# Coupling of permeabilized microorganisms for efficient enantioselective reduction of ketone with cofactor recycling†

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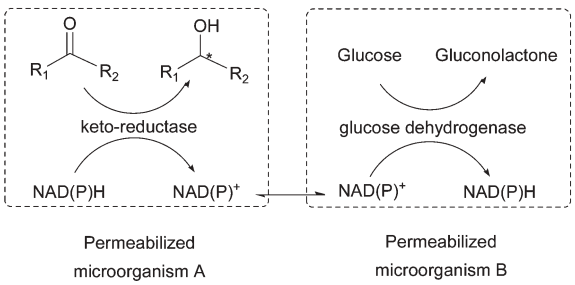
**A novel, simple and efficient cofactor recycling method for enantioselective bioreduction has been developed by the use of permeabilized cells of a reductase-containing microorganism and a glucose dehydrogenase-containing microorganism.**

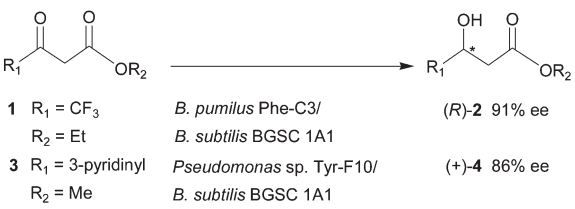
Enantioselective enzymatic oxidations and reductions are important reactions in asymmetric synthesis and industrial production of enantiopure synthons and pharmaceutical intermediates. A major challenge for practical application is the need for a stoichiometric amount of the expensive nicotinamide cofactor NAD(P)H or $NAD(P)^+$ involved in most enzyme-catalyzed oxidoreductions. This need is generally met by an additional chemical, electrochemical, or enzymatic reaction that regenerates the necessary cofactor,[1] with enzymatic regeneration approaches being favored. In the "coupled substrates" concept, a single alcohol dehydrogenase is used for the reduction of a carbonyl-containing substrate and for the simultaneous oxidation of an alcohol co-substrate to regenerate NAD(P)H.[2–3] In the "coupled enzymes" concept, a second enzyme is utilized for cofactor regeneration, such as formate dehydrogenase[4] and glucose dehydrogenase (GDH)[5] for the regeneration of NADH and NADPH, respectively. These concepts have been applied with isolated enzymes[3–5] as well as whole cells containing necessary enzyme(s).[2,6] While approaches based on isolated enzymes are expensive, approaches based on whole cells depend on the amount of available intracellular cofactor which may be limiting and cannot be altered by the addition of extracellular cofactor.

The cytoplasmic membrane of a microorganism can be made permeable for NAD(P)H and $NAD(P)^+$ without the release of the desired enzyme by controlled treatment with an organic solvent.[7–8] Such permeabilized cells in essence constitute an *in vitro* biocatalysis system, with one or more required enzymes "immobilized" inside the permeable cell. The use of these previously permeabilized cells for biotransformation is advantageous: compared with whole cells, permeabilized cells enable the use of the externally added cofactor for efficient catalysis and cofactor recycling, easy substrate access, and easy product release; compared with the isolated enzyme, permeabilized cells are cheap, easily available in large amounts, and active for a longer period. Despite these obvious attractions of permeabilized cells as biocatalysts, there are only a few examples of using them for bioreductions and cofactor recycling. These include the use of a permeabilized microorganism coupled with an isolated enzyme[9] and of a single permeabilized microorganism containing two necessary enzymes.[10–11] While the former required an isolated enzyme, the latter gave a very low total turnover number (TTN) of the cofactor. Here we describe a new concept based on "coupled permeabilized microorganisms" for efficient enantioselective reduction with cofactor recycling (Scheme 1): permeabilized organisms A and B are utilized for the desired reduction and cofactor regeneration, respectively; and the cofactor is added externally and recycled in such a system.

To demonstrate the principle, enantioselective reduction of ethyl 3-keto-4,4,4-trifluorobutyrate **1** giving the hydroxyester (*R*)-**2** was selected as the target reaction (Scheme 2), since the product is a useful intermediate for the preparation of the antidepressant Befloxatone.[12] *Bacillus pumilus* Phe-C3 was chosen as the reduction microorganism. This strain was found to contain a NADPH-dependent ketoreductase that catalyzed the reduction of **1** giving (*R*)-**2** in 91% ee.[13] *Bacillus subtilis* BGSC 1A1 containing a glucose dehydrogenase (GDH)[14] was used as the cofactor-regeneration microorganism. GDH catalyzes the oxidation of glucose to gluconolactone while regenerating NAD(P)H from $NAD(P)^+$. The initial product gluconolactone is spontaneously hydrolyzed to gluconate. This *non*-enzymatic hydrolysis makes the overall reaction strongly exothermic and thus favorable for the regeneration of NAD(P)H. To permeabilize the cells,[7]




