ARTICLE

OBC
www.rsc.org/obc

The first fluorogenic assay for detecting a Baeyer–Villigerase activity in microbial cells

María C. Gutiérrez, Arthur Sleegers, Helen D. Simpson, Véronique Alphand and Roland Furstoss *

Groupe Biocatalyse et Chimie Fine UMR-CNRS 6111, Université de la Méditerranée, Faculté des Sciences de Luminy, case 901, 163 Avenue de Luminy, 13288 Marseille, Cedex 9, France. E-mail: furstoss@luminy.univ-mrs.fr

Received 16th June 2003, Accepted 14th July 2003
First published as an Advance Article on the web 16th September 2003

The first fluorogenic assay allowing for detection of microbial enzymes able to perform Baeyer–Villiger oxidation is described. This is based on the use of 4-oxopentyl umbelliferyl ether **1** as a fluorogenic substrate. When Baeyer–Villigerases active against this test ketone are present in the selected whole cells, **1** is transformed into 3-hydroxypropyl umbelliferyl ether **3**, which, in a subsequent step, releases the fluorescent product umbelliferone. Different microorganisms, known to be endowed with Baeyer–Villigerase activity, were assayed.

Introduction

The use of a biotransformation strategy for achieving fine organic synthesis is nowadays recognized as an indisputable fact.[1] This is due to both the fantastic potential of enzymes as (chiral) catalysts and, also, to the fact that such approaches can be regarded as attractive "green chemistry" alternatives to chemical reactants or catalysts. In this context, the main issue is obviously the selection of an appropriate enzyme. Recently, several High Throughput Screening (HTS) techniques have been elaborated, which allow the very rapid screening of libraries of thousands of potential enzyme sources within collections of wild type microorganisms or libraries of clones obtained *via* molecular biology techniques.[2,3] Obviously, the crucial key point of such screening approaches lies in the availability of an appropriate assay allowing selection of the proper biocatalyst.

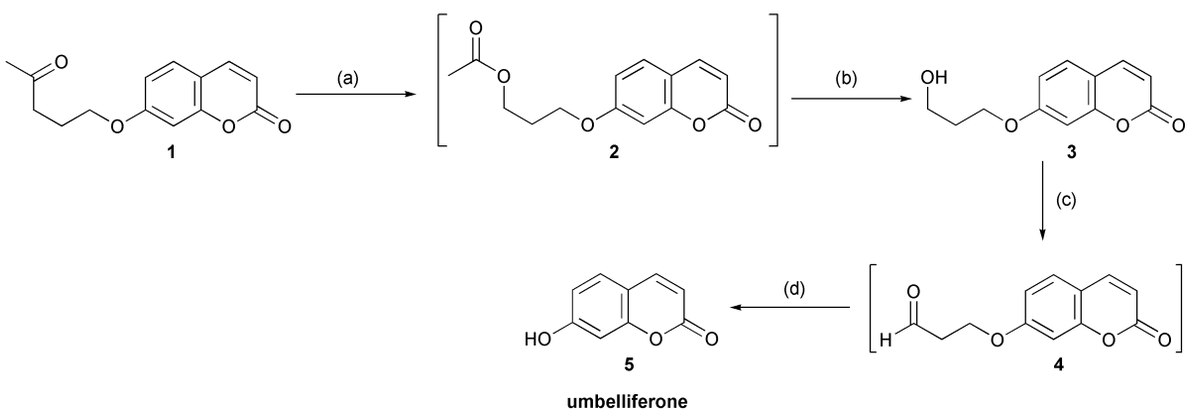
We have been interested for several years in studying asymmetric Baeyer–Villiger (BV) oxidation (a type of reaction which still proves to be very difficult, if not impossible, using conventional chemistry)[4] by way of a biocatalytic approach.[5–8] To the best of our knowledge, a first (colorimetric) assay, which only applies to monocyclic ketones and is limited to the millimolar concentration range of test substrate, has been recently described for the detection of BV monooxygenase (BVMO) activity.[9] Due to the intrinsic limitations of this first assay, we decided to try to set up a more sensitive Baeyer–Villigerase assay, keeping in mind that such an assay should also be (a) reliable, (b) enzyme specific and (c) fast and easy to use.

A process involving a fluorogenic test substrate should meet these requirements best. Elegant applications of such a strategy have been documented previously, for example by Reymond and coworkers for detecting alcohol dehydrogenase,[10] as well as lipase or esterase, aldolase and epoxide hydrolase activity.[11] In this paper, we describe the first fluorimetric test allowing for the detection of Baeyer–Villigerase activity in microbial whole-cells.

Results and discussion

Assay principle

Our strategy was based on the use of ketone **1** as a fluorogenic test substrate. The principle of the procedure is illustrated in Scheme 1. It consists of a two-step process involving: step 1: the biocatalysed Baeyer–Villiger oxidation of **1** by a putative native Baeyer–Villigerase present in the cell (followed by the spontaneous, or enzyme catalysed, hydrolysis of the thus primarily formed acetate **2** into the alcohol intermediate **3**); step 2: oxidation of this alcohol into the aryloxyaldehyde **4**,[12] which, upon spontaneous β-elimination, is known to lead to the fluorescent umbelliferone (**5**). This second step could be performed by using an oxidative enzyme, *i.e.* an alcohol dehydrogenase in the presence of added NADH and of bovine serum albumin (BSA)



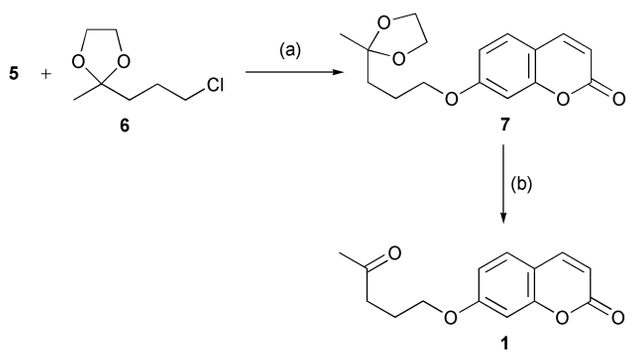
Scheme 1 Principle of the fluorogenic assay: (a) enzymatic BV oxidation; (b) spontaneous or enzymatic hydrolysis; (c) enzymatic (HLADH–NAD^+) or chemical (TEMPO–NaOCl) oxidation; (d) β-elimination.

