UV irradiation and a two-substrate application

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Abstract

The stereodifferentiation of the *baker's yeast* reduction of β-ketoesters is improved by exposing this reaction to 300–400 nm UV light and via a new method entitled 'two-substrate application'. Both routes are accomplished by selectively interfering with those oxidoreductases that compete for the same substrate. While the irradiation enhances the enantioselectivity of the *baker's yeast* upon reducing the substrate 3-oxo-*n*-valeric ethylester inhibits it the conversion of ethyl acetoacetate, whereas other homologues are not influenced. Corresponding in vitro reductions with the *baker's yeast* enzymes *alcohol dehydrogenase I* (ADH I), L-*lactate dehydrogenase* (L-LDH), and *β-ketoacylreductase* are light-resistant, whereby the L-LDH reacts in the presence of *β*-nicotinamide adenine dinucleotide (NADH). The catalytic activity of pre-irradiated ADH I is reduced because the light changes the conformation of the isolated enzyme according to CD spectroscopy. The natural enzyme cofactor NADH protects the ADH I in the in vitro reduction experiment from the near UV light by absorbing it for conducting its own photochemistry. In the *baker's yeast* reduction of ethyl acetoacetate, apparently four oxidoreductases compete for this substrate.

Keywords: Photochemistry; Biotransformation; *Baker's yeast*; Reduction of ketoesters; *Baker's yeast* oxidoreductases: alcohol dehydrogenase I (ADH I); β-Ketoacylreductase; L-Lactate dehydrogenase (L-LDH); β-NADH; Two-substrate application; Circular dichroism (CD); Light enhancement; UV light protection

1. Introduction

1.1. Background

Biotransformation constitutes a valuable method in asymmetric organic synthesis [1] and especially *baker's yeast* is a frequently applied catalyst [2–4]. The reduction reactions performed therewith are very efficient, however if the different oxidoreductases of the yeast compete for the same substrate the stereodifferentiation becomes moderate and difficult to predict [3–6]. Although some of these oxidoreductases were characterized, their exact number is not yet known [1,7]. In view of the practical relevance of this reaction, efforts were undertaken to enhance the functionality and applicability of the *baker's yeast* reduction through variation of the conditions [1–4,6,7].

1.2. Objectives

Target is the development of new strategies with broad validity for improving the effectiveness of the reduction of ketoesters with *baker's yeast* [8a]. This is based on the concept that the activity of a certain enzyme may either directly be enhanced [9] or that the equilibrium of those competing oxidoreductases is influenced. Besides with other methods, this goal is here supposed to be achieved by irradiation at 300–400 nm. In this wavelength range the destructive effects occurring on proteins in the far UV region [10] as well as undesirable blue light effects on the yeast *Saccharomyces cerevisiae* [11–14] should be excluded. The chromophores will either be the non-peptide receptors of the yeast [15,16] or those amino acids with a UV-tailing absorption above 300 nm [17,18]. Measurable characteristics as the reaction rate and

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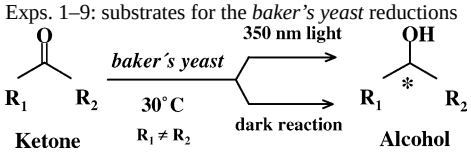
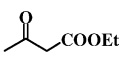
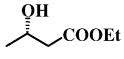
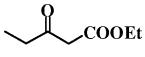
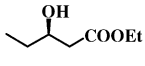
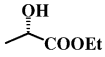
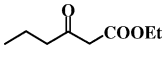
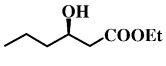
Table 1
Exps. 1–9: substrates for the *baker's yeast* reductions



Substrate	Product	Abs. config.	Ref.
 ethyl acetoacetate (1)	 3-hydroxybutyric ethylester (5)	*S*	[3]
 3-oxo-n-valeric ethylester (2)	 3-hydroxy-n-valeric ethylester (6)	*R*	[3]
 ethylpyruvate (3)	 ethyl lactate (7)	*S*	[3]
 3-oxo-n-caproic ethylester (4)	 3-hydroxy-n-caproic ethylester (8)	*R*	[5]

the enantioselectivity are the criteria for assessing the success when exposing this reaction to the light. Pursuing both values by gas chromatography (GC) should allow the semi-quantitative detection of a light-effect. Substrates are the homologous α- and β-ketoesters **1**–**4**, listed in Table 1 [3–5], because they do either not absorb in the wavelength range of the light source (substrates **1**, **2** and **4**) or there was no competitive photoreaction found (**3**) [8c].

2. Experimental

2.1. Abbreviations and definitions

ADH I: *baker's yeast* alcohol dehydrogenase I (EC 1.1.1.1); BSTFA: *N*,*O*-bis-(trimethylsilyl)-trifluoracetamide; *b.y.*: *baker's yeast*; CR: continued reaction, named, e.g. 'CR light + dark', mentioning the illumination condition of the main reaction at first, followed by the condition of its continued portion (see Section 2.3.1); def.: definition, ee: enantiomeric excess; ether: diethyl ether; Exp.: experiment; GC$^{1-3}$: GC method employed to determine the ee (see Section 2.3.1); GRP: general reaction procedure; L-LDH: L-lactate dehydrogenase from *b.y.* (cytochrome b_2) (EC 1.1.2.3); m.r.: main reaction; β-NAD(P)H: reduced form of β-nicotinamide adenine dinucleotide (phosphate); prod.: product; prp.: preparative; resp.: respectively; r.t.: reaction time; TFAA: trifluoracetic anhydride; t.o. = product/(starting material + product) × 100, defined as reaction turnover in % (see Section 2.3.1).

2.2. Instrumentation and measurement

2.2.1. Materials

Merck: ADH I from *b.y.* (180 U/mg); (*S*)-(−)-ethyl lactate. Sigma: ADH I from *b.y.* (200 U/mg); L-LDH from *b.y.* (0.1–0.6 units/mg), suspension in 3.2 M NH_4SO_4, pH 6; β-NADH, disodium salt (98%); β-NADPH, tetrasodium salt (90%). Hefe DHW Vital Gold, Deutsche Hefe Werk Hamburg, Nürnberg: *baker's yeast*. Professor E. Schweizer [19]: β-ketoacylreductase of the *baker's yeast's* fatty acid synthase complex. Millipore: Millidisk filter SLSR 025NS, pore size 0.5 μm; water: deionized by a Milli-Q-System. Argon: purity 4.8. Ether: purified on Alox; all substrates were distilled to a ≥98.6% purity (GC).

2.2.2. Equipment

Irradiations: Preparative photochemical Rayonet reactor type RS, RPR-208 with eight lamps, each 24 W, RUL 350 nm, λ_{max} = 350 nm, emission range 300–400 nm; the irradiation vessels were equipped with a cooling finger and made from quartz, unless stated otherwise. *Dark reactions:* The irradiation vessel was coated with aluminum foil; a dark-control was conducted to each irradiation. *Temperature:* 30 ± 0.2 °C were maintained by a cryomate, a thermocouple element (NiCrNi, 0.5 × 250 mm), and a plotter. *Analytics:* ^{1}H NMR: 400 MHz Bruker AM-400, in $CDCl_3$, shift reagent was Eu(tfc)$_3$, unless stated otherwise. D NMR: 60 MHz Bruker, in $CHCl_3$. IR: Perkin-Elmer 580, film on KBr. $[\alpha]_D^{23}$: Zeiss Old 5-polarimeter; 1 dm cell, c in g/100 ml, in $CHCl_3$ unless stated otherwise. UV: Omega 10, Bruins Instruments or Cary 17, in CH_3CN unless stated otherwise, λ_{max} in nm, ε in l mol^{-1} cm^{-1}; Exps. 14 and 15 only: Cary 2300, 1 mm round cell. CD: JASCO J-20, 1 mm round cell, path-length applied in dm, sensitivity 0.001°. GC/MS: MD 5970 Hewlett-Packard with an I.E. of 70 eV, GC: 30 m Carbowax 20M column. Analytical GC: hydrogen (0.6 atm) as carrier gas. Preparative GC: 8 m Volaspher A4 column, 60–80 mesh, coated with 20% Carbowax 20M, nitrogen as carrier gas.

2.2.3. Methods

The enantiomeric excess was determined through different techniques: (a) ^{1}H NMR with the shift reagents Eu(tfc)$_3$ or Eu(hfc)$_3$, (b) optical rotation [α], (c) HPLC: 3-hydroxybutyric ethylester (**5**) was derivatized by 3,5-dinitrophenylisocyanate [20], and (d) GC, whereby different methods (GC$^{1-3}$) were employed for sample preparation.

GC^1: 3-hydroxybutyric ethylester (**5**) was derivatized by (*S*)-(−)-1-phenyl-ethylisocyanate [2,21]; analysis on a 25 m SE-54 column at 60→260 °C, 6 °C/min. *GC^2:* 3-hydroxybutyric ethylester (**5**) and ethyl lactate (**7**) were derivatized by TFAA in *n*-hexane or cyclohexane within 1 h at room temperature; analysis on a 50 m fused silica-capillary column (Ø = 0.25 mm) coated with 90% OV 1701 + 10% permethylated-β-C′-Dextrin [22] at 70 or 80 °C isotherm for **5** and 75 °C isotherm for **7**. *GC^3:* ethyl lactate (**7**) was derivatized by BSTFA in *n*-hexane within 1 h at room temperature;

analysis at 75 °C isotherm on that column described under method GC[2] [22].