**Scheme 1**




**Scheme 2**

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**Table 1** Reduction of **1** to (*R*)-**2** and of **3** to (+)-**4** with NADPH recycling by the use of two permeabilized microorganisms[a]

| Sub. | Phe-C3 [g/L] | Tyr-F10 [g/L] | BGSC 1A1 [g/L] | Glucose [mM] | Activity[b] [U/g cdw] | Prod. | Yield[c] [%] |
|---|---|---|---|---|---|---|---|
| **1** | 6.7[d] | | | 55 | 5.4 | (*R*)-**2** | 46 |
| **1** | 6.7 | | 13 | 55 | 4.8 | (*R*)-**2** | 75 |
| **1** | 6.7 | | 27 | 55 | 6.9 | (*R*)-**2** | 79 |
| **1** | 6.7 | | 27 | 110 | 7.0 | (*R*)-**2** | 96 |
| **3** | | 6.7[d] | | 55 | 7.5 | (+)-**4** | 75 |
| **3** | | 6.7 | 13 | 55 | 5.9 | (+)-**4** | 86 |
| **3** | | 6.7 | 27 | 55 | 7.1 | (+)-**4** | 87 |
| **3** | | 6.7 | 27 | 110 | 7.2 | (+)-**4** | 98 |

[a] The reaction was carried out in 10 mL Tris-buffer (pH = 7.0) with substrate (20 mM), $NADP^+$ (0.20 mM), and glucose at different concentrations. [b] Activity was determined over the first 30 min. [c] Yield at 72 h for (*R*)-**2** and at 48 h for (+)-**4**, respectively. [d] Biotransformation with whole cells.

microorganisms were exposed to 5–7% toluene and 5 mM EDTA for 30 min (*B. pumilus* Phe-C3) and 3 h (*B. subtilis* BGSC 1A1), respectively.‡ Permeabilized cells were harvested by centrifugation, a procedure which removed toluene, EDTA, and also the cofactor originally present in the cells. Permeabilized *B. subtilis* BGSC 1A1 cells (6.7 g cdw/L) showed a specific activity of 5.0 U/g cdw (U = μmol/min, cdw = cell dry weight) for the generation of NADPH from $NADP^+$ (2 mM) by oxidation of glucose (55 mM). On the other hand, permeabilized *B. pumilus* Phe-C3 (6.7 g cdw/L) demonstrated an activity of 9.7 U/g cdw for the reduction of **1** (20 mM) in the presence of NADPH (20 mM). Therefore, permeabilized *B. pumilus* Phe-C3 and *B. subtilis* BGSC 1A1 were combined in a mass ratio of 1 : 2 or 1 : 4 for efficient cofactor regeneration and bioreduction of **1** (20 mM). The less expensive form of the cofactor, $NADP^+$ (0.20 mM), was added for the generation and regeneration of NADPH in the presence of glucose (55 or 110 mM). As shown in Table 1, bioreduction with permeabilized cells gave higher yields than that with the equivalent whole cells. The specific activity increased with an increasing cell mass ratio of *B. subtilis* BGSC 1A1, and the final product yield was also dependent on the amount of glucose added. Some NADPH oxidase activity was found in permeabilized cells, as a result of which an excess of glucose is required for the regeneration of more NADPH to complete the reduction of **1**. (*R*)-**2** was formed in 96% yield (19.2 mM) and 91% ee with a cell ratio of 1 : 4, 0.20 mM $NADP^+$, and 110 mM glucose. This corresponds to a cofactor TTN of 96.

To demonstrate the generality of this approach, we selected the enantioselective reduction of methyl 3-keto-(3′-pyridyl)-propionate **3** as the new target (Scheme 2). The corresponding enantiopure hydroxyester **4** is a useful intermediate for the preparation of the GPIIb/IIIa antagonist RWJ-5330830.[15] *Pseudomonas* sp. Tyr-F10 was found to catalyze this reduction giving (+)-**4** in 87% ee,[13] thus being chosen as the reduction microorganism. Since the responsible ketoreductase is also dependent on NADPH, *B. subtilis* BGSC 1A1 was used again for the cofactor regeneration. Permeabilized cells of *Pseudomonas* sp. Tyr-F10 were prepared according to the procedure used for *B. pumilus* Phe-C3,‡ and bioreduction of **3** (20 mM) with two coupled microorganisms was performed in the same conditions as described for the reduction of **1**. As shown in Table 1, the reduction of **3** with the permeabilized microorganisms also resulted in higher yields than that of the whole cell transformation. (+)-**4** was obtained in 86% ee and 98% yield (19.6 mM) with a TTN of 98 for NADPH. This result demonstrated the generality of the "coupled permeabilized microorganisms" approach and the general usefulness of *B. subtilis* BGSC 1A1 as cofactor-regenerating microorganism in this approach.