DOI: 10.1039/b306687h

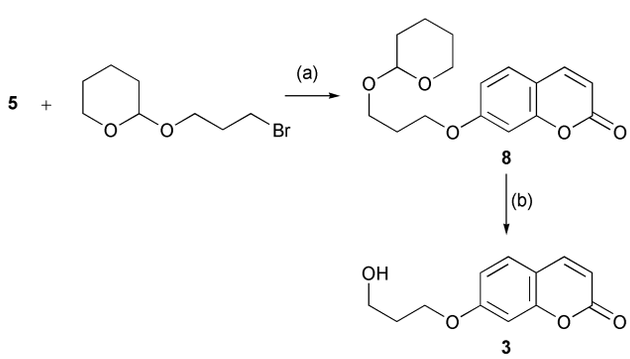
as previously described by Reymond.[10] However, due to the rather slow reaction rate of this second step, we also were interested in exploring the possibility of performing this oxidation using a chemical oxidant, an approach which we hoped would make this second step more efficient, easier and cheaper to carry out on a large scale (*i.e.* for directed evolution approaches implying thousands of strains for example). We here describe the results we have obtained along these lines.

Preliminary experiments

In order to define the most appropriate experimental conditions, we decided to check separately each one of the two steps of the proposed procedure. This necessitated first the synthesis of the test ketone **1**, as well as the preparation of an authentic sample of the (putative) alcohol intermediate **3**. We then studied separately: (a) the biotransformation of **1** using different bacterial strains; (b) the optimisation of the parameters involved in the oxidation of **3**. The test ketone **1**, *i.e.* 7-(4-oxopentoxy)-2*H*-benzopyran-2-one, was synthesised from umbelliferone (**5**) as described in Scheme 2. Compound **3**, *i.e.* 7-(3-hydroxypropoxy)-2*H*-1-benzopyran-2-one, was synthesised by condensation of **5** with tetrahydropyranyl 3-bromopropyl ether, as described in Scheme 3.



Scheme 2 Synthesis of test compound **1**. (a) NaH, DMF, 110 °C, 12 h, N_2, 75%; (b) Amberlite IR-120; acetone, r.t., 81%.



Scheme 3 Synthesis of alcohol **3**. (a) NaH, DMF, 70 °C, 12 h, N_2, 70%; (b) *p*-TsOH, MeOH–H_2O, r.t., 64%.

Step 1: biotransformation of 1 using different bacterial strains

The main drawback of this type of assay is the possibility that the test ketone would not be a substrate for the concerned enzymes. In our case, this point deserved special attention because only a few linear ketones have been described as Baeyer–Villigerase substrates.[5,7,13] Biotransformation of **1** (at a typical 1 mM substrate concentration) was therefore checked with a set of different bacteria previously used for enzymatic BV oxidation.[14–16] These included the wild type strains: *Acinetobacter calcoaceticus* NCIMB 9871,[17] *Acinetobacter sp.* TD63, *Pseudomonas sp.* NCIMB 9872[18] and *Arthrobacter sp.* M5,[19] as well as a recombinant *E. coli* TOP10 [pQR239] host recently constructed to overexpress the well known cyclohexanone monooxygenase (CHMO) from *A. calcoaceticus* NCIMB 9871.[20,21] Analytical (or small preparative scale) biotransformations of **1** using these different bacteria were performed and monitored by HPLC. Our results indicate that **1** was efficiently oxidised by most of these strains. Thus, (a) *Acinetobacter sp.* TD63 afforded a 90% isolated yield of alcohol **3** after 16 hours; (b) *Pseudomonas sp*. NCIMB 9872 produced **3** with an excellent analytical yield (90%) after 7 hours (after 24 hours this alcohol concentration decreased slightly); (c) *Arthrobacter sp*. M5 performed total oxidation of **1** after 4 hours, leading to an 80% analytical yield of **3**. (As in the case of *Pseudomonas sp.*, the concentration of **3** decreased with time and the product disappeared within 24 h). In some cases, traces of the primarily formed ester intermediate **2** were also detected indicating that, as expected, spontaneous hydrolysis was indeed occurring. However, **1** was not oxidised by the wild type strain of *A. calcoaceticus* NCIMB 9871, nor by the recombinant *E. coli* TOP10 [pQR239], indicating that, most probably, **1** is not a substrate for this specific enzyme. Thus, 85% of **1** (analytical HPLC) could be detected after 24 hours biotransformation in spite of the fact that, for both strains, the Baeyer–Villigerase activity was checked to be present against bicyclo[3.2.0]hept-2-en-6-one,[14] a well known substrate of CHMO.

Step 2: oxidation of alcohol 3

As far as the second step of this assay is concerned, two possibilities were envisaged: (a) the use of an enzymatic approach (as already described[11] for the detection of other types of enzymatic reactions); (b) the application of a chemical oxidation process.

Enzymatic oxidation of 3. The possibility of achieving the oxidation of **3** using an alcohol dehydrogenase was explored. Different commercial enzymes (including yeast alcohol dehydrogenases (YAD), horse liver alcohol dehydrogenase (HLADH) as well as the *Lactobacillus kefir* alcohol dehydrogenase) were tested. In all cases but one (the *L. kefir* enzyme), a time-dependent appearance of fluorescence was observed upon oxidation of **3**. HLADH proved to be the most efficient. This was further optimised and it appeared that special attention had to be paid to the buffer composition and pH. Two types of buffers, *i.e.* a 20 mM borate buffer as well as a 100 mM glycine–NaOH buffer, were assayed with the different enzymes. The best results were always found in the latter buffer. Furthermore, experiments conducted in the glycine–NaOH buffer showed that appearance of fluorescence, *i.e.* formation of umbelliferone **5**, occurred within about 1.5 hours at pH 7.7, but was roughly four times faster at pH 9.6. However, the maximum fluorescence intensity obtained at pH 9.6 was not higher than 50% of the theoretical maximum (Fig. 1). Since we have verified that ketone **1** was perfectly stable under these experimental conditions, this discrepancy can be due to either incomplete oxidation of **3** (as confirmed by TLC analysis) and/or to noticeable decomposition of **5**. Test experiments using commercial **5** indeed indicated a decrease of fluorescence of about 25% per hour under these experimental conditions (Fig. 1). Interestingly, however, this decrease slowed down and became negligible after about 2 hours.