2.3. General reaction procedures (GRP)

2.3.1. GRP 1: general procedure for the baker's yeast reduction of ketoesters

The reactions were conducted in a 500 ml irradiation vessel with outlet and at 30 °C. For comparing related experiments, the *baker's yeast* was taken from the same package(s). 32.5 g D-(+)-saccharose dissolved in 122 ml H_2O and 13 g *baker's yeast* were generally employed. For pre-incubation, this mixture was stirred for 25 min under the subsequent reaction conditions. The substrate added thereafter was allowed to react for 9–12 h. Mostly before terminating a main reaction (m.r.), approximately 5 ml of it were transferred into a small quartz irradiation vessel with outlet for continuing a portion of this reaction (CR) (see Section 2.1) for another 10–14 h under individual conditions. The turnover in % (t.o.) (see Section 2.1) was determined based on GC measurements. The therefore regularly withdrawn 2 ml GC samples were quenched with acetone, then filtered (Millidisk) and under addition of NaCl with ether extracted. *Work-up:* Quenching with acetone was followed by filtration over celite and concentration. The aqueous layer was with ether extracted under addition of NaCl. After drying (Na_2SO_4), the residue was first purified by Kugelrohr distillation (110–140 °C, 12–25 mm) to the crude product and then by preparative GC to the colorless and liquid product alcohol whose purity was determined by GC. The products were identified by IR and ^{1}H NMR spectroscopy and the UV spectra once recorded for compounds **1**–**8** (Exps. 1, 5, 8 and 9). The enantiomeric excess could be determined for most of the products (Section 2.2.3).

2.3.2. GRP 2: reductions with baker's yeast under argon

The procedure follows in principal GRP 1. Only, through a gas inlet tube the equipment, the pre-incubation and the subsequent reaction became intensely flushed with argon (∼50 atm inertgas within 24 h). The main reactions under argon were labeled, e.g. as dark, Ar and their extensions, e.g. as CR light + dark, Ar (see Section 2.1).

2.3.3. GRP 3: reductions with oxidoreductases isolated from baker's yeast

The employed ADH I was from Merck, and for comparing related experiments were the enzymes and NAD(P)H taken from the same charge or starting solution. The reaction mixtures were stirred in a 2 ml irradiation vessel at 30 °C. The reaction progress was pursued by periodically withdrawn GC samples (50 μl) that were quenched with acetone, filtered (Millidisk), and then extracted with ether under addition of NaCl. The conversion was analyzed by GC and the ee values of the product alcohols determined based on method GC[2] (Section 2.2.3), unless stated otherwise.

2.3.4. GRP 4: investigation of irradiated ADH I by means of CD and UV spectroscopy

The ADH I was obtained from Merck, unless stated otherwise. To be able to compare related experiments, they were fed of the same starting solution. The samples were stirred in a 20 ml irradiation vessel at 30 °C and filtered (Millidisk) prior to a measurement.

2.4. Experiments

2.4.1. Experiment 1: baker's yeast reduction of ethyl acetoacetate (**1**) to (S)-(+)-3-hydroxybutyric ethylester (**5**)

Procedure and analytics according to GRP 1; product identification by IR [23,24] and ^{1}H NMR [23–25], UV absorptions (in *n*-hexane) of **1**: ε (240) = 3890[1] with a shoulder at 295 nm and **5**: no absorption above 250 nm; GC: 32 m Carbowax 20M column at 70→240 °C, 4 °C/min; prp. GC at 120 °C.

2.4.1.1. Exp. 1A: irradiation. 1.862 g (14.3 mmol) **1** were irradiated for 10.5 h, t.o.: 38.8%; 1.06 g crude, 113 mg of 97% pure **5**; ee (m.r.): 33.4% (^{1}H NMR), 34%[2] ([α] = +14.8° (*c* = 1.00)), 32.3% (GC[**1**]) and 33.5% (GC[2]). Two continued reactions (CR) (def. in Section 2.3.1) were conducted: CR under irradiation for altogether 23.17 h, labeled as 'CR light + light', t.o.: 44.8%; ee (CR): 35.7% (GC[**1**]) and 44% (HPLC) [20]; CR in the dark for altogether 23 h, labeled as 'CR light + dark', t.o.: 75.2%; ee (CR): 60.7% (GC[**1**]) and 64% (HPLC) [20].

2.4.1.2. Exp. 1B: dark-control. 1.866 g (14.3 mmol) **1** reacted in 11.17 h, t.o.: 84.7%; 1.23 g crude, 424 mg of 99.3% pure **5**; ee (m.r.): 71.2% (^{1}H NMR), 76.6% (see footnote 2) ([α] = + 33.3° (*c* = 0.99)), 67.8% (GC[**1**]), 70% (GC[2]) and 70% (HPLC) [20]; CR in the dark for 23 h, labeled as 'CR dark + dark', t.o.: 99%, 75.8% ee (GC[2]).

2.4.2. Experiment 2: baker's yeast reduction of ethyl acetoacetate (**1**) to (S)-(+)-**5** under argon

Procedure and analytics according to GRP 1 and 2 and Exp. 1.

2.4.2.1. Exp. 2A: irradiation under argon. Under argon, 1.863 g (14.3 mmol) **1** were irradiated for 9.42 h, t.o.: 98%; 0.29 g crude, 90 mg of 99.6% pure **5**; ee (m.r.): 46% (^{1}H NMR), 53.3% (see footnote 2) ([α] = + 23.2° (*c* = 0.70)) and 55.4% (GC[**1**]). CR light + light, Ar (def. in Section 2.3.2) for 25.33 h, t.o.: 100%, 55.6% ee (GC[**1**]); CR light + dark, Ar for 25.25 h, t.o.: 100%, 55.5% ee (GC[**1**]).

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[1] ε (2439 Å) = 8100 (in hexane), ε (2439 Å) = 5300 (in ether), ε (2457 Å) = 1900 (in EtOH) [26].

[2] The ee values determined for **5** were referred to (a) $[\alpha]_D^{25}$ = +43.5° (*c* = 1.00; $CHCl_3$), 100% ee [25]; (b) $[\alpha]_D^{20}$ = +41.3° (*c* = 1.00; $CHCl_3$), 97% ee [2].

2.4.2.2. Exp. 2B: irradiation with no argon. 1.862 g (14.3 mmol) **1** were irradiated for 9.72 h, t.o.: 30.2%; 0.89 g crude, 90 mg of 97.5% pure **5**; ee (m.r.): 35.2% (^{1}H NMR), 35.6% (see footnote 2) ([α] = + 15.5° (*c* = 1.01)) and 29.3% (GC**1**). CR light + light for 22.67 h, t.o.: 65.8%, 44.3% ee (GC**1**); CR light + dark for 23 h, t.o.: 96.2%, 61.3% ee (GC**1**).

2.4.2.3. Exp. 2C: dark-control. 1.863 g (14.3 mmol) **1** reacted in 10.58 h, t.o.: 75.8%; 1.24 g crude, 150 mg of 99.3% pure **5**; ee (m.r.): 68.3% (^{1}H NMR), 78.4% (see footnote 2) ([α] = + 34.1° (*c* = 1.00)) and 72.5% (GC**1**). CR dark + dark for 24.67 h, t.o.: 98%, 78.5% ee (GC**1**).

*2.4.3. Experiment 3: supplementary baker's yeast reductions of **1** to (S)-(+)-**5** under argon*

Procedure and analytics according to GRP 1 and 2 and Exp. 1.

2.4.3.1. Exp. 3A: irradiation under argon. Under argon, 1.862 g (14.3 mmol) **1** were irradiated for 8.92 h, t.o.: 84.7%; 0.37 g crude, 105 mg of 99% pure **5**; ee (m.r.): 52% (^{1}H NMR), 47.1% (see footnote 2) ([α] = + 20.5° (*c* = 0.92)) and 43.3% (GC**1**). CR light + light, Ar for 23.08 h, t.o.: 99%, 52.6% ee (GC**1**).

2.4.3.2. Exp. 3B: dark-control under argon. Under argon, 1.862 g (14.3 mmol) **1** reacted in 9.42 h, t.o.: 99%; 0.46 g crude, 180 mg of 99.6% pure **5**; ee (m.r.): 65.3% (^{1}H NMR), 72.6% (see footnote 2) ([α] = + 31.6° (*c* = 1.02)) and 66.6% (GC**1**). CR dark + dark, Ar for 22.63 h, t.o.: 100%, 66.5% ee (GC**1**).

2.4.3.3. Exp. 3C: dark-control with no argon. 1.862 g (14.3 mmol) **1** reacted in 9.87 h, t.o.: 63.3%; 1.19 g crude, 310 mg of 99.5% pure **5**; ee (m.r.): 57.2% (^{1}H NMR), 70.6% (see footnote 2) ([α] = +30.7° (*c* = 1.02)) and 64.8% (GC**1**). CR dark + dark for 23.08 h, t.o.: 99%, 69.1% ee (GC**1**).