To increase the TTN of the cofactor and product concentration, bioreduction of **1** was performed with higher cell densities of permeabilized *B. pumilus* Phe-C3 (20 g cdw/L) and *B. subtilis* BGSC 1A1 (40 g cdw/L), higher substrate concentration (120 mM), and excess amount of glucose (1.7 M) added at different time points. With the initial addition of 0.20 mM $NADP^+$, cofactor TTN of 435 and a product concentration of 87 mM were obtained at 30 h. As shown in Fig. 1 A–C, decrease of the initial $NADP^+$ concentration from 0.20 to 0.12 mM had nearly no influence on the reduction and gave (*R*)-**2** in 82 mM. This, however, increased the cofactor TNN to 683. Further decrease of the initial concentration of $NADP^+$ to 0.04 mM reduced the product concentration to 39 mM, but led to a cofactor TTN of 975 at 30 h. Bioreduction with an initial $NADP^+$ concentration of 0.01 mM for 43 h further increased the TTN to 1620 (Fig. 1F).§ Obviously, higher TTN could be achieved by using even less $NADP^+$. To increase the product concentration under a given high cofactor TTN, new portions of cofactor may be added to continue the reduction. Moreover, higher product concentration and higher cofactor TTN were obtained by performing the biotransformation for a longer period with more glucose and substrate, as demonstrated in Fig. 1 G *vs.* C. Bioreduction of **1** (140 mM) with 0.12 mM $NADP^+$ added initially and 3.6 M glucose added at different time points afforded (*R*)-**2** in 114.4 mM (21 g/L) and a TTN of NADPH of 953 at 68 h.

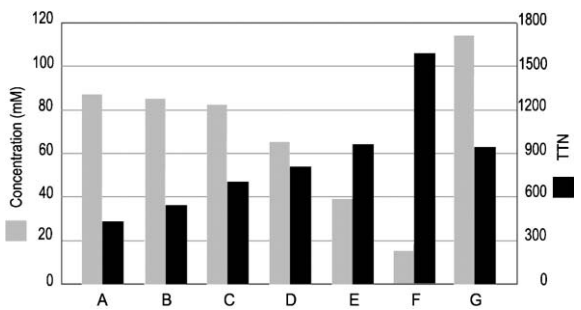


**Fig. 1** TTN of NADPH and concentration of (*R*)-**2** in the reduction of ethyl 3-keto-4,4,4-trifluorobutyrate **1** with permeabilized cells of *B. pumilus* Phe-C3 (20 g cdw/L) and *B. subtilis* BGSC 1A1 (40 g cdw/L). A–E: 120 mM **1**, 1.7 M glucose, 30 h. A, 0.20 mM $NADP^+$; B, 0.16 mM $NADP^+$; C, 0.12 mM $NADP^+$; D, 0.08 mM $NADP^+$; E, 0.04 mM $NADP^+$; F: 120 mM **1**, 2.4 M glucose, 0.01 mM $NADP^+$, 43 h; G: 140 mM **1**, 3.6 M glucose, 0.12 mM $NADP^+$, 68 h.

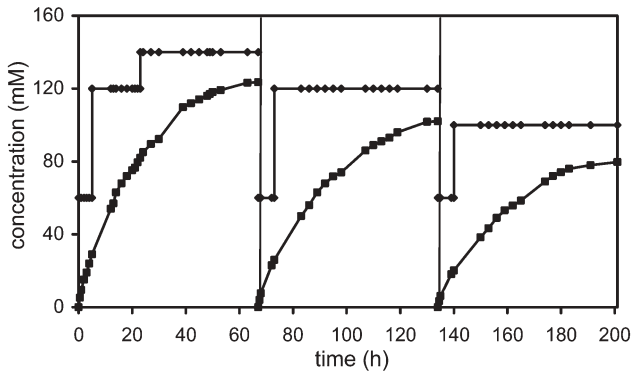


**Fig. 2** Repeated use of permeabilized cells of *B. pumilus* Phe-C3 and *B. subtilis* BGSC 1A1 for efficient bioreduction of ethyl 3-keto-4,4,4-trifluorobutyrate **1** with NADPH recycling: ◆ – cumulative substrate **1**; ■ – product (*R*)-**2**.