Chemical oxidation of 3. As pointed out above, we also were interested in exploring the possibility of performing this second step (oxidation of **3**) by using a chemical reagent instead of an enzyme. An improvement of this step would be of general application to the type of assays described by Reymond *et al.*, whatever enzymatic activity was looked for, *i.e.* lipase, esterase... The problem was, therefore, to find a chemical reactant allowing the oxidation of alcohol **3** into aldehyde **4** in aqueous solution. Moreover, it was necessary to find a method where no over-oxidation of **4** (into the corresponding carboxylic acid) would occur, since this acid itself does not undergo β-elimination.

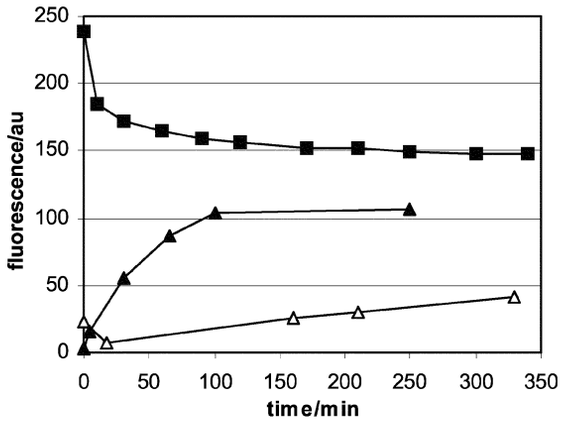


Fig. 1 Fluorescent signal observed upon enzymatic oxidation of alcohol **3**. Fluorescence signal (arbitrary units) observed at λ_{ex} 360 ± 20 nm and λ_{em} 460 ± 20 nm during enzymatic oxidation of alcohol **3** (10 μM) to umbelliferone **5** in a 100 mM glycine–NaOH buffer. HLADH (5 μg mL^{-1}), NAD^+ (1 mM) and BSA (2 mg mL^{-1}): ▲ pH 9.6, △ pH 7.7. Fluorescence signal (arbitrary units) observed, using the same conditions, with 10 μM umbelliferone (**5**): ■ pH 9.6.

Albeit numerous reactants enabling oxidation of a primary alcohol are available, methods allowing the performance of such a reaction in the aqueous phase are surprisingly scarce. Preliminary experiments led us to select the TEMPO–NaOCl oxidative combination.[22] Thus, a bicarbonate buffer (0.5 M, pH 9.2) solution of **3** containing BSA (1.3 mg mL^{-1}) was distributed in a 96 well plate and both a methylene chloride solution of TEMPO and a 3% aqueous sodium hypochlorite solution were added to each well. We thus could observe very fast appearance of fluorescence. This is due to oxidation of **3** followed by spontaneous β-elimination of the intermediate formed (aryloxyaldehyde **4**), thus leading to formation of umbelliferone **5**. Again, optimisation of this process indicated a noticeable influence of the pH value, as well as of the ionic strength, on the reaction rate and signal intensity. Various aqueous (pH 9.2) bicarbonate buffer solutions of different ionic strength were tested, indicating that the reaction rate increased with ionic strength. Oxidation conducted in a bicarbonate buffer of 550 mM concentration showed the highest rate (Fig. 2), whereas use of higher amounts of oxidant (or co-oxidant) did not produce any significant increase of fluorescence. The best experimental conditions were: 550 mM bicarbonate buffer (pH 9.2), 1.3 mg mL^{-1} of BSA, 1.3 mM

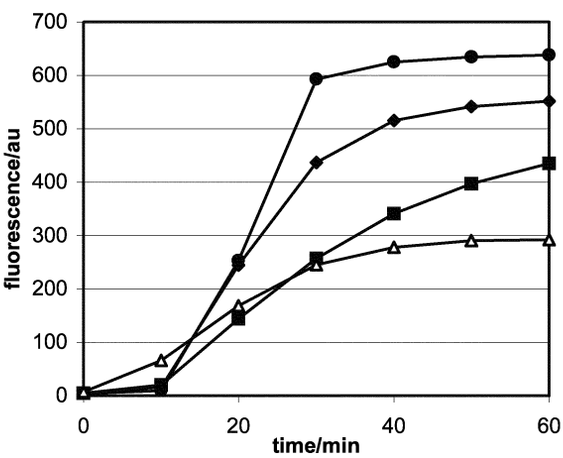


Fig. 2 Fluorescent signal observed upon chemical oxidation of alcohol **3**. Fluorescence signal (arbitrary units) observed upon chemical oxidation of alcohol **3** (0.05 mM), using TEMPO (CH_2Cl_2)–NaClO in bicarbonate buffers (pH 9.2) of different ionic strength: ● 550 mM, ◆ 165 mM, ■ 55 mM. Enzymatic oxidation of alcohol intermediate **3** (0.05 mM), Δ in a 100 mM glycine–NaOH buffer, pH 9.6, HLADH–NAD^+.

solution of TEMPO in CH_2Cl_2 and of a (3%) aqueous NaOCl solution. Test ketone **1** appeared to be stable in these conditions. Using these experimental conditions, chemical oxidation of **3** appeared to be noticeably faster and more efficient than the enzymatic process. This led to a nearly 3 times more intense fluorescent response, this being reached after only 30 minutes as compared to 90 minutes for the enzymatic process. However, as in the above described enzymatic approach, again only 50% of the theoretical fluorescence intensity was reached.