*2.4.4. Experiment 4: baker's yeast reduces ethyl acetoacetate (**1**) to (S)-(+)-**5** in 53% D_2O*

Procedure and analytics according to GRP 1 and Exp. 1; Exp. 4 was conducted in a Pyrex irradiation vessel.

2.4.4.1. Exp. 4A: irradiation in D_2O/H_2O. 1.862 g (14.3 mmol) **1** were irradiated for 11.25 h while 53% of the solvent were D_2O, t.o.: 32.7%; 0.33 g crude, 36 mg of 94.5% pure **5**; ee (m.r.): 31.7% (^{1}H NMR), 37% (see footnote 2) ([α] = + 16.1° (*c* = 0.37)) and 29.7% (GC^2). CR light + light for 21.92 h, t.o.: 65.4%, 39.8% ee (GC^2); CR light + dark for 22 h, t.o.: 44.8%, 44.6% ee (GC^2). Deuterium content of **5** determined by GC–MS-coupling (Table 2) and by D NMR: D^1,D^2:D^3 = 10.4:1 (Fig. 1).

2.4.4.2. Exp. 4B: dark-control in D_2O/H_2O. 1.862 g (14.3 mmol) **1** reacted in 9.75 h while 53% of the solvent were D_2O, t.o.: 43.5%; 0.93 g crude, 165 mg of 97.5% pure **5**; ee (m.r.): 43.5% (^{1}H NMR), 49.9% (see footnote 2) ([α] = +21.7° (*c* = 0.91)) and 46.8% (GC^2). CR dark + dark for 23.92 h, t.o.: 73%, 66% ee (GC^2). Deuterium content of **5** determined by GC–MS-coupling (Table 2) and by D NMR: D^1,D^2:D^3 = 10.3:1 (Fig. 1).

Table 2
Deuteriation of (*S*)-(+)-**5** in % according to GC–MS-coupling

m^+	117	118	119	120
Number of included deuterium atoms	0	1	2	3
Exp. 4A	29	46	22	3
Exp. 4B	28	46	24	2–3

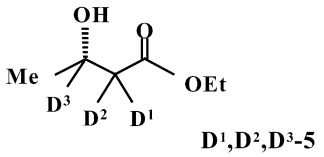


Fig. 1. Deuteriation of (*S*)-(+)-**5** via keto–enol tautomerism (D^1,D^2) and by the *yeast* reduction (D^3).

2.4.4.3. Exp. 4C: irradiation. 1.862 g (14.3 mmol) **1** were irradiated for 10.83 h, t.o.: 56.8%; 0.95 g crude, 215 mg of 99.3% pure **5**; ee (m.r.): 58% (^{1}H NMR), 57.9% (see footnote 2) ([α] = +25.2° (*c* = 1.03)) and 53.9% (GC^2). CR light + light for 22.67 h, t.o.: 64.1%, 58% ee (GC^2); CR light + dark for 22.75 h, t.o.: 91.7%, 73.2% ee (GC^2).

2.4.4.4. Exp. 4D: dark-control. 1.862 g (14.3 mmol) **1** reacted in 9 h, t.o.: 62.1%; 1.15 g crude, 382 mg of 99.5% pure **5**; ee (m.r.): 74.6% (^{1}H NMR), 71% (see footnote 2) ([α] = +30.9° (*c* = 1.01)) and 69.4% (GC^2). CR dark + dark for 22.92 h, t.o.: 87.7%, 73.7% ee (GC^2).

*2.4.5. Experiment 5: baker's yeast reduction of 3-oxo-n-valeric ethylester (**2**) to (R)-(−)-3-hydroxy-n-valeric ethylester (**6**)*

Procedure and analytics according to GRP 1; product identification by IR [27] and ^{1}H NMR [3,27,28], UV absorptions of **2**: ε (210) = 91.9 and **6**: ε (210) = 77.4; GC: 32 m Carbowax 20M column at 70→240 °C, 4 °C/min; prp. GC at 120 °C isotherm.

2.4.5.1. Exp. 5A: irradiation. 2.059 g (14.3 mmol) **2** were irradiated for 7.75 h, t.o.: 73.5%; 0.98 g crude, 438 mg of 100% pure **6**; ee (m.r.): 43% (^{1}H NMR, Eu(hfc)$_3$) [28], 45% (^{1}H NMR), 48.1%[3] ([α] = −16.6° (*c* = 1.00)) and 46% (see footnote 3) ([*a*] = −15.9° (*c* = 1.00)). CR light + light for 23.92 h, t.o.: 82%; CR light + dark for 23.75 h, t.o.: 91.7%.

2.4.5.2. Exp. 5B: dark-control. 2.060 g (14.3 mmol) **2** reacted in 7.75 h, t.o.: 80.6%; 1.44 g crude, 514 mg of 99.7%

[3] $[\alpha]_D$ = −34.6° (*c* = 5.0; $CHCl_3$), 100% ee [3,27,29].

pure **6**; ee (m.r.): 37% (^{1}H NMR, Eu(hfc)$_3$) [28] and 33.4% (^{1}H NMR), 42.5% (see footnote 3) ([α] = −14.7° (*c* = 1.00)) and 42.3% (see footnote 3) ([α] = −14.6° (*c* = 1.00)). CR dark + dark for 24.17 h, t.o.: 91.7%.

*2.4.6. Experiment 6: simultaneous baker's yeast reduction of ethyl acetoacetate (**1**) to (S)-(+)-**5** and 3-oxo-n-valeric ethylester (**2**) to (R)-(−)-**6***

Procedure and analytics according to GRP 1, Exps. 1 and 5.

*2.4.6.1. Exp. 6A: dark-control with ethyl acetoacetate (**1**).* 1.862 g (14.3 mmol) **1** reacted in 8.58 h, t.o.: 57.8%; turnover (t.o. in %, def. in Section 2.1) followed by GC:

Reaction time (h)	0.5	1	2	3.83	5.25	6.5	7.67	8.58	22.83
Turnover	3.8	7.1	14.5	26.7	35	45.5	59.5	57.8	75.8$

$CR dark + dark.

1.38 g crude, 292 mg of 99.4% pure **5**; ee (m.r.): 68.3% (^{1}H NMR), 69.7% (see footnote 2) ([α] = +30.3° (*c* = 1.00)) and 66.1% (GC^2). CR dark + dark for 22.83 h, t.o.: 75.8%, 70.5% ee (GC^2).

*2.4.6.2. Exp. 6B: dark-control with 3-oxo-n-valeric ethylester (**2**).* 2.061 g (14.3 mmol) **2** reacted in 8 h, t.o.: 81.3%; turnover (t.o. in %) followed by GC:

Reaction time (h)	0.5	1	2	3	5	7	8	26.3
Turnover	11.3	19.1	36.4	50	63.7	76.3	81.3	99$

$CR dark + dark.

1.58 g crude, 524 mg of 96.6% pure **6**; ee (m.r.): 34.6% (^{1}H NMR, Eu(hfc)$_3$) [28] and 39.9% (see footnote 3) ([α] = −13.8° (*c* = 1.02)). CR dark + dark for 26.30 h, t.o.: 99%.

2.4.6.3. Exp. 6C: two-substrate reduction in the dark. 1.862 g (14.3 mmol) **1** and 2.062 g (14.3 mmol) **2** reacted in 7.92 h, t.o.: 22.5% **5** and 57.1% **6**; turnover (t.o. in %) followed by GC:

Reaction time (h)	0.5	1	2	3	5	7	7.92	24.5
Turnover for **5**	1.7	3.4	6.4	9.4	15.1	20.3	22.5	51.5$
Turnover for **6**	6.7	11.9	21.1	29.4	43.5	53.2	57.1	84$

$CR dark + dark.

2.7 g crude prod., 83 mg of 96% pure **5**, 271 mg of 93.7% pure **6**; ee values (m.rs.): 75% for **5** (^{1}H NMR), 79% for **6** (^{1}H NMR, Eu(hfc)$_3$) [28], 75.2% (see footnote 2) for **5** ([α] = +32.7° (*c* = 0.58)) and 81.5% (see footnote 3) for **6** ([α] = −28.2° (*c* = 1.03)), and 72% ee (GC^2) for **5**. CR dark + dark for 24.50 h, t.o.: 51.5% **5**, 84% **6**, 80.7% ee (GC^2) for **5**.

2.4.6.4. Exp. 6D: two-substrate reduction under irradiation. 1.863 g (14.3 mmol) **1** and 2.059 g (14.3 mmol) **2** were irradiated for 7.25 h, t.o.: 17.2% **5** and 55.6% **6**; turnover (t.o. in %) followed by GC:

Reaction time (h)	0.5	1	2	3	5	7.25	26.83	26.92
Turnover for **5**	1.5	2.8	5.2	7.8	12.3	17.2	47.8$	22.6€
Turnover for **6**	6.8	12.3	22.3	31.8	45.2	55.6	85.5$	68€

$CR light + dark; € CR light + light.

