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Efficient NADPH Recycling in Enantioselective Bioreduction of a Ketone with Permeabilized Cells of a Microorganism Containing a Ketoreductase and a Glucose 6-Phosphate Dehydrogenase

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Abstract: We have demonstrated, for the first time, the efficient recycling of NADPH in a bioreduction with permeabilized cells of a single microorganism. Permeabilized cells of *Bacillus pumilus* Phe-C3 containing an NADPH-dependent ketoreductase and a glucose 6-phosphate dehydrogenase (G-6-PDH) were successfully used for the reduction of ethyl 3-oxo-4,4,4-trifluorobutanoate (**1**) to give (*R*)-ethyl 3-hydroxy-4,4,4-trifluorobutanoate (**2**) in 95% ee with the recycling of NADPH for 4220 times from the externally added $NADP^+$. The permeabilized cells were shown to be stable and active for a long period, allowing for high product concentration with high cofactor TTN by continuing the bioreduction with renewed addition of $NADP^+$. This provides with not only a practical synthesis of (*R*)-**2** but also a useful method applicable to many microbial oxidoreductions, since G-6-PDH is a very common enzyme existing in many microorganisms.

Keywords: cofactor recycling; enzyme catalysis; glucose 6-phosphate dehydrogenase; ketoreductase; permeabilized cells; reduction

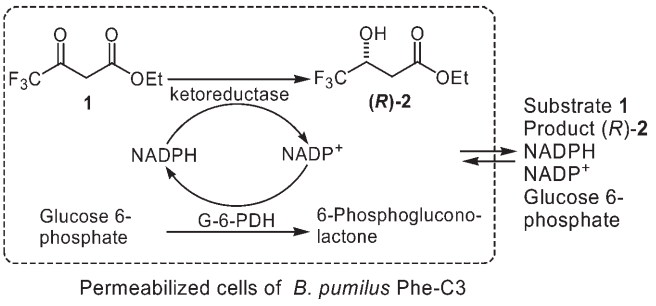
Enantioselective reductions are important reactions in the asymmetric syntheses of enantiopure compounds that are useful synthons and pharmaceutical intermediates. Enzymes can catalyze these reactions with high enantioselectivity,[1] providing a useful and green alternative to chemical catalysis.[2] The practical application of bioreductions depends on the efficient and economic supply of the expensive nicotinamide cofactor NAD(P)H which is required in stoichiometric amounts. In general, the cofactor can be recycled by the coupling of a desired enzymatic reaction with a chemical, electrochemical, or enzymatic reaction that regenerates the necessary cofactor.[3] The enzymatic regeneration by "coupled substrates"[4] and "coupled enzymes"[5] approaches is preferred so far. Cofactor recycling has been achieved with isolated enzyme(s)[4,5] and whole cells containing the necessary enzyme(s).[6,7] While the use of isolated enzyme(s) is still expensive, the use of whole cells depends on the amount of available intracellular cofactor which may be limiting and cannot be altered by adding extracellular cofactor unless the cell membranes are damaged in special cases during biotransformation.[7]

Microbial cells can be made permeable for the cofactor NAD(P)H and $NAD(P)^+$ by controlled treatment with an organic solvent and/or a detergent,[8,9] while keeping the necessary enzymes inside cells. The use of such permeabilized cells as biocatalysts allows for the utilization of externally added cofactor, the easy access of substrate, and the easy release of product. Moreover, enzymes inside permeabilized cells often retain high activity for a long period. For these reasons, permeabilized cells have been explored for bioreduction with cofactor recycling: permeabilized cells of *Alcaligenes eutrophus* containing a hydrogenase were used to regenerate NADH from NAD^+ for the reduction of ketones with HLADH;[10] permeabilized cells of *Gluconobacter oxydans* containing an NADH-dependent ketoreductase were used either with a formate dehydrogenase as coupled enzyme or with 2-butanol as coupled substrate for the reduction of ketones and cofactor regeneration,[11] and permeabilized *Bacillus megaterium* cells containing an L-valine dehydrogenase and a glucose dehydrogenase were reported for the reductive amination of α-ketoisovalerate with NADH recycling.[12] All these ap-

proaches are very interesting, but the total turnover number (TTN) of the cofactor was too low to be applicable in practical synthesis. Moreover, they all dealt with NADH recycling. Here, we report the first example using a single permeabilized microorganism for efficient bioreduction with NADPH recycling and the achievement of high cofactor TTN with glucose 6-phosphate dehydrogenase (G-6-PDH), a common enzyme existing in many microorganisms, as cofactor regenerating enzyme.