Assay validation

In order to validate this assay, the biotransformation experiments on **1** performed using the five described strains and checked by analytical HPLC as described above, were analysed using the following typical procedure. Biotransformations using wild type bacteria were carried out directly in the culture medium, after the appropriate growth period. In the case of the recombinant strain, harvested cells were centrifuged and re-suspended in the same volume of phosphate buffer (50 mM, pH 7.5); together with 0.05% of glycerol. Ketone **1** was dissolved in the minimum volume of ethanol and was added to each biotransformation medium to obtain a final concentration of 1 mM. Aliquots (1 mL) were withdrawn at different time intervals, centrifuged and frozen. They were thawed at room temperature just before testing and placed into a (polypropylene) 96 well plate (5 μl per well). All samples were tested by performing in parallel both enzymatic and chemical oxidation procedures. The results obtained over a 1.5 hour period are presented in Fig. 3. As can be seen, both types of methodologies, enzymatic or chemical based oxidation, afforded comparable qualitative results. However, as a general feature, chemical oxidation led to a faster and more intense response. The fluorescence intensity increased in all cases up to a maximum, which however was always lower than the value obtained from the control experiment achieved using a 1 mM (50% aqueous MeCN) solution of **3** in the absence of cells. This might be due to adsorption of the produced alcohol inside the cell, which would protect it from oxidation. We have found that addition of inactivated cells into the standard alcohol **3** solution prior to addition of the oxidant indeed led to a noticeable decrease of the fluorescence signal intensity. As can be seen in Fig. 3, all samples of **1** biotransformed by *Acinetobacter sp.* TD63 and *Pseudomonas sp*. NCIMB 9872 produced intense fluorescent responses due to efficient biooxidation of **1** by these strains. Fluorescence appearance, as well as decay, was in perfect agreement with the results obtained by HPLC analysis. Thus, samples obtained upon biotransformation of **1** by *Arthrobacter sp.* led to a signal whose intensity decreased after 3 hours and totally disappeared after 24 hours. Blank experiments conducted using the non-induced cells (lacking this enzymatic activity) did not exhibit a noticeable fluorescent signal. As expected from HPLC analysis, the recombinant *E. coli* TOP10 [pQR239] bacteria overexpressing the well known CHMO enzyme, did not lead to any noticeable fluorescent response, due to the fact that **1** is not oxidised by this enzyme. This result interestingly illustrates the potential bottleneck of any such screening assay based on the use of a specific test compound which, as a general feature, can lead to so called "false negative answers" if the test compound is not recognised as a substrate by a specific enzyme. In principle, another limitation of such an assay could of course be the fact that other types of enzymes would lead to "false positive responses". Although this hypothesis cannot be totally eliminated, this seems highly unlikely due to the two-step nature of this assay, where appearance of fluorescence (in step 2) necessitates prior formation (in step 1) of a fluorogenic (and stable) intermediate, *i.e.* the alcohol **3**. Fluorescence detected in the first step is not considered as a positive result. In any case, further more detailed analysis is

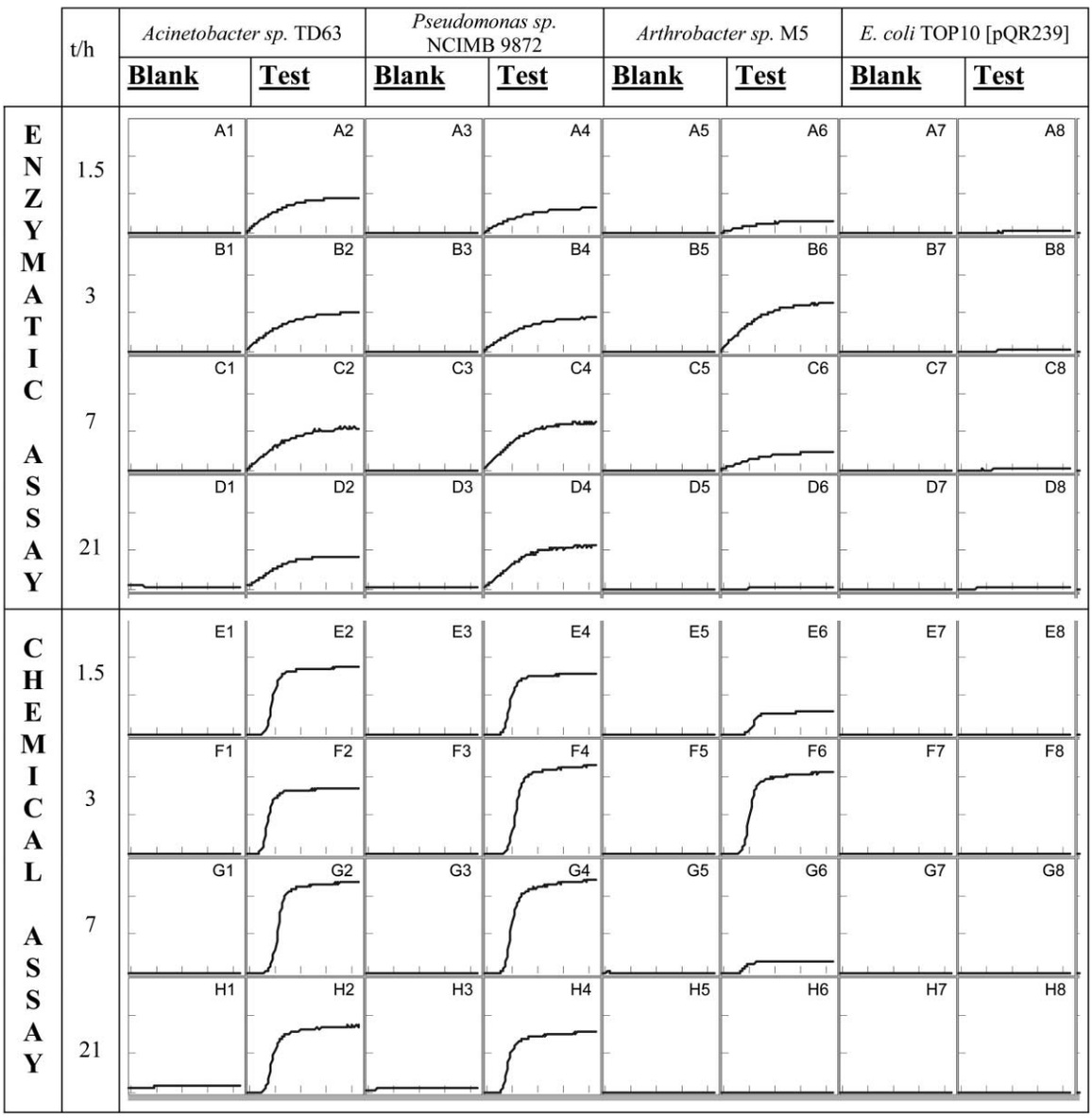


Fig. 3 On line observation of fluorescence appearance in a 96 well plate. Fluorescence signal (arbitrary units) observed: Lines A–D: upon enzymatic oxidation. Lines E–F: upon chemical oxidation. Columns 1, 3, 5, 7: blank experiments. Columns 2, 4, 6, 8: test experiments.

obviously needed once positive answers are obtained in any such assay.