2.20 g crude prod., 56 mg of 88.8% pure **5**, 245 mg of 95.9% pure **6**; ee values (m.rs.): 65% for **5** (^{1}H NMR) and 86.1% for **6** (^{1}H NMR, Eu(hfc)$_3$) [28], 65.5% (see footnote 2) for **5** ([α] = +28.5° (*c* = 0.32)) and 91% (see footnote 3) for **6** ([α] = −31.5° (*c* = 1.07)), and 62.6% ee (GC^2) for **5**. CR light + light for 26.92 h, t.o.: 22.6% **5** and 68.03% **6**, 61% ee (GC^2) for **5**; CR light + dark for 26.83 h, t.o.: 47.8% **5** and 85.5% **6**, 73.4% ee (GC^2) for **5**.

*2.4.7. Experiment 7: simultaneous baker's yeast reduction of **2** to (R)-(−)-**6** and of an excess of **1** to (S)-(+)-**5** (1:1.5)*

Procedure and analytics according to GRP 1, Exps. 1 and 5.

*2.4.7.1. Exp. 7A: dark-control with an excess of ethyl acetoacetate (**1**).* 2.796 g (21.5 mmol) **1** reacted in 7.25 h, t.o.: 30.4%; turnover (t.o. in %, def. in Section 2.1) followed by GC:

Reaction time (h)	0.5	1	1.5	2.42	4.42	5.5	6.5	7.25	23
Turnover	3.2	5.7	7.8	10.3	20.8	23	27.9	30.4	57.1$

$CR dark + dark.

1.36 g crude, 90 mg of 96.9% pure **5**; ee (m.r.): 37.4% (^{1}H NMR), 39.1% (see footnote 2) ([α] = + 17.0° (*c* = 0.83)) and 36.2% (GC^2). CR dark + dark for 23 h, t.o.: 57.1%, 52.2% ee (GC^2).

*2.4.7.2. Exp. 7B: dark-control with 3-oxo-n-valeric ethylester (**2**).* 2.062 g (14.3 mmol) **2** reacted in 7.25 h, t.o.: 74.1%; turnover (t.o. in %) followed by GC:

Reaction time (h)	0.5	1	2	3.5	5.33	6.75	7.25	23.5
Turnover	12	20.8	36.8	51.3	64.1	73.5	74.1	95.2$

$CR dark + dark.

1.03 g crude, 385 mg of 98.2% pure **6**; ee (m.r.): 53.6% (^{1}H NMR, Eu(hfc)$_3$) [28], 48.2% (^{1}H NMR) and 51.7% (see footnote 3) ([α] = −17.9° (*c* = 1.06)). CR dark + dark for 23.5 h, t.o.: 95.2%.

*2.4.7.3. Exp. 7C: two-substrate reduction in the dark with an excess of **1**.* 2.794 g (21.5 mmol) **1** and 2.062 g (14.3 mmol) **2** reacted in 7.33 h, t.o.: 7.3% **5**, 36.6% **6**, turnover (t.o. in %) followed by GC:

Reaction time (h)	0.5	1	1.5	2.5	3.5	5	6.5	7.33	24.5
Turnover for **5**	1.6	2.7	3.4	4.6	5.4	6.6	7.2	7.3	7.8[$]
Turnover for **6**	9	14.2	18	23.8	28	33.1	35.8	36.6	37.7[$]

[$]CR dark + dark.

1.80 g crude prod., 37 mg of 74.3% pure **5**, 106 mg of 89.4% pure **6**; ee values (m.rs.): 46% for **5** (^{1}H NMR), 86.7% for **6** (^{1}H NMR, Eu(hfc)$_3$) [28], 46% (see footnote 2) for **5** ([α] = +20.0° (*c* = 0.50)) and 82.9% (see footnote 3) for **6** ([α] = −28.7° (*c* = 0.99)), 52% ee (GC^2) for **5**. CR dark + dark for 24.50 h, t.o.: 7.8% **5**, 37.7% **6**, 50% ee (GC^2) for **5**.

*2.4.7.4. Exp. 7D: two-substrate reduction under irradiation with an excess of **1**.* 2.794 g (21.5 mmol) **1** and 2.062 g (14.3 mmol) **2** were irradiated for 7.33 h, t.o.: 6.5% **5**, 37.5% **6**; turnover (t.o. in %) followed by GC:

Reaction time (h)	0.5	1	1.5	2.5	3.5	5	6.5	7.33	24.33	24.25
Turnover for **5**	0.9	1.6	2	3.2	4.2	5.4	6.2	6.5	8.4[$]	8.6[€]
Turnover for **6**	6.3	10.4	13.6	20.9	26	31.7	35.7	37.5	45.2[$]	43.7[€]

[$]CR light + dark; [€] CR light + light.

2.01 g crude prod., 79 mg of 77% pure **5**, 154 mg of 91.8% pure **6**; ee values (m.rs.): 41.9% for **5** (^{1}H NMR), 87.3% for **6** (^{1}H NMR, Eu(hfc)$_3$) [28], 38.4% (see footnote 2) for **5** ([α] = +16.7° (*c* = 0.51)) and 90.5% (see footnote 3) for **6** ([α] = −31.3° (*c* = 1.06)) with 40.6% ee (GC^2) for **5**. CR light + light for 24.25 h, t.o.: 8.6% **5** and 43.7% **6**, 41% ee (GC^2) for **5**; CR light + dark for 24.33 h, t.o.: 8.4% **5** and 45.2% **6**, 48.6% ee (GC^2) for **5**.

*2.4.8. Experiment 8: baker's yeast reduction of ethylpyruvate (**3**) to (S)-(−)-ethyl lactate (**7**)*

Procedure and analytics according to GRP 1; product identification by IR [23] and ^{1}H NMR [23], UV absorptions of **3**: ε (329) = 19.7[4] and **7**: ε (212) = 92.8; GC: 30 m PS-240 column with 5 min isotherm at 40 °C, then 40→240 °C, 4 °C/min; prp. GC at 100 °C isotherm.

2.4.8.1. Exp. 8A: dark-control. 1.661 g (14.3 mmol) **3** reacted in 8.50 h, t.o.: 100%; 0.82 g crude, 259 mg of 99.8% pure **7**; ee (m.r.): 86.3%[5b] ([α] = −12.7° (*c* = 1.03; acetone)), 77.1% (see footnote 5b) [α] = −1.4° (*c* = 1.00), 89.2% (GC^3).

2.4.8.2. Exp. 8B: irradiation. 1.662 g (14.3 mmol) **3** were irradiated for 9 h, t.o.: 100%; 0.62 g crude, 316 mg of 99.7% pure **7**; ee (m.r.): 86.3% (see footnote 5b) ([α] = −12.7° (*c* = 0.64; acetone)), 86.4% (see footnote 5b) [α] = −1.6° (*c* = 1.08), 90.8% (GC^3).

*2.4.9. Experiment 9: baker's yeast reduction of 3-oxo-n-caproic ethylester (**4**) to (R)-(−)-3 -hydroxy-n-caproic ethylester (**8**)*

Procedure and analytics according to GRP 1; product identification by IR [3] and ^{1}H NMR [3], UV absorptions of **4**: ε (246) = 1282[6] with a shoulder at 300 nm and **8**: no absorption above 260 nm; GC: 30 m PS-240 column at 60→260 °C, 6 °C/min; prp. GC at 130 °C isotherm.

2.4.9.1. Exp. 9A: dark-control. 2.265g (14.3 mmol) **4** reacted in 9 h, t.o.: 29.3%; 1.32 g crude, 90 mg of 98.1% pure **8**; ee (m.r.): 95.5% (^{1}H NMR), [α] = −25.0° (*c* = 0.91)[7]; CR dark + dark for 23.43 h, t.o.: 31.3%.

2.4.9.2. Exp. 9B: irradiation. 2.262 g (14.3 mmol) **4** were irradiated for 9.25 h, t.o.: 27.5%; 0.49 g crude, 37 mg of 95.8% pure **8**; ee (m.r.): 92.6% (^{1}H NMR), [α] = −24.8° (*c* = 0.51) (see footnote 7); CR light + light for 24.08 h, t.o.: 28.7%; CR light + dark for 24 h, t.o.: 28.8%.

[4] (a) ε (340) = 19.9 (in CH_3CN), 14.8 (in heptane) [30]; (b) ε (340) = 5.4 (in EtOH) [31].

[5] (a) $[\alpha]^{20}_D$ = +9.6° (*c* = 1.2; acetone), 92% ee [23]; (b) [α] = −14.2° (*c* = 1.05; acetone) and [α] = −1.8° (*c* = 1.05; $CHCl_3$) were found for purchased and distilled (*S*)-(−)-ethyl lactate (**7**) (99% purity by GC), whose ee is 100% according to GC^3 [8a]. The enantiomeric excess determined for **7** via optical rotation were referred to these [α] value being assigned to 100% ee.

[6] ε (248) = 10 200 (in EtOH) [32].

[7] The here determined ee values did exceed the published one: [α] = −22.1° (*c* = 1.04; $CHCl_3$) 90% ee [5].

*2.4.10. Experiment 10: ADH I reduces ethyl acetoacetate (**1**) to (S)-(+)-**5***

Procedure according to GRP 3, analytics as in GRP 1 and Exp. 1; ee determined by GC[2].