Enantioselective reduction of ethyl 3-oxo-4,4,4-trifluorobutanoate (**1**) to give the corresponding product (*R*)-**2** is a very interesting reaction (Scheme 1), since the product is a useful intermediate in the synthesis of the antidepressant Befloxatone.[13] We previously found that *Bacillus pumilus* Phe-C3[14] containing an NADPH-dependent ketoreductase catalyzed this reaction affording (*R*)-**2** in 91% ee.[15] This enzyme demonstrated the highest enantioselectivity observed for this bioreduction known thus far.[16] Bioreduction of **1** (20 mM) with resting cells of *Bacillus pumilus* Phe-C3 (6.7 g cdw/L, cdw = cell dry weight) showed that the addition of 2% glucose increased the initial activity from 2.7 to 12 U/g cdw (U = μmol/min) *via* the recycling of NADPH inside the cells. However, such cofactor recycling is not efficient, and biotransformation for 5 h yielded (*R*)-**2** in only 42% yield. To develop an efficient cofactor recycling system, experiments with the soluble, cell-free extracts of this strain was carried out to discover the necessary NADPH regenerating enzyme. No glucose dehydrogenase but rather G-6-PDH was found in the cells. Thus, permeabilized cells of *B. pumilus* Phe-C3 containing the ketoreductase and G-6-PDH were chosen to explore efficient enantioselective bioreduction with NADPH recycling.

Cells of *B. pumilus* Phe-C3 were prepared by growth on glucose and phenylalanine in ½ Evans medium.[15] The cells were harvested at a cell density of 2.5 g cdw/L and stored at −80 °C. For permeabilization,[8] cells were suspended in 100 mM Tris-buffer (pH = 8.0) and treated with 5% toluene and 5 mM EDTA at 25 °C for 30 min and at 4 °C for 1 h. Permeabilized cells were harvested by centrifugation to remove toluene and EDTA,



Scheme 1. Bioreduction of **1** with NADPH recycling by permeabilized cells of *B. pumilus* Phe-C3.

a procedure which also removed the cofactor originally presented in the cells. Biotransformations with permeabilized cells was examined in Tris-buffer (pH = 7.0) on a 5-mL scale. Permeabilized cells (6.7 g cdw/L) themselves showed no activity for the reduction of **1** (20 mM), since no NADPH was present. As given in Table 1, addition of 20 mM NADPH restored the activity to 10 U/g cdw in the first 30 min, and gave the desired product (*R*)-**2** in 96% ee. The reduction, however, could not be completed and yielded only 4.4 mM product at 5 h. Some NADPH was consumed, possibly by NADPH oxidase inside permeabilized cells. With initial addition of only 1 mM NADPH and 59 mM glucose 6-phosphate (G-6-P), the reduction was nearly complete at 5 h, yielding 93% (18.6 mM) of (*R*)-**2** in 95% ee. This suggests clearly the recycling of NADPH within the biocatalytic system, with a TTN of 18. It is interesting to see that both activity and final product yield are much higher than those with resting cells. The TTN of the cofactor was significantly increased by using less cofactor (1.0 – 0.1 mM) and more substrate (60 mM): (*R*)-**2** was obtained in 95% ee and 67% yield (40 mM) at an initial NADPH concentration of 0.1 mM, corresponding to a cofactor TTN of 401.

To reduce the cost of the cofactor in this approach, the less expensive $NADP^+$ was added for the cofactor regeneration. As shown in Table 1, bioreduction and NADPH recycling were as efficient as those with the addition of NADPH. A TTN of 402 was simply reached by the use of 60 mM **1**, 0.1 mM $NADP^+$, and 200 mM G-6-P. Further addition of 20 mM **1** and 100 mM G-6-P at 5 h increased the TTN to 504 and the concentration of (*R*)-**2** to 50 mM. The activities in the first 30 min reached 17 – 25 U/g cdw, and these values are higher than that with intact cells. Obviously, the cofactor concentration inside intact cells is lower than 0.1 mM, limiting the bioreduction activity. As shown in Figure 1, all reactions were fast in the first 4 h and became slow afterwards. Addition of more G-6-P at 5 h significantly increased the reaction rates, since this contributed to the regeneration of more NADPH. The activity between 5 – 7 h in this case is as high as that for the first two hours, and this indicates also the high stability of the permeabilized cells. No more reduction could be observed after 24 h, possibly due to the decomposition of the cofactor. The half-life time for NADPH was reported to be 34 h in the presence of 100 mM Tris, but decreased to 21 h in the presence of 100 mM G-6-P.[5b] Therefore, it is necessary to reduce the concentration of G-6-P for achieving longer reaction periods. Interestingly, the product ee increased to 95% ee from the 91% ee obtained with intact cells. The reason for this could be the lack of NADH-dependent ketoreductase activity in permeabilized cells, since no NADH or NAD^+ was present.