Conclusion

We have described in this work the first fluorimetric assay allowing detection of Baeyer–Villigerase activity displayed by whole bacterial cells. This two-step assay is based on the use of the test ketone **1** which we have selected and synthesised. In this context, we have set up a new methodology which allows the replacement of the previously described enzymatic methodology, based on the use of an alcohol dehydrogenase (HLADH) catalysed oxidation, by a chemical oxidation methodology, thus leading to an about three times faster and more intense response. We have shown that, when applied to bacterial strains known to be able to perform the Baeyer–Villiger oxidation of the test ketone **1**, *i.e.*: *Acinetobacter sp.* TD63, *Pseudomonas sp.* NCIMB 9872 and *Arthrobacter sp.* M5, it was possible to detect this enzymatic activity. Concentrations as low as 50 μM of test alcohol **3** proved to be sufficient for qualitative detection. The use of cheap chemical reactant and co-oxidant (aqueous NaOCl solution) is an additional advantage of this method. This (first) fluorogenic assay clearly could be of value in the development of high throughput screening (HTS) strategies aimed at detecting novel Baeyer–Villigerase activities in large collections of microorganisms.

Experimental

General procedures and materials

Gas chromatography analyses were performed with a Shimadzu GC-14A chromatograph equipped with an Optima 5 fused silica capillary column (diameter 0.5 μm, length 30 m) (Macherey-Nagel GmbH & Co., Duren, Germany) with helium as the carrier gas. HPLC analyses were achieved in a Shimadzu chromatograph equipped with a UV-detector ($\lambda = 310$ nm) and a Hypersil DBS C_{18} (250 × 4.6 mm, 5 μ) column using acetonitrile–H_2O (50%, 0.5 mL min^{-1}) as eluent. Fluorescence measurements were performed in individual HTS multiwell™ storage plates, each containing 96 U bottomed wells made of polypropylene (Costar and Falcon) with a Cytofluor Series 4000 (Perseptive Biosystems, filters λ_{ex} 360 ± 20 nm, λ_{em} 460 ± 20 nm) and a Fluoroskan Ascent FL (Labsystems type 374, filters λ_{ex} 360 ± 20 nm, λ_{em} 460 ± 20 nm) fluorescence-plate reader. Melting points were determined on a Büchi apparatus

and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 250 spectrometer in $CDCl_3$ solutions. Chemical shifts (δ) are quoted in ppm with Me_4Si as reference and J values are in Hz. Microorganisms were cultured in a 2 L fermentor (Setric).

The overexpressing strain *E. coli* TOP10 [pQR 239], the wild type strains *Acinetobacter sp.* TD63 and *Arthrobacter sp*. M5 were generous gifts from Professor J. Ward (University College, London), Professor P. W. Trudgill (University College of Wales) and Professor D. B. Janssen (University of Groningen), respectively. The wild type strains *Acinetobacter calcoaceticus* NCIMB 9871 and *Pseudomonas sp.* NCIMB 9872 were obtained from the National Collection of Industrial and Marine Bacteria (UK). Stock cultures were grown on nutrient agar at 30 °C and stored at 4 °C, except the recombinant strain, kept at −20 °C in a 40% glycerol solution.

The alcohol dehydrogenases from *Lactobacillus kefir* (05643), HLADH (05645) and YADH (05640), NAD^+, BSA, 5-chloro-2-pentanone, 3-[(tetrahydro-2*H*-pyran-2-yl)oxy]propyl bromide and umbelliferone were purchased from Fluka, baker's yeast alcohol dehydrogenase (A-7011) and 2,2,6,6-tetramethyl-1-piperidinyloxyl free radical (TEMPO) from Sigma-Aldrich. Solvents were distilled prior to use. Reactions with NaH were carried out under nitrogen atmosphere.

Syntheses

2-(3-Chloropropyl)-2-methyl-1,3-dioxolane (6). A mixture of 5-chloro-2-pentanone (19.6 g, 158 mmol), ethylene glycol (49.7 g, 800 mmol) and *p*-toluenesulfonic acid monohydrate (300 mg, 1.6 mmol) was heated in 500 mL of toluene under reflux with a Dean–Stark trap for 24 hours. The mixture was then washed with 10% aq. $NaHCO_3$ solution (3 × 30 mL), followed by brine (3 × 30 mL). The organic phase was dried over anhydrous Na_2SO_4. After filtration the solvent was removed under reduced pressure and the residual liquid was distilled to give 24 g (92%) of compound **6**: colorless liquid; δ_H(250 MHz; $CDCl_3$; Me_4Si): 1.35 (3 H, s, C*H*$_3$), 1.85 (4 H, m, C*H*$_2$-C*H*$_2$), 3.60 (2 H, t, *J* 7, C*H*$_2$Cl), 3.95 (4 H, m, OC*H*$_2$C*H*$_2$O).