2.4.10.1. Exp. 10A: irradiation. Upon irradiation, 19.2 mg **1** were reduced to **5** by 70.2 mg ADH I and 70.4 mg NADH in 0.7 ml H_2O, r.t.: 23.42 h, t.o.: 92.6%, 95.7% ee; CR light + dark for 23.17 h, t.o.: 97.1%, 93.2% ee.

2.4.10.2. Exp. 10B: dark-control. 19 mg **1** were reduced to **5** by 70.3 mg ADH I and 70.1 mg NADH in 0.7 ml H_2O, r.t.: 23.17 h, t.o.: 96.2%, 95.7% ee.

*2.4.11. Experiment 11: ADH I reduces ethylpyruvate (**3**) to (S)-(−)-**7***

Procedure according to GRP 3, analytics as in GRP 1; starting solution: 100.3 mg **3**, 140.4 mg ADH I, and 140.3 mg NADH in 1.4 ml H_2O; 100% ee was determined by method GC[3] for both reactions in two extra experiments under the same conditions.

2.4.11.1. Exp. 11A: irradiation. Upon irradiation, 0.7 ml of the starting solution reacted in 23.83 h to **7**, t.o.: 91%.

2.4.11.2. Exp. 11B: dark-control. 0.7 ml of the starting solution reacted in 23.92 h to **7**, t.o.: 93.5%.

2.4.12. Experiment 12: UV spectrum of L*-lactate dehydrogenase (*L*-LDH) (a) and its application for reducing ethyl acetoacetate (**1**) to (S)-(+)-**5** (b)*

(a) UV spectrum of the *baker's yeast* L-LDH (Sigma): 0.35 μl of a 5 units suspension were gradually diluted in H_2O, d = 1 cm [8a,c]. (b) Procedure according to GRP 3, analytics as in GRP 1 and Exp. 1; ee determined by GC[2]; starting solution: 32.1 mg **1** and 300.9 mg NADH dissolved in the 3 ml of the undiluted L-LDH suspension (50 units).

2.4.12.1. Exp 12A: irradiation. Upon irradiation, a half of the starting solution reacted in 23 h to **5**, t.o.: 42.7%, 67% ee.

2.4.12.2. Exp. 12B: dark-control. A half of the starting solution reacted in 22.83 h to **5**, t.o.: 42.6%, 66.9% ee.

*2.4.13. Experiment 13: β-ketoacylreductase reduces ethyl acetoacetate (**1**) to (R)-(−)-3-hydroxybutyric ethylester (−)-(**5**)*

Procedure according to GRP 3, analytics as in GRP 1 and Exp. 1; ee determined by GC[2]; starting solution: 38.6 mg **1**, 140.4 mg NADPH and 300 μl of the enzyme suspension [19] were dissolved in 1.1 ml H_2O.

2.4.13.1. Exp. 13A: irradiation. Upon irradiation, 50% of the starting solution reacted in 24.08 h to (*R*)-(−)-**5**, t.o.: 13.7%, 97.6% ee.

2.4.13.2. Exp. 13B: dark-control. Fifty percent of the starting solution reacted in 23.83 h to (*R*)-(−)-**5**, t.o.: 12.3%, 98.2% ee.

2.4.14. Experiment 14: CD and UV spectra of irradiated ADH I

Procedure according to GRP 4; the UV and CD spectra were recorded after each experiment.

2.4.14.1. Exps. 14A–D. Starting solution: 350.9 mg ADH I were diluted in 50 ml H_2O. Ten milliliters of the starting solution were kept in the dark (14A) and another 10 ml were irradiated (14C), both for 23.6 h. Simultaneously, 10 ml of the starting solution were kept in the dark (14B) and another 10 ml were irradiated (14D), both for 47.8 h. The ADH I was from the same charge used for Exp. 15.

2.4.14.2. Exps. 14E and F. Starting solution: 40.5 mg ADH I (Sigma) were diluted in 50 ml H_2O. Ten milliliters of that solution were irradiated (14E) and another 10 ml were kept in the dark (14F), both for 20.4 h.

*2.4.15. Experiment 15: reduction of ethyl acetoacetate (**1**) to (S)-(+)-**5** with pre-irradiated ADH I*

Procedure according to GRP 3, analytics as in GRP 1 and Exp. 1; starting solution: 280.7 mg ADH I dissolved in 2.8 ml H_2O; the ADH I was from the same charge used for Exp. 14; ee determined by method GC[2].

2.4.15.1. Exp. 15A: irradiation for approximately 2 days. 0.7 ml of the starting solution were irradiated for 23.7 h. After 70.1 mg NADH and 19.3 mg **1** were added, this mixture reacted under irradiation in 22.8 h to **5**, t.o.: 64.1%, 95.6% ee.

2.4.15.2. Exp. 15B: irradiation for approximately 1 day. Upon irradiation, 19.6 mg **1** reacted with 0.7 ml of the starting solution plus 70.5 mg NADH to **5**, r.t.: 22.8 h, t.o.: 89.3%, 95.5% ee.

2.4.15.3. Exp. 15C: dark-control for approximately 2 days. 0.7 ml of the starting solution were kept in the dark for 23.7 h. After 70.3 mg NADH and 20.4 mg **1** were added, this mixture reacted in 22.8 h to **5**, t.o.: 88.5%, 93.9% ee.

2.4.15.4. Exp. 15D: dark-control for approximately 1 day. 20.6 mg **1** were reduced to **5** by 0.7 ml of the starting solution plus 70.6 mg NADH, r.t.: 22.8 h, t.o.: 92.6%, 95.4% ee.

*2.4.16. Experiment 16: attempted ADH I reduction of 3-oxo-n-valeric ethylester (**2**)*

Procedure according to GRP 3, analytics as in GRP 1.

2.4.16.1. Exp. 16A: dark-control. 19.3 mg **2**, 70.1 mg ADH I and 70.2 mg NADH in 0.7 ml H_2O formed 2% unidentified product, r.t.: 23.6 h.

2.4.16.2. Exp. 16B: irradiation. Upon irradiation, 19.6 mg **2**, 69.2 mg ADH I and 69.9 mg NADH in 0.7 ml H_2O formed 2% unidentified product, r.t.: 24.6 h.

*2.4.17. Experiment 17: attempted ADH I reduction of 3-oxo-n-caproic ethylester (**4**)*

Procedure according to GRP 3, analytics as in GRP 1.

2.4.17.1. Exp. 17A: dark-control. 20.6 mg **4** in 0.7 ml H_2O were not reduced by 70.5 mg ADH I and 70.8 mg NADH, r.t.: 23.75 h.

2.4.17.2. Exp. 17B: irradiation. Upon irradiation, 21.3 mg **4** in 0.7 ml H_2O were not reduced by 71.2 mg ADH I and 70.4 mg NADH, r.t.: 23.75 h.

*2.4.18. Experiment 18: β-ketoacylreductase reduces 3-oxo-n-valeric ethylester (**2**) to **6***

Procedure according to GRP 3, analytics as in GRP 1 and Exp. 5. 19.5 mg **2** in 0.55 ml H_2O were reduced to **6** by 150 μl of the enzyme suspension [19] and 70.1 mg NADPH, r.t.: 22.83 h, t.o.: 80%.

*2.4.19. Experiment 19: β-ketoacylreductase reduces ethylpyruvate (**3**) to **7***

Procedure according to GRP 3, analytics as in GRP 1 and Exp. 8. 12.6 mg **3** in 0.55 ml H_2O were reduced to **7** by 150 μl of the enzyme suspension [19] and 70.8 mg NADPH, r.t.: 22.83 h, t.o.: 90.9%.

*2.4.20. Experiment 20: β-ketoacylreductase reduces 3-oxo-n-caproic ethylester (**4**) to **8***

Procedure according to GRP 3, analytics as in GRP 1 and Exp. 9. 20.3 mg **4** in 0.55 ml H_2O were reduced to **8** by 150 μl of the enzyme suspension plus 70.7 mg NADPH [19], r.t.: 22.5 h, t.o.: 86.2%.

3. Results and discussion

3.1. Baker's yeast reduction upon irradiation

Irradiating the *baker's yeast* reduction of ethyl acetoacetate (**1**) with 300–400 nm UV light inhibits the yeast's activity (Fig. 2, Exp. 1A, □): Besides a lower reaction rate, the enantiomeric excess (ee) of product (*S*)-(+)-3-hydroxybutyric ethylester (**5**) is after 10.5 h only 32 instead of the 68% achieved in the dark-control (Exp. 1B, △). If a portion of the main reaction (1A) is further irradiated (CR light + light, Exp. 1A, □), the enantiomeric excess is still only 36% after a total of 23.2 h. Is another portion of 1A instead left in the dark (CR light + dark, Exp. 1A, ■), the yeast recovers strongly by reaching 76% of the turnover and 80% of the ee of the also continued dark-control (CR dark + dark, Exp. 1B, ▲). The light-inhibited enzyme generates obviously the *S*-configuration ('*S*-enzyme') and its replenishment experiences a significant recovery in darkness.