To further increase the cofactor TTN in bioreduction, an even lower amount of $NADP^+$ was initially added, G-6-P was supplied in 30 – 60 mM at different time points,

Table 1. Reduction of ethyl 3-keto-4,4,4-trifluorobutanoate **1** to (*R*)-**2** with NADPH recycling by permeabilized cells of *B. pumilus* Phe-C3 (6.7 g cdw/L) with the initial addition of G-6-P and NADPH or $NADP^+$.

1 [mM]	NADPH [mM]	$NADP^+$ [mM]	G-6-P [mM]	Activity[a] [U/g cdw]	(*R*)-**2**[b] [mM]	Yield[b] [%]	ee[b] [%]	TTN [mol/mol]
20[c]				12	8.4[d]	42[d]	91[d]	
20	20			10	4.4[d]	22[d]	96[d]	
20	1.0		59	35	18.6[d]	93[d]	95[d]	18
60	1.0		200	30	58.8	98	96	58
60	0.3		200	25	49.8	83	96	165
60	0.1		200	18	40.2	67	95	401
60		1.0	200	25	58.8	98	95	59
60		0.3	200	25	49.8	83	95	166
60		0.1	200	17	40.2	67	95	402
60 + 30[e]		0.3	200 + 100[e]	25	68.4	76	94	228
60 + 20[f]		0.1	200 + 60[f]	17	50.4	63	95	504

[a] Average activity in the first 30 min.
[b] At 24 h.
[c] Control experiment with resting cells in the presence of 2% glucose.
[d] At 5 h.
[e] 30 mM **1** and 100 mM G-6-P were added at 5 h.
[f] 20 mM **1** and 60 mM G-6-P were added at 5 h.

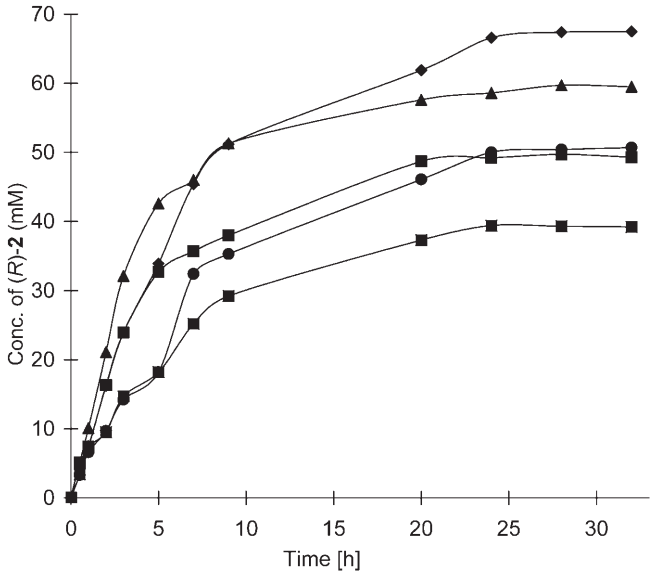


Figure 1. Reduction of ethyl 3-keto-4,4,4-trifluorobutanoate **1** with NADPH recycling by permeabilized cells of *B. pumilus* Phe-C3 (6.7 g cdw/L) with initial addtion of 60 mM **1**, 200 mM G-6-P, and 0.1–1.0 mM $NADP^+$. (▲)1.0 mM $NADP^+$. (■) 0.3 mM $NADP^+$. (◆) 0.3 mM $NADP^+$; 30 mM **1** and 100 mM G-6-P were added at 5 h. (■) 0.1 mM $NADP^+$. (◆) 0.1 mM $NADP^+$; 20 mM **1** and 60 mM G-6-P were added at 5 h.

and a higher cell density (20 g cdw/L) was used. As given in Table 2, the TTN was improved to 3410 and 4060 with the initial $NADP^+$ concentration of 0.01 and 0.005 mM, respectively. Addition of more substrate increased the TTN further to 4220.