The permeabilized cells are rather stable and can be reused. As shown in Fig. 2, reduction of substrate **1** (140 mM) with permeabilized cells of *B. pumilus* Phe-C3 (27 g cdw/L) and *B. subtilis* BGSC 1A1 (53 g cdw/L) in the presence of $NADP^+$ (0.20 mM) and glucose (2.6 M) afforded 89% of (*R*)-**2** (124 mM, 23 g/L) at 67 h. The cells were harvested, resuspended into buffer to the same cell density as before, and used for the second bioreduction experiment under the same conditions. This gave about 80% of the activity of the first batch and 102 mM (*R*)-**2** from 120 mM substrate. Using the permeabilized cells for the third time bioreduction resulted in the formation of 80 mM of (*R*)-**2**, again showing 80% of the productivity seen with the previous batch.

In conclusion, we have developed a new concept for efficient cofactor recycling in biooxidoreduction based on "coupled permeabilized microorganisms". Our method has several distinctive features: 1) it is general, simple, and easy to process; 2) permeabilized cells retain high activity and high stability, and they can be repeatedly used; 3) the product can be obtained in high concentration; 4) NADPH was recycled for 1620 times. Considering the $NADP^+$ price of 7 EUR/g, this TTN is in the practical range for the preparation of chiral pharmaceutical intermediates with high value. Moreover, higher TTN could be simply reached by using even less cofactor.

Further development of the "coupled permeabilized cells" approach will involve the improvement of the cofactor TTN by developing highly efficient cofactor-regenerating microorganisms *via* overexpressing the GDH in suitable hosts and the exploration of other oxidoreductions. A large variety of oxidoreductase-containing native or recombinant microorganisms could be combined with a few very effective cofactor-regenerating organisms, resulting in efficient biocatalysis–cofactor regeneration couples for a wide range of oxidoreductions.

## Notes and references

‡ Preparation of permeabilized cells: The frozen cells of *Bacillus pumilus* Phe-C3, *Bacillus subtilis* BGSC 1A1, and *Pseudomonas* sp. Tyr-F10 (cell preparation: see Supplementary Information) were separately thawed, washed with 100 mM Tris-buffer (pH = 8.0), and resuspended in 100 mM Tris-buffer (pH = 8.0) to a cell concentration of 10 g cdw/L. For *B. pumilus* Phe-C3 and *Pseudomonas* sp. Tyr-F10, toluene (5%) and EDTA (5 mM) were added and the mixture was shaken at 25 °C and 300 rpm for 30 min. For *B. subtilis* BGSC 1A1, 7% toluene and EDTA (5 mM) were added and the mixture was shaken at 25 °C and 300 rpm for 3 h. The resulting mixtures for all three strains were incubated at 0 °C for 1 h, respectively. The permeabilized cells were harvested by centrifugation at 4 °C and 5000 rpm for 10 min and directly used for the biotransformation.

§ Bioreduction of ethyl 3-keto-4,4,4-trifluorobutyrate **1** with cofactor recycling by permeabilized cells of *B. pumilus* Phe-C3 and *B. subtilis* BGSC 1A1: To a suspension of permeabilized cells of *B. pumilus* Phe-C3 (20 g cdw/L) and *B. subtilis* BGSC 1A1 (40 g cdw/L) in 10 mL Tris-buffer (pH = 7.0) were added $NADP^+$ (0.08 mg, 0.10 μmol, 0.01 mM), substrate **1** (110 mg, 0.6 mmol, 60 mM), and glucose (0.80 g, 4.4 mmol, 440 mM). The mixtures were shaken at 25 °C and 300 rpm. Additional substrate **1** was added at 5 h (97 mg, 0.53 mmol, 60 mM), and more glucose was supplied at 5 h (0.70 g, 3.9 mmol, 440 mM), 14 h (0.68 g, 3.8 mmol, 440 mM), 19 h (0.16 g, 0.91 mmol, 110 mM), 24 h (0.16 g, 0.9 mmol, 110 mM), 29 h (0.63 g, 3.5 mmol, 440 mM) and 38 h (0.61 g, 3.4 mmol, 440 mM). Aliquots (300 μL) were taken at different time points. After centrifugation, 100 μL supernatant was diluted with 400 μL Tris-buffer and extracted with 500 μL chloroform containing 2 mM hexadecane as the internal standard. The organic phase was separated, dried over $Na_2SO_4$, filtrated, and analyzed by GC to determine the concentration and ee of **2** (analytic conditions: see Supplementary Information). After 43 h reaction, the volume of reaction mixtures was reduced to 7.0 mL due to sample taken. 16.2 mM product (*R*)-**2** was formed in 92% ee, with a cofactor TTN of 1620.


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