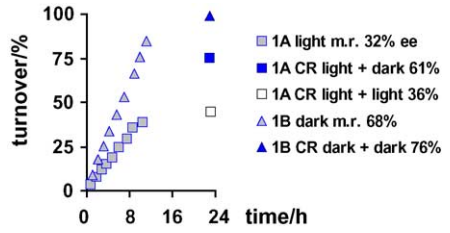


Fig. 2. [8] Exp. 1. Irradiation of the *b.y.* reduction of **1** to (*S*)-(+)-**5** (1A, □), and dark-control (1B, △).

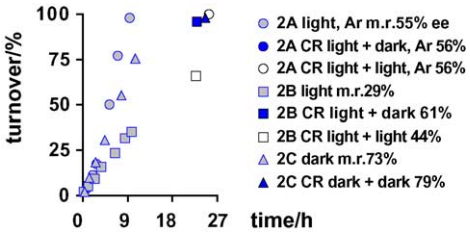


Fig. 3. Exp. 2. Irradiating the *b.y.* reduction of **1** to (*S*)-(+)-**5** with (2A, ○) and with no Ar (2B, □), plus dark-control (2C, △) (see footnote 8).

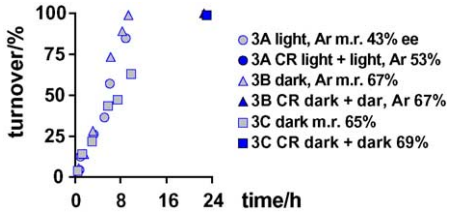


Fig. 4. Exp. 3. Ar-flushed *b.y.* reductions of **1** to (*S*)-(+)-**5** in light (3A, ○) and darkness (3B, △), plus dark-control (3C, □) (see footnote 8).

3.2. Irradiating the baker's yeast reduction under argon

Under argon, the *baker's yeast* reduction of substrate **1** is accelerated due to the Pasteur effect [33] and therefore already complete after approximately 9 h (Figs. 3 and 4, Exps. 2A, ○ , 3A, ○ and 3B, △). The light damage is here less severe (2A) because the enantiomeric excess is 55% instead of the only 29% achieved in the regular irradiation (Exp. 2B, □). That improvement under argon points the inhibition (1A and 2B) to the light-triggered development of an aggressive

[8] The ee values listed in Figs. 2–7 and 13 for the main reactions (m.r.) belong to their latest GC samples (Section 2.3.1).

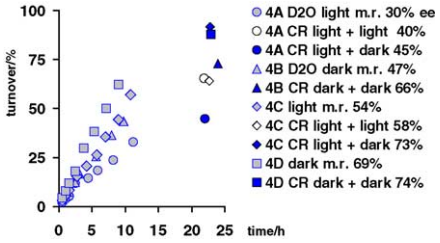


Fig. 5. Exp. 4. *Baker's yeast* reductions of **1** to (*S*)-(+)-**5** in partially (53%) D_2O under irradiation (4A, ○) and in darkness (4B, △), plus irradiation (4C, ◇) and dark-control (4D, □) in H_2O (see footnote 8).

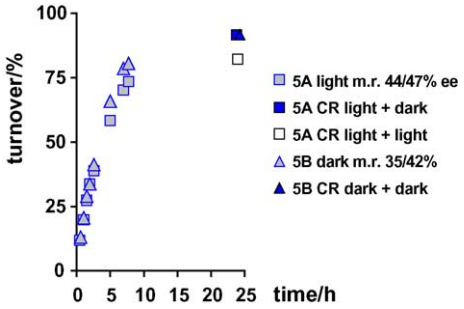


Fig. 6. Exp. 5. Irradiating the *b.y.* reduction of **2** to (*R*)-(−)-**6** (5A, □), and dark-control (5B, △) (see footnote 8).

oxygen species, such as singlet oxygen or the superoxide anion that may both influence the enzyme's activity. If singlet oxygen is formed, it may deactivate the enzyme by oxidizing its amino acids [15,16,35–40]. This option is reviewed by employing D_2O as partial (53%) solvent (Fig. 5, Exp. 4), since a damage through singlet oxygen should be amplified therein [41]. Although the irradiated reduction of substrate **1** is indeed delayed in the presence of D_2O (4A, ○ and B, △), a potential singlet-oxygen effect cannot be distinguished from the also on this reaction occurring slowdown caused by the kinetic deuterium isotope effect. An inhibition due to the superoxide anion, that is e.g. formed when the light affects present NADH [42–44], may become feasible if this species is not quenched by the natural *superoxide dismutase* (*SOD*) of the *baker's yeast* [45]. Remarkably, one argon-flushed irradiation experiment (2A) lies with 55% under the 73% ee of its dark-control (2C), while another one (3A) achieves only 64% of the enantiomeric excess of its corresponding inertgas reaction in the dark (3B). Unless there is residual oxygen left in the reaction mixture[9], this could be due to an additional photo-effect on the enzyme's amino acids, as the one observed when irradiating L-LDH from bovine heart at 300 nm [34]. This idea appears to receive support from that in a Pyrex vessel conducted Exp. 4 (Fig. 5) where the light-inhibition on the yeast's activity is after around 9 h only 29% (4C and D), whereas the damage was 46% in a quartz tube (1A and B).

3.3. UV light enhances the enantioselectivity

An increase of the stereoselectivity by UV light is accomplished for the *baker's yeast* reduction with the homologous ketoester 3-oxo-*n*-valeric ethylester (**2**). When irradiating its reduction to (*R*)-(−)-3-hydroxy-*n*-valeric ethylester (**6**) (Fig. 6, Exp. 5A, □), the reaction rate is somewhat worse than in darkness (Exp. 5B, △). Instead is the enantiomeric excess of product **6** with 44 resp. 47% (5A) higher than the 35 resp. 42% achieved in the dark-control (5B). The observed advance is therewith explained that the photosensitive '*S*-enzyme' (Exp. 1) is also involved in this transformation and suffers the light inhibition here too. The resulting higher contribution of the unaffected '*R*-enzyme', which is considered to be the β-ketoacylreductase of the *baker's yeast* fatty acid synthase complex [46a], enhances so the enantiomeric excess of the product. This method has synthetic potential if the constellation of the enzymes involved is appropriate for a particular substrate.

3.4. Improved stereoselectivity via a 'two-substrate application'

The previous results affirm again that several oxidoreductases may participate in the *baker's yeast* reduction of ketoesters [1,7]. The efficiency of this reaction should therefore be improved by deliberately disengaging a disturbing enzyme forming the undesirable enantiomer. This idea is realized by adding two substrates, which the *baker's yeast* transforms with opposite stereochemistry, simultaneously to the same reaction. Through it the e.g. unwanted '*S*-enzyme' converts less of the actually desired '*R*-substrate', and if that also applies to the '*R*-enzyme' will the enantiomeric excess of both products be enhanced. Adding both substrates, ethyl acetoacetate (**1**) and 3-oxo-*n*-valeric ethylester (**2**), simultaneously and in the ratio 1:1 to a *baker's yeast* reduction raises the enantiomeric excess for (*R*)-(−)-3-hydroxy-*n*-valeric ethylester (**6**) to 79% (6C), whereas 35% ee were achieved in the conversion with only one substrate (6B) (Table 3). The further increase to 86% ee upon irradiation (6D) results from the light effect found in Exp. 5, while also (*S*)-(+)-3-hydroxybutyric ethylester (**5**) achieves a slightly higher ee upon this experimental variant (6C). The light-sensitive '*S*-enzyme' (see Exp. 1) is also here involved for the inhibition on the irradiated reduction of substrate **1** (6D). However, despite applying ethyl acetoacetate (**1**) in excess (1.5:1) there is no further improvement obtained (Exp. 7). For a general application of this method, at least one of the involved enzymes must be able to accept two substrates at once.

3.5. Light-resistant baker's yeast reductions

Interestingly, there is no light-effect observed for the *baker's yeast* reductions of the homologous ketoesters

[9] Degassing the reaction mixture by the freeze-pump technique is here not advised.

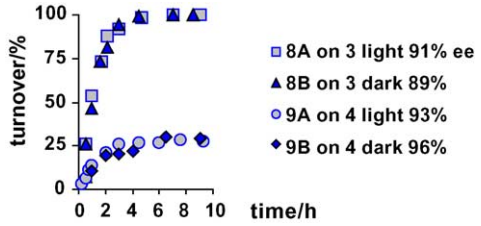


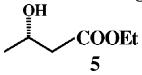
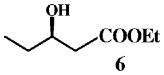
Fig. 7. Exps. 8 and 9. Irradiation of the *b.y.* reductions of **3** to (*S*)-(−)-**7** (8A, □) and **4** to (*R*)-(−)-**8** (9A, ○), plus dark-controls (8B, ▲, 9B, ♦) (see footnote 8).

ethylpyruvate (**3**) to (*S*)-(−)-ethyl lactate (**7**) (Fig. 7, Exps. 8A, □ and B, ▲) [3] and 3-oxo-*n*-caproic ethylester (**4**) to (*R*)-(−)-3-hydroxy-*n*-caproic ethylester (**8**) (Fig. 7, Exps. 9A, ○ and B, ♦) [5]. Conclusively, the photosensitive '*S*-enzyme' found in Exp. 1 seems not to be involved in these transformations here, unless the inhibition depends also on the substrate's geometry.