7-[(4,4-Ethylenedioxy)pentoxy]-2*H*-1-benzopyran-2-one (7). A mixture of 7-hydroxy-2*H*-1-benzopyran-2-one (**5**) (8.4 g, 52 mmol), NaH (1.7 g, 70 mmol, 60% suspension in oil) and 2-(3-chloropropyl)-2-methyl-1,3-dioxolane (**6**) (8.5 g, 52 mmol) was heated in 50 mL anhydrous DMF for 12 h at 110 °C under nitrogen atmosphere. The residual NaH was then destroyed by a few drops of water and the mixture was evaporated at reduced pressure. The residue was diluted with 200 mL AcOEt and was washed with 10% NaOH solution (3 × 20 mL) and then with brine (3 × 20 mL). The organic phase was dried over anhydrous Na_2SO_4, then filtrated and evaporated. A crude solid was obtained and the recrystallisation gives 11.3 g (75%) of compound **7**: colourless solid, mp 86 °C (from MeOH); (Found: C 65.85; H 6.23. Calc. for $C_{16}H_{18}O_5$: C 66.23; H 6.20%); ν_{max} (CCl_4)/cm^{-1}: 2980, 1740, 1600, 1275, 1120, 830; δ_H(250 MHz; $CDCl_3$; Me_4Si): 1.38 (3 H, s, C*H*$_3$), 1.90 (4 H, m, C*H*$_2$-C*H*$_2$), 3.98 (4 H, m, OC*H*$_2$-C*H*$_2$O), 4.05 (2 H, t, *J* 6.0, C*H*$_2$O), 6.25 (1 H, d, *J* 9.5, H-3), 6.80 (1 H, d, *J* 8.3, H-6), 6.84 (1 H, s, H-8), 7.38 (1 H, d, *J* 8.3, H-5), 7.65 (1 H, d, *J* 9.5, H-4).

7-(4-Oxopentoxy)-2*H*-1-benzopyran-2-one (1). 6.6 g (23 mmol) of 7-[(4,4-ethylenedioxy)pentoxy]-2*H*-1-benzopyran-2-one (**7**) and 28 g of acid Amberlite IR 120 were stirred in 200 mL of acetone at room temperature for 12 h. The mixture was then filtered and the liquid phase was evaporated at reduced pressure. Recrystallisation of the residue yields 4.6 g (81%) of compound **1**: white solid, mp 79 °C (toluene); (Found: C 68.35; H 5.82. Calc. for $C_{14}H_{14}O_4$: C 68.31; H 5.69%); ν_{max} (CCl_4)/cm^{-1}: 2980, 1740, 1710, 1600, 1275, 1120, 830; δ_H(250 MHz; $CDCl_3$; Me_4Si): 2.10 (2 H, m, C*H*$_2$), 2.20 (3 H, s, C*H*$_3$), 2.69 (2 H, t, *J* 7.0, CO-C*H*$_2$), 4.04 (2 H, t, *J* 6.0, C*H*$_2$O), 6.25 (1 H, d, *J* 9.5, H-3), 6.80 (1 H, d, *J* 8.3, H-6), 6.84 (1 H, s, H-8), 7.38 (1 H, d, *J* 8.3, H-5), 7.65 (1 H, d, *J* 9.5, H-4); δ_C(62.5 MHz; $CDCl_3$; Me_4Si): 208, 162, 161, 156, 144, 129, 113, 113, 112, 101, 68, 40, 30, 23.

7-{3-[(Tetrahydro-2*H*-pyran-2-yl)oxy]propoxy}-2*H*-1-benzopyran-2-one (8). A mixture of 7-hydroxy-2*H*-1-benzopyran-2-one (**5**) (2.92 g, 18 mmol), NaH (0.6 g, 25 mmol, 60% suspension in oil) and 3-[(tetrahydro-2*H*-pyran-2-yl)oxy]propyl bromide (4.0 g, 18 mmol) was heated in 10 mL anhydrous DMF under nitrogen atmosphere for 12 h at 70 °C. Residual metal hydride was then hydrolysed with a few drops of water. The mixture was diluted with 100 mL of AcOEt and the organic layer was washed with 3 × 10 mL of 10% NaOH aq. solution and 3 × 10 mL of brine. The organic phase was then dried over anhydrous Na_2SO_4 and the solvent was removed at reduced pressure. Chromatography on a silica gel column yielded 4.0 g (70%) of compound **8**: colourless oil; ν_{max} (CCl_4)/cm^{-1}: 2920, 1730, 1600, 1110, 1020; δ_H(250 MHz; $CDCl_3$; Me_4Si): 1.8–1.5 (6 H, m, C*H*$_2$-cycle), 2.10 (2 H, m, C*H*$_2$), 3.60 (2 H, m, OC*H*$_2$-cycle), 3.90 (2 H, m, C*H*$_2$O), 4.60 (1 H, br s, O$_2$C*H*), 6.25 (1 H, d, *J* 9, H-3), 6.80 (1 H, d, *J* 8, H-6), 6.85 (1 H, s, H-8), 7.37 (1 H, d, *J* 8, H-5), 7.65 (1 H, d, *J* 9, H-4).

7-(3-Hydroxypropoxy)-2*H*-1-benzopyran-2-one (3). 3.0 g (10 mmol) of 7-{3-[(tetrahydro-2*H*-pyran-2-yl)oxy]propoxy}-2*H*-1-benzopyran-2-one (**8**) and 190 mg (1 mmol) of *p*-toluenesulfonic acid monohydrate were dissolved in 40 mL of MeOH–H_2O (95 : 5). The mixture was stirred at room temperature for 12 h. 0.8 mL of triethylamine were then added to neutralise the mixture and the solvent was evaporated under reduced pressure. The residue was diluted with 100 mL of AcOEt and the organic layer was washed with 3 × 10 mL of 10% NaOH aq. solution and also with 3 × 10 mL of brine. The organic phase was dried over anhydrous Na_2SO_4, then filtrated and evaporated. Recrystallisation afforded 1.4 g (64%) of compound **3**. Colorless solid, mp 79 °C (toluene); ν_{max} (CCl_4)/cm^{-1}: 3400 (bb), 2920, 1730, 1670, 1600, 1340, 1100; δ_H(250 MHz; $CDCl_3$; Me_4Si): 1.74 (1 H, br t, O*H*), 2.09 (2 H, m, C*H*$_2$), 3.87 (2 H, m, C*H*$_2$-OH), 4.18 (2 H, t, *J* 6, C*H*$_2$-O), 6.25 (1 H, d, *J* 9, H-3), 6.84 (1 H, d, *J* 8, H-6), 6.86 (1 H, s, H-8), 7.36 (1 H, d, *J* 8, H-5), 7.64 (1 H, d, *J* 9, H-4); δ_C(62.5 MHz; $CDCl_3$; Me_4Si): 162, 161, 156, 144, 129, 113, 113, 112, 101, 66, 60, 32.