A potential problem in the case of using less cofactor to achieve high TTN is the low product concentration. This was solved by adding a small amount of $NADP^+$ at several different time points to continue the biotransformation. Figure 2 showed the bioreduction of **1**

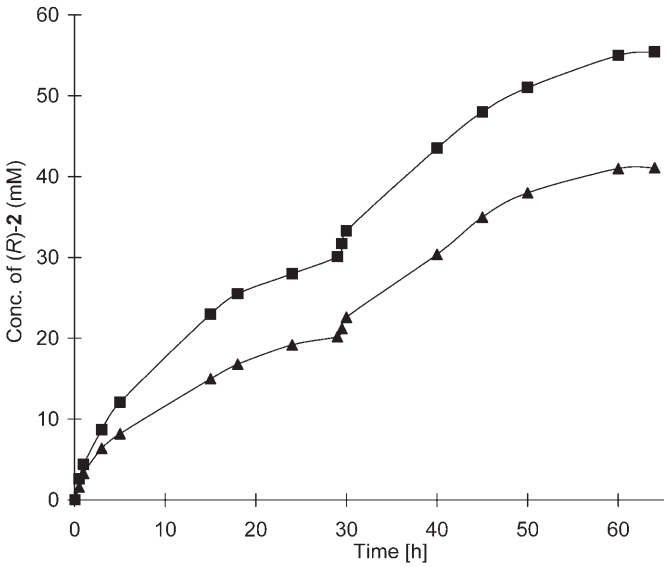


Figure 2. Reduction of ethyl 3-oxo-4,4,4-trifluorobutanoate (**1**) (60 mM) with NADPH recycling by permeabilized cells of *B. pumilus* Phe-C3 (20 g cdw/L) with the addition of 0.005–0.01 mM $NADP^+$ twice and 30–60 mM G-6-P at different time points (total concentration 480 mM). (■) 0.01 mM $NADP^+$ was added at the beginning and at 29 h. (▲) 0.005 mM $NADP^+$ mM was added at the beginning and at 29 h.

Table 2. Reduction of ethyl 3-oxo-4,4,4-trifluorobutanoate (**1**) to (*R*)-**2** with NADPH recycling by permeabilized cells of *B. pumilus* Phe-C3 (20 g cdw/L) with the initial addition of 0.005–0.01 mM $NADP^+$ and G-6-P.

1 [mM]	$NADP^+$ [mM]	G-6-P[a] [mM]	Activity[b] [U/g cdw]	Time [h]	(*R*)-**2** [mM]	Yield [%]	ee [%]	TTN [mol/mol]
60	0.01	390	4.7	48	34.1	57	94	3410
60	0.005	390	2.5	48	20.3	34	94	4060
60 + 20[c]	0.005	390	2.5	48	21.1	26	94	4220
60	0.01 + 0.01[d]	480	4.8	62	55.2	92	95	2760
60	0.005 + 0.005[e]	480	2.7	62	41.0	68	95	4100

[a] G-6-P was added in 30–60 mM at different time points.
[b] Activity was determined over the first 30 min.
[c] 20 mM substrate was added at 15 h.
[d] 0.01 mM $NADP^+$ was added at the beginning and at 29 h.
[e] 0.005 mM $NADP^+$ was added at the beginning and at 29 h.

(60 mM) with permeabilized cells by adding $NADP^+$ at the beginning and at 29 h. The reduction in the second period (29–64 h) is as fast as that in the first period (0–29 h), resulting in the same TTN of the cofactor. With the addition of 0.005 mM $NADP^+$ twice, (*R*)-**2** was obtained in 95% ee and in 41 mM (68% yield). This corresponds to the recycling of NADPH for 4100 times from the totally added 0.01 mM $NADP^+$. The experiments also demonstrated that permeabilized cells were stable and active for a long time. In principle, the product concentration could be further increased by continuing the reaction with new addition of $NADP^+$.

In conclusion, we have achieved a practical synthesis of (*R*)-**2** by enantioselective reduction of **1** with permeabilized cells of *B. pumilus* Phe-C3. More importantly, we have demonstrated the first example of efficient recycling of NADPH in a bioreduction by the use of a permeabilized microorganism containing a ketoreductase and a G-6-PDH. Our method is simple, the cofactor can be recycled for 4220 times, and the product can be obtained in high concentration. Our approach should be generally applicable to other cofactor-dependent oxidoreductions with microorganisms containing the necessary oxidoreductase, since G-6-PDH is a very common enzyme and possibly exists in those microorganisms. At the present stage, the relatively expensive G-6-P is used in excess for cofactor regeneration. Further improvement will involve the use of glucose 6-sulfate, a cheap substrate known for G-6-PDH,[17] to regenerate NADPH.