3.6. Reductions performed by isolated baker's yeast oxidoreductases

Searching for the mechanism of the light-damage to the *baker's yeast* (Exp. 1), corresponding reduction experiments are conducted with isolated yeast enzymes in the presence of NAD(P)H, whereby ethyl acetoacetate (**1**) or ethylpyruvate (**3**) are the substrates. There is no light-effect found however if the ADH I transforms substrates **1** (Fig. 8, Exps. 10A, △ and B, ▲) and **3** (Fig. 8, Exps. 11A, □ and B, ■) or the L-lactate dehydrogenase (cytochrome b_2) converts substrate **1** in the presence of NADH to (*S*)-(+)-**5** (Fig. 8, Exps. 12A, ○ and B, ●), and also not when the (β-ketoacylreductase [19] reduces **1** to (*R*)-(−)-**5** (Fig. 8, Exps. 13A, ◇ and B, ♦). Either the light-sensitive '*S*-enzyme' is a different oxidoreductase or the here employed enzyme preparations have other characteristics after their isolation than they had in the intact yeast cell. Moreover are also the reaction conditions not entirely the same, like, e.g. the oxygen concentration. Interestingly, ethyl acetoacetate (**1**) is here reduced by isolated, commercial *baker's yeast* L-lactate dehydrogenase (cytochrome b_2) [47] in the presence of NADH to 43% and with an enantiomeric excess of 67% (Exp. 12). The mitochondrial lactate dehydrogenase (L-LDH) isolated from *baker's yeast* is one NAD-independent exception [48,49] requiring instead flavin mononucleotide (FMN) and protoheme for function, while the cofactor-free apoprotein was found to be inactive [35–37,50,51]. For its UV spectrum (Fig. 9) is the here employed, commercial L-LDH flavin-free showing only a reduced protoheme γ-band at 423 nm [8a,35–37,51]. Because the reduction experiment with substrate **1** was repetitively carried out with success [8a,c], NADH is assumed to be here the electron donating group maintaining the L-LDH's activity [37,52]. For comparison, in an oxidation experiment the catalytic activity of the flavin-free apoprotein was restored by the addition of FMN [53].

Table 3
Exp. 6: enantiomeric excess (in %) for the two-substrate application

Condition	^{1}H NMR	GC	[α]	Exp.
Dark reaction, single substrate				
 **5**	68	66	70	6A
 **6**	35	n.d.	40	6B
Dark reaction, two substrates				6C
5	75	72	75	
6	79	n.d.	82	
Irradiation two substrates				6D
5	65	63	66	
6	86	n.d.	91	

3.7. UV light affects the tertiary structure of isolated ADH I

The effect of the near UV irradiation on isolated *baker's yeast* alcohol dehydrogenase I is examined here by spectroscopic methods (Exp. 14). When this enzyme is exposed to 300–400 nm UV light its 280 nm CD absorption band, representing its tertiary structure [54,55], is clearly reduced after approximately 24 h (Fig. 10b, Exp. 14C) and has

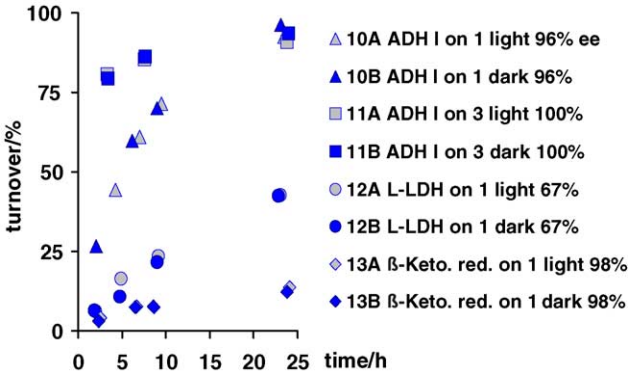


Fig. 8. Exps. 10–13. Irradiation of the reductions with ADH I on **1** to (*S*)-(+)-**5** (10A, △) and **3** (*S*)-(−)-**7** (11A, □), L-LDH on **1** to (*S*)-(+)-**5** (12A, ○), and β-ketoacylreductase [19] on **1** to (*R*)-(−)-**5** (13A, ◇), and dark-controls (10B, ▲, 11B, ■, 12B, ●, 13B, ♦) (see footnote 8).

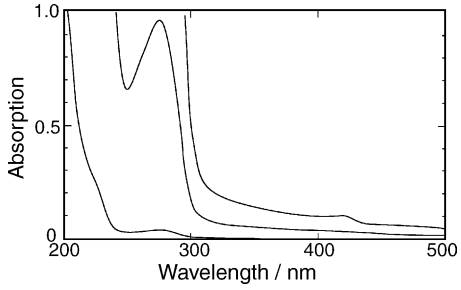


Fig. 9. Exp. 12. UV spectrum of the *baker's yeast* L-lactate dehydrogenase (L-LDH) from Sigma [8a].

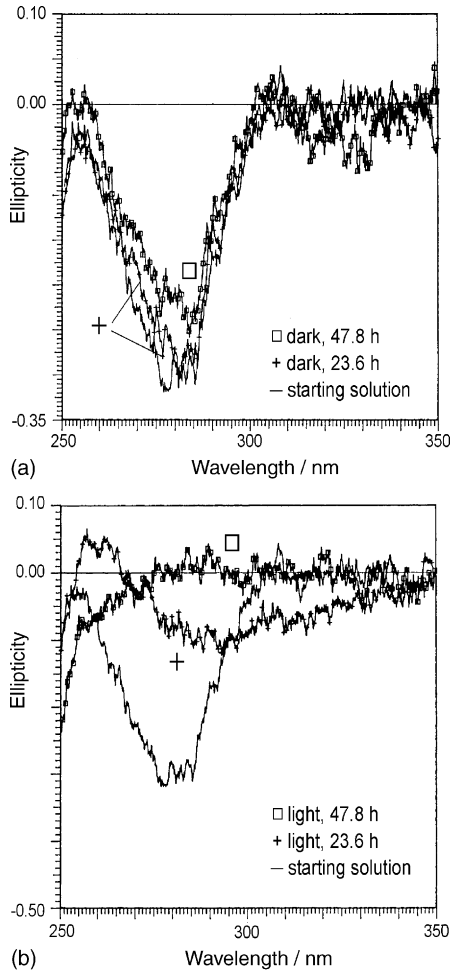


Fig. 10. (a) Exps. 14A and B. CD spectra of ADH I kept in the dark for 23.6 h (14A, +) and 47.8 h (14B, □). (b) Exps. 14C and D. CD spectra of ADH I irradiated for 23.6 h (14C, +) and 47.8 h (14D, □).

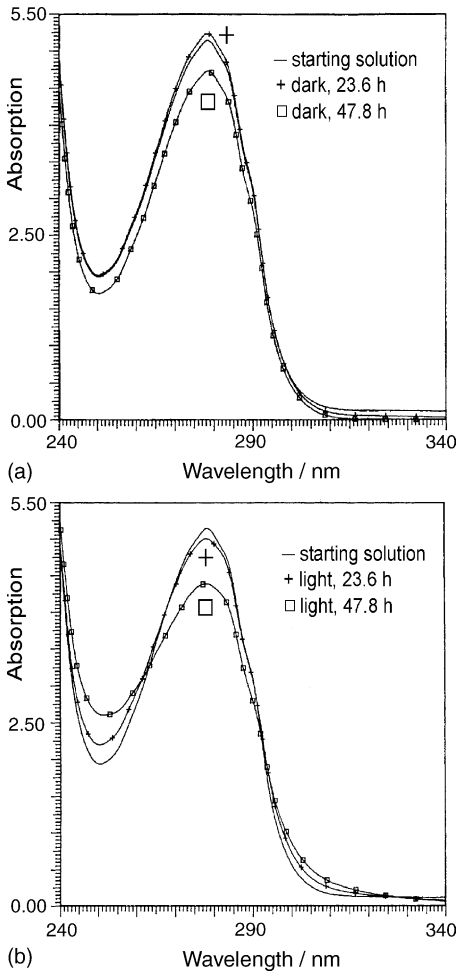


Fig. 11. (a) Exps. 14A and B. UV spectra of ADH I kept in the dark for 23.6 h (14A, +) and 47.8 h (14B, □). (b) Exps. 14C and D. UV spectra of ADH I irradiated for 23.6 h (14C, +) and 47.8 h (14D, □).

vanished after around 2 days (Fig. 10b, Exp. 14D). Responsible chromophor should be the enzyme's amino acids tryptophane (trp) or cystine (cys-cys), whose UV-tailing absorptions are furthest extended into the wavelength range of the light [17,18]. As the irradiation has no real impact on its UV absorption band at 280 nm (Fig. 11, Exps. 14C and D), the enzyme's light-induced loss of chirality may only be conformational with preferentially trp, or another aromatic amino acid, twisting out of its chiral environment. Because also the 215 nm CD absorption of the ADH I is not influenced after 20.4 h of irradiation (Fig. 12, Exp. 14E), the enzyme's secondary structure assigned to this CD band [56] should not have been altered either. The light-induced loss of the CD absorption at 280 nm (Fig. 10b) may therefore not be due to a photoreaction changing the secondary structure of the enzyme, what should exclude a light effect on the ADH I's disulfide bridge [57] just as a photooxidation of its trp units [10,58].