7-(3-Acetoxypropoxy)-2*H*-1-benzopyran-2-one (2). A mixture of 7-(3-hydroxypropoxy)-2*H*-1-benzopyran-2-one (**3**, 50 mg, 0.23 mmol), acetic anhydride (0.5 mL), glacial acetic acid (2 mL) and *p*-toluenesulfonic acid monohydrate (6 mg) was stirred for 16 h at 60 °C. The reaction mixture was then evaporated to dryness and the crude product was chromatographed on a silica gel column to obtain 40 mg (67%) of compound **2**. Colourless oil; δ_H(250 MHz; $CDCl_3$; Me_4Si): 2.10 (3 H, s, COC*H*$_3$), 2.18 (2 H, q, *J* 6, C*H*$_2$), 4.10 (2 H, t, *J* 6, C*H*$_2$-O), 4.25 (2 H, t, *J* 6, C*H*$_2$-OCO), 6.25 (1 H, d, *J* 9, H-3), 6.80 (1 H, d, *J* 8, H-6), 6.85 (1 H, s, H-8), 7.37 (1 H, d, *J* 8, H-5), 7.64 (1 H, d, *J* 9, H-4); δ_C(62.5 MHz; $CDCl_3$; Me_4Si): 170, 162, 161, 156, 144, 129, 113, 113, 112, 101, 66, 60, 28, 21.

Microbial culture conditions

Recombinant *Escherichia coli*. *E. coli* TOP10 [pQR 239] contains a pBAD plasmid into which the CHMO gene from *Acinetobacter calcoaceticus* NCIMB 9871 has been cloned. The expression of the CHMO gene was induced by L-(+)-arabinose. The recombinant bacterium was cultivated essentially as described previously.[14,20] The medium (pH 6.8) contained 10 g L^{-1} each of glycerol, yeast extract, peptone, NaCl, and 100 mg L^{-1} of ampicillin. A 2 L fermentor was filled with 1 L of culture medium and was inoculated with 50 mL of a 12 h pre-culture at

37 °C. The fermentor was stirred at 500 rpm with an air flow of 16 L h^{-1}. L-(+)-arabinose (0.05% w/v) was added to induce the enzymatic activity into the exponential growth phase (A_{600}: 2–3). After a further 4 h growth period the cells were harvested by centrifugation at 4 °C and used for biotransformation.

Wild type strains: *A. calcoaceticus* NCIMB 9871, *Acinetobacter sp.* TD63, *Arthrobacter sp.* M5 and *Pseudomonas sp.* NCIMB 9872. The culture media were a 1 L typical minimal mineral medium (4 g Na_2HPO_4, 2 g KH_2PO_4, 3 g $(NH_4)_2SO_4$, 0.1 g $CaCl_2$, 0.01 g $FeSO_4$, 1 mL trace elements solution and 0.2 g yeast extract, pH 7) supplemented with 1.5 g of 1,2-cyclohexanediol (*Acinetobacter* strains) or 1.5 g of cyclopentanol (*Pseudomonas sp.*) or 0.5 g of acetophenone (*Arthrobacter sp.*) as the only carbon source. After inoculation with a 6–24 h preculture, cells were grown for 15–20 h at 30 °C.

Biotransformation conditions

Biotransformations using wild type bacteria were carried out directly in the culture medium, after a growth period. In the case of *E. coli* TOP10 [pQR 239], harvested cells were centrifuged and re-suspended in the same volume of phosphate buffer (50 mM, pH 7.5). An addition of glycerol (0.05%) was also necessary for biotransformations.[14]

Analytical experiments. The biotransformations were routinely performed on a 50 mL scale in 250 mL Erlenmeyer flasks in an agitated water bath maintained at 30 °C. Ketone **1** was pre-dissolved in the minimum volume of ethanol and was added to the biotransformation medium to obtain a final concentration 1 mM. 1 mL aliquots of the biotransformation media were taken at the different biocatalysis times and cells were removed by centrifugation. The biooxidations were monitored by HPLC analysis of samplings. After addition of methanol, the supernatant was filtrated over a 0.45 μm Teflon filter. Quantitative evaluation of ketone and alcohol concentration was determined using a calibration curve.

Preparative scale experiments. Preparative scale biotransformations were achieved in a 2 L fermentor using experimental conditions identical to those used for the culture. Each 0.5 g of ketone **1**, dissolved in a minimum volume of EtOH, was added into the whole cell media of *Acinetobacter sp.* TD63 and *A. calcoaceticus* NCIMB 9871, respectively. After 16 h, the biotransformation with *Acinetobacter sp.* TD63 cells was stopped and continually extracted using AcOEt. The extracts were dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure to yield 0.4 g (90%) of alcohol **3**. On the other hand, after 24 h no oxidation of test ketone **1** was observed for the biotransformation by *A. calcoaceticus* NCIMB 9871.

Fluorescence measurements

Standard solutions, 1 mM, of test ketone **1**, alcohol **3** and umbelliferone **5** were diluted from the 10 mM stock solutions in 50% aq. MeCN. Enzymes were also diluted from the commercial solutions in the different buffers to prepare different concentration solutions. 100 mM borate buffer, pH 8.8; 100 mM glycine–NaOH buffers, pH 9.6 and pH 7.7; 55 mM, 165 mM and 550 mM bicarbonate buffers, pH 9.2; and 3% NaClO aq. solution were prepared. BSA and NAD^+ were diluted into the borate and glycine–NaOH buffers to a final concentration 2 mg mL^{-1} and 0.7 mg mL^{-1}, respectively, in the enzymatic assays. BSA was also diluted into the bicarbonate buffers to a final concentration 1.3 mg mL^{-1} and 1.3 mM TEMPO solution in dichloromethane was prepared in the chemical assays. The 200 or 100 μL assays were followed in individual "U" bottomed wells of polypropylene 96 well plates, with a fluorescence plate-reader (filters λ_{ex} 360 ± 20 nm, λ_{em} 460 ± 20 nm) at 1200 rpm.