Experimental Section

Standard Protocol for Preparing Permeabilized Cells of *Bacillus pumilus* Phe-C3

Bacillus pumilus Phe-C3 was grown in 140 mL of ½ Evans liquid medium containing 2% glucose and 5 mM phenylalanine in a shaking flask at 25 °C and 300 rpm. The cells were harvested at 36 h with a cell density of 2.5 g cdw/L and cell pellets were stored at −80 °C.

Frozen cells were thawed, suspended in 50 mL of 100 mM Tris-buffer (pH = 8.0) to a density of 6.7 g cdw/L, and centrifuged at 4 °C and 5000 rpm for 10 min. Cell pellets were resuspended in 30 mL of 100 mM Tris-buffer (pH = 8.0) to a density of 10 g cdw/L. Toluene (1.5 mL, 5% v/v) and EDTA (55.8 mg, 5.0 mM) were added, and the mixture was shaken at 300 rpm and 25 °C for 30 min. The incubation was continued at 4 °C for 1 h, followed by centrifugation at 4 °C and 5000 rpm for 10 min. After removal of the supernatant, the permeabilized cells were used immediately for biotransformations.

Typical Example of Bioreduction of Ethyl 3-Oxo-4,4,4-trifluorobutanoate (1) with Permeabilized Cells of *B. pumilus* Phe-C3

To a suspension of permeabilized cells of *B. pumilus* Phe-C3 (20 g cdw/L) in 5 mL Tris-buffer (pH = 7.0) were added $NADP^+$ (0.02 mg, 5.0 μL solution of 4 mg/mL; 0.025 μmol, 0.005 mM), substrate **1** (55.2 mg, 0.3 mmol, 60 mM), and glucose 6-phosphate (102 mg, 0.3 mmol, 60 mM). The mixture was shaken at 25 °C and 300 rpm. More glucose 6-phosphate was added at 5 h (86 mg, 0.25 mmol, 60 mM), 14 h (82 mg, 0.24 mmol, 60 mM), 24 h (73 mg, 0.21 mmol, 60 mM), 29 h (69 mg, 0.20 mmol, 60 mM), 30 h (30.6 mg, 0.09 mmol, 30 mM), 41 h (57 mg, 0.17 mmol, 60 mM), 46 h (27 mg, 0.08 mmol, 30 mM), 51 h (49 mg, 0.14 mmol, 60 mM), and total concentration of glucose 6-phosphate added is 480 mM. Another portion of 0.005 mM $NADP^+$ was added at 29 h (0.014 mg, 3.5 μL solution of 4.0 mg/mL; 0.018 μmol, 0.005 mM). At different time points, 200 μL aliquots were taken out for analysis. After centrifugation, 100 μL supernatant were mixed with 400 μL Tris-buffer (pH = 7.0), and the product was extracted into 500 μL chloroform containing 2 mM hexadecane as the internal standard, followed by drying over Na_2SO_4. The sample was analyzed by GC to quantify the concentration and ee of ethyl 3-hydroxy-4,4,4-trifluorobutanoate [(*R*)-**2**]. After 65 h reaction, 2.0 mL reaction mixture remained due to sample taking.

Ethyl 3-hydroxy-4,4,4-trifluorobutanoate [(*R*)-**2**] was formed in 95% ee and in 41 mM (15.3 mg, 0.082 mmol, 68%

yield), and the total turnover number of NADPH reached 4100.

The concentration of **2** was analyzed by GC on a Chrompack Optima-5 column (25 m × 0.32 mm). Temperature program: 50 °C for 10 min, increase to 280 °C at a rate of 10 °C/min, hold at 280 °C for 2 minutes; retention time: 4.03 min for 2, 1.89 and 7.02 min for 1, 13.04 min for hexadecane.

The ee of **2** was determined by GC with a Lipodex-A column (25 m × 0.25 mm). Temperature program: 40 °C to 120 °C at 5 °C /min, increase to 170 °C at 45 °C /min; retention time: 11.89 min for (*S*)-2, 12.21 min for (*R*)-2.

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