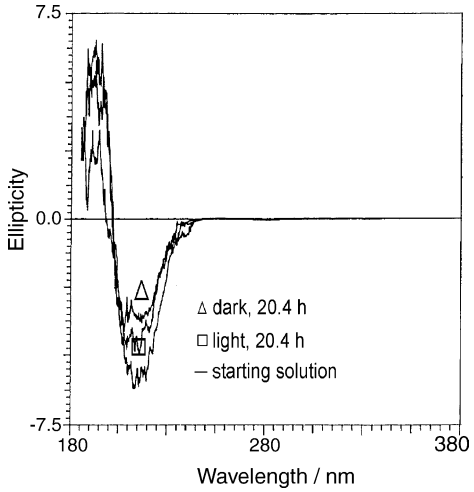


Fig. 12. Exps. 14E and F. 215 nm CD absorption band of ADH I (Sigma) irradiated for 20.4 h (14E, □) and dark-control for 20.4 h (14F, △).

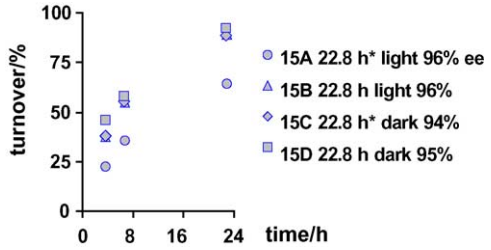


Fig. 13. Exp. 15. Reduction of **1** to (*S*)-(+)-**5** with pre-irradiated ADH I (15A, ○), regular irradiation (15B, △), and dark-controls (15C, ◇ and D, □). The symbol (*) indicates the addition of substrate **1** and NADH after the 23 h pre-treatment of the ADH I (15A and C).

3.8. Irradiation impacts the activity of ADH I

Remarkably, although the 300–400 nm irradiation affects the tertiary structure of isolated ADH I (Exp. 14) it does not influence its catalytic function (Exp. 10). To resolve this conflict, a reduction experiment is conducted where isolated ADH I gets pre-irradiated for almost one day to induce those structure changes in the enzyme that were previously documented by means of CD spectroscopy (Fig. 10b, Exp. 14C and D). When in an in vitro reduction experiment carried out under irradiation the substrate ethyl acetoacetate (**1**) and the cofactor NADH are added to the already pre-irradiated ADH I, there is indeed a lower reaction rate found in the first GC sample, which then remains rather constant throughout the entire reaction (Fig. 13, Exp. 15A, ○). This is neither observed for the corresponding reduction where the ADH I is initially kept in darkness for nearly one day before performing the subsequent dark reaction for 22.8 h (Exp. 15C, ◇), nor with both control experiments (Exps. 15B, △ and D, □). This result with the pre-irradiated ADH I is the experimental proof that the by CD spectroscopy visualized structure changes do actually affect the enzyme's functionality. That the enantioselectivity is not different upon pre-irradiation (15A) supports the previously expressed idea (see Section 3.7), that the light-triggered changes are merely conformational. As three of the 27 trp units of the ADH I are located in its catalytic domain [16,59], their conformational changes may narrow the access to the enzyme's active site and so decline the reaction rate, however leaving the stereo-selectivity unaffected.

3.9. The role of NADH in the irradiation of ADH I

Because isolated *baker's yeast* alcohol dehydrogenase I is here proven to be photosensitive (Exp. 14), its experimentally observed light resistance in the in vitro reduction experiment with substrate **1** (Exps. 10 and 15B) is referred to the presence of the enzyme cofactor NADH. For its maxima at 280 and 340 nm, the NADH has a stronger UV absorption than the ADH I [8,60,61] and should therefore shield the enzyme from the near UV light by absorbing it for conducting its own photochemistry [8b,42–44]. Furthermore, the in vitro reduction with ADH I could get affected by the aggressive superoxide anion, which is the intermediate of the NADH phototransformation to NAD^+ in the near UV range [42–44]. Since such a light-effect is not observed (Exps. 10 and 15B), the photochemistry of NADH is submitted to a re-investigation [8]. It reveals that NAD^+ and therewith a larger quantity of the superoxide anion are only formed under sufficiently aerobic conditions. As the in vitro reduction experiments with the ADH I are carried out here in a fairly oxygen-poor environment (Exps. 10 and 15B), the actual formation of the superoxide anion remains uncertain.

3.10. Four enzymes compete for ethyl acetoacetate

This research contributes to general efforts undertaken to characterize those *baker's yeast* enzymes that compete simultaneously for one substrate [1,7]. For instance, ethyl acetoacetate (**1**) appears here to be accepted by four oxidoreductases at once. Enzyme I is the photosensitive '*S*-enzyme' upon irradiating the *baker's yeast* reduction of substrate **1** at 300–400 nm. Speculatively, this could be the β-3-hydroxybutyrate dehydrogenase [6,7,46b] if the present oxygen is able to mediate the light-inhibition. Should the light-effect moreover depend on the geometry of the employed substrate (see Section 3.5), also the *baker's yeast* L-lactate dehydrogenase (L-LDH) would come as an explanation in question (Exp. 12) [47,62]. Enzyme II is the ADH I, whose catalytic activity is in vitro not impaired by the light based on the protective function of NADH (Exps. 10 and 15B), but which is however photosensitive if irradiated alone (14C, D and 15A). The isolated enzyme transfers ethyl acetoacetate (**1**) (Exp. 10) and ethylpyruvate (**3**) (Exp. 11) to product alcohols with *S*-configuration, while 3-oxo-*n*-valeric ethylester (**2**) (Exp. 16) and 3-oxo-*n*-caproic ethylester (**4**) (Exp. 17) are no substrates. Enzyme III is likely the β-ketoacylreductase of the *yeast's* fatty acid synthase complex [46a]. Isolated [19], this enzyme accepts all substrates used here (Table 1, Exps. 13 and 18–20), however without light inhibition when converting ethyl acetoacetate (**1**) to its *R*-configurated alcohol (−)-**5**. (Exp. 13). Enzyme IV gives *S*-configurated product alcohols and is for the low enantiomeric excess of 35% responsible that is observed in the dark-control when the yeast reduces 3-oxo-*n*-valeric ethylester (**2**) (Exps. 5B, 6B, and 7B). It differs from Enzyme I in terms of light-sensitivity, from Enzyme II because 3-oxo-*n*-valeric ethylester (**2**) is a substrate, and from Enzyme III because it forms alcohols with *S*-configuration. Enzyme IV may be identical to the ADH IV [16].

3.11. Conclusion

The *baker's yeast* reduction of α- and β-ketoesters is irradiated with light of the near UV range (300–400 nm) for improving the effectiveness of this biotransformation. By interfering with the equilibrium of those oxidoreductases that compete for the same substrate, the light enhances the

enantioselectivity when reducing 3-oxo-*n*-valeric ethylester. Since there is a light inhibition only then observed if ethyl acetoacetate is the substrate, the mitochondrial respiration of the yeast is obviously not influenced by the light. According to experiments carried out with homologous substrates, only one of the participating oxidoreductases ('*S*-enzyme') is photosensitive. When continuing an initially light-inhibited reduction experiment in the dark, a significant recovery suggests that the inhibition of the enzyme's replenishment is not permanent. The *baker's yeast* reduction under argon is generally accelerated due to the Pasteur effect. The enhancement observed when flushing the irradiated yeast reduction of ethyl acetoacetate with argon refers the destructive light-effect to the participation of singlet oxygen or the superoxide anion. That this improvement under argon is not complete may be due to an additional, direct photo-effect on those enzyme's amino acids with a particularly long UV tailing absorption, unless the reason is residual oxygen in the solution. The stereodifferentiation of reductions with *baker's yeast* is significantly increased by a new method entitled 'two-substrate application', where an enzyme impairing the enantioselectivity gets disengaged by a simultaneously added, second substrate. Pre-irradiating the ADH I without NADH and substrate prior to the reduction reaction inhibits the enzyme's activity which should be a result of those light-induced conformational changes in the enzyme's tertiary structure that are discovered by means of CD spectroscopy. In vitro reductions performed in the presence of NADH are light-resistant since the cofactor protects the enzyme by consuming the light for conducting its own photochemistry. Irradiating the reductions with isolated *baker's yeast* enzymes in the presence of NADH under oxygen-poor conditions prevents a sufficient formation of the aggressive superoxide anion species that may otherwise be harmful to the enzyme. FMN-free *baker's yeast* L-lactate dehydrogenase (L-LDH) is usually inactive, but the purchased one shows here a reasonable reduction activity (43% conversion, 67% enantiomeric excess) that is referred to the presence of NADH, which appears to be the electron-donating group here. At least four yeast oxidoreductases seem to be simultaneously involved in the conversion of the substrate ethyl acetoacetate.

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