Calibration experiments. In order to ascertain the accuracy of our calculation of umbelliferone **5** concentration in the selected experimental conditions, we verified that the fluorescence intensity could be converted into concentration values according to a calibration curve.

Standard solutions, 0.04, 0.1, 0.2, 0.4, 1, 2 and 5 mM, of umbelliferone (**5**) were prepared from the 10 mM stock solution of **5** in 50% aq. MeCN. The fluorescent responses were analysed in both the test media. Thus it appeared in both media that, up to about 20 μM, the relation was linear. However, higher concentration values led to a non-proportional increase of fluorescence signals due to inherent quenching. Thus, determination of the accurate concentration of **5** became increasingly imprecise at concentrations between 20 and 100 μM.

Typical procedure for the enzymatic assay (100 μL). 90 μL of NAD^+–BSA solution and 5 μL of HLADH solution (0.1 mg mL^{-1}) were added in this order into each well containing: 5 μL of 1 mM alcohol **3** standard solution (*control experiments*) or 5 μL of an aliquot of the biotransformation media (*test experiments*). In *blank experiments* each well also contained 5 μL of an aliquot of the biotransformation media, but no addition of the HLADH solution was performed and 5 μL of 100 mM glycine–NaOH buffer solution were added in its place. The 96 well plate was incubated (25 °C, 1200 rpm), and fluorescence output was measured. After 90 min, the maximum fluorescence was reached.

Typical procedure for the chemical assay (100 μL). 45 μL of 1.3 mM TEMPO solution, then 45 μL of 550 mM bicarbonate buffer (containing BSA, 1.3 mg mL^{-1}) and 5 μL of 3% aq. NaOCl solution were added in this order into each individual well containing: 5 μL of 1 mM alcohol **3** standard solution (*control experiments*) or 5 μL of an aliquot of the biotransformation media (*test experiments*). In *blank experiments* the wells also contained 5 μL of an aliquot of the biotransformation medium, but the oxidant and co-oxidant solutions were not added, so 45 μL of CH_2Cl_2 and 5 μL of distilled water were added in replacement. The multi-well plate was agitated (1200 rpm, 25 °C) and fluorescence was measured. After 30 min, the chemical reaction was finished and the maximum fluorescent response was reached.

Acknowledgements

This work was partly funded by the Project BIO4-CT98-0267 and QLK3-CT2001-00403 as part of the European Community Framework Programmes. We would like to express our thanks to Professor J. Ward, Professor P. W. Trudgill and Professor D. B. Janssen for having provided us with the bacterial strains. One of the authors (M.C.G.) would like to express her gratitude to the Foundation Ramón Areces for providing a postdoctoral fellowship to carry out the research work.

References

1 See, for instance: D. C. Demirjian, P. C. Shah, F. Morís-Varas, in *Biocatalysis: from Discovery to Application*, Ed. W. C. Fessner, Springer, Berlin, 1999, vol. 200, pp. 1–29.
2 M. Olsen, B. Iverson and G. Georgiou, *Curr. Opin. Biotechnol.*, 2000, **11**, 331–337.
3 D. Wahler and J. L. Reymond, *Curr. Opin. Biotechnol.*, 2001, **12**, 535–544.
4 For a recent publication in chemical asymmetric Baeyer–Villiger oxidation see, for example: A. Watanabe, T. Uchida, K. Ito and T. Katsuki, *Tetrahedron Lett.*, 2002, **43**, 4481–4485 and references therein.
5 V. Alphand, R. Furstoss, in *Enzyme Catalysis in Organic Synthesis: a Comprehensive Handbook*, Ed. K. Danz, H. Waldmann, VCH, Weinheim, 1995, vol. 2, pp. 745–772.

6 A. Willetts, *Trends Biotechnol.*, 1997, **15**, 55–62.
7 M. D. Mihovilovic, B. Müller and P. Stanetty, *Eur. J. Org. Chem.*, 2002, **22**, 3711–3730.
8 V. Alphand, G. Carrea, R. Wohlgemuth, R. Furstoss and J. M. Woodley, *Trends Biotechnol.*, 2003, **21**, 318–323.
9 A. B. Watts, J. Beecher, C. S. Whitcher and J. A. Littlechild, *Biocatal. Biotransfor.*, 2002, **20**, 209–214.
10 G. Klein and J. L. Reymond, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 1113–1116.
11 D. Wahler and J. L. Reymond, *Curr. Opin. Chem. Biol.*, 2001, **5**, 152–158.
12 R. Pérez-Carlón, N. Jourdain and J. L. Reymond, *Chem. Eur. J.*, 2000, **6**, 4154–4162.
13 N. M. Kamerbeek, A. J. J. Olsthoorn, M. W. Fraaije and D. B. Janssen, *Appl. Environ. Microbiol.*, 2003, **69**, 419–426.
14 H. D. Simpson, V. Alphand and R. Furstoss, *J. Mol. Catal. B: Enzym.*, 2001, **16**, 101–108.
15 V. Alphand and R. Furstoss, *J. Org. Chem.*, 1992, **57**, 1306–1309.
16 M. C. Gutiérrez, V. Alphand and R. Furstoss, *J. Mol. Catal. B: Enzym.*, 2003, **21**, 231–238.
17 N. A. Donoghue and P. W. Trudgill, *Eur. J. Biochem.*, 1975, **60**, 1–7.
18 M. Griffin and P. W. Trudgill, *Biochem. J.*, 1972, **129**, 595–603.
19 J. Havel and W. Reineke, *Appl. Environ. Microbiol.*, 1993, **59**, 2706–2712.
20 S. D. Doig, L. M. O'Sullivan, S. Patel, J. M. Ward and J. M. Woodley, *Enzyme Microb. Technol.*, 2001, **28**, 265–274.
21 S. D. Doig, H. Simpson, V. Alphand, R. Furstoss and J. M. Woodley, *Enzyme Microb. Technol.*, 2003, **32**, 347–355.
22 A. E. J. de Nooy, A. C. Besemer and H. van Bekkum, *Synthesis*, 1996, 1153–1174.