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# Bioreduction of methyl *o*-chlorobenzoylformate at 500 g $L^{-1}$ without external cofactors for efficient production of enantiopure clopidogrel intermediate

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ABSTRACT

Biocatalytic reduction of methyl *o*-chlorobenzoylformate (CBFM) provides a green and direct access to methyl (*R*)-*o*-chloromandelate [(*R*)-CMM], an intermediate for a platelet aggregation inhibitor named clopidogrel. As much as 500 g $L^{-1}$ of CBFM was stoichiometrically converted into enantiopure (*R*)-CMM at 20 °C by using a whole-cell catalyst coexpressing an aldo-keto reductase from *Bacillus* sp. and a glucose dehydrogenase (GDH). In addition to the high productivity of 812 g $L^{-1}$ $d^{-1}$, this new whole-cell reduction is attractive by eliminating the need of an added external cofactor.



Optically active α-hydroxy esters are ubiquitous structural motifs in numerous biologically interesting natural and unnatural compounds and have been widely used as important building blocks in pharmaceutical industry.[1] Among them, methyl (*R*)-*o*-chloromandelate [(*R*)-CMM] is a key chiral intermediate for the synthesis of (*S*)-clopidogrel, a 'heavy bomb' drug with platelet aggregation inhibiting activity, which has been widely administered to atherosclerotic patients with the risk of a heart attack or stroke caused by blood clots. As one of the most intensively investigated prescription antiplatelet medicines, Plavix® ((*S*)-clopidogrel bisulfate) claims the title of second-best selling drug in the world with the global sales of $10 billion per year. (*R*)-CMM can be prepared from the (*R*,*S*)-*o*-chloromandelic acid through diastereomeric crystallization,[2] enzymatically enantioselective hydrolysis of corresponding racemic nitrile compounds[3–6] or ester compounds.[7–10] A more straightforward and attractive method for obtaining the key enantiomer would be asymmetric reduction of methyl chlorobenzoylformate (CBFM) which has an inherent advantage of achieving up to 100% theoretical yield. Ru-catalyzed asymmetric hydrogenation of CBFM has been developed with the hydroxy product of 92% ee at most;[11,12] in the meantime, reductases are interesting and promising for the asymmetric reduction of CBFM due to their high enantioselectivity and environmental compatibility, rounding off the chemist's toolbox. For example, Ema et al. identified a carbonyl reductase from *Saccharomyces cerevisiae* and expressed it in *Escherichia coli* (*E. coli*), which was subsequently employed for CBFM reduction at a substrate concentration of 198 g $L^{-1}$ with 1.2 M $NADP^+$, giving (*R*)-CMM in >99% ee.[13] Whole cells of *Saccharomyces cerevisiae* could also catalyze the reduction of CBFM to (*R*)-CMM with good enantioselectivity of 96.1% ee.[14] Asymmetric reduction of CBFM mediated by an NADH-dependent carbonyl reductase from *Thermus thermophilus* yielded (*R*)-CMM with 95% and 92% ee using archaeal GDH and *Bacillus stearothermophilus* alcohol dehydrogenase for cofactor regeneration.[15] However, the number of enzymes isolated and characterized exhibiting CBFM reduction activity is still limited so far and the often observed low tolerance of biocatalysts against high substrate concentration or the requirement of large amount of cofactor during biocatalytic reductions represents an impediment en route to true preparative applicability.[16,17]

In our previous work, an NADPH-dependent aldo-keto reductase (YtbE) cloned from a ketone reductase-producing *Bacillus* sp., strain no. EUC0013,[18] has been identified with the capability of reducing various aromatic ketones, α- and β-keto esters with high enantioselectivity.[19] Herein, its applicability in the production of (*R*)-CMM via asymmetric reduction of CBFM was presented to further evaluate the power of the versatile reductase. Consequently, a new access to optically pure (*R*)-CMM at an extremely high substrate concentration of 500 g $L^{-1}$ was developed even without external cofactor.

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As we were interested in developing an efficient biocatalyst-based process for enantiopure (*R*)-CMM, a survey on the catalytic activity of purified YtbE toward CBFM was conducted by spectrophotometric assays. This reductase was unexpectedly active in the reduction of CBFM (4.42 U $mg^{-1}$) for which the $k_{cat}/K_m$ was determined as 1.87 $mM^{-1}$ $s^{-1}$, about 5 times higher than that for ethyl pyruvate.[19] To determinate the enantioselectivity, the purified reductase was employed for the reduction of 10 mM CBFM with an NADPH regeneration system consisting of glucose dehydrogenase (GDH), glucose and $NADP^+$. The HPLC analysis of the product revealed that YtbE exhibits splendid enantioselectivity for CBFM reduction, giving (*R*)-CMM in >99% ee. It is worth mentioning that the purified reductase showed merely moderate activity toward methyl benzoylformate (0.21 U $mg^{-1}$), producing the corresponding (*R*)-alcohol in only 44% ee, which indicates the significant role of the *ortho*-chloro substituent in improving both the enzyme activity and enantioselectivity.[20] The unexpectedly good performance of YtbE in CBFM reduction inspired us to further investigate this stereoselective reaction as a valuable case of biocatalytic application.

The cofactor dependency of redox enzymes and the high price of cofactors make an efficient regeneration system a prerequisite for practical bioreduction process.[21] Simultaneous expression of the required enzymes in one microorganism is an efficient approach to circumvent cofactor challenges and to simplify the process.[22,23] Here, a glucose dehydrogenase (GDH) from *Bacillus subtilis* was introduced for NADPH regeneration. A one-plasmid strategy with YtbE and GDH genes ligated into one plasmid (pET28a) was applied and *E. coli* strain BL21(DE3) was used as host organism. The functional expression of both enzymes was determined by measuring their activities in cell-free extract, resulting in YtbE and GDH activities 540 U and 1125 U per gram of lyophilized cells, respectively.

For the analysis of the capability to produce (*R*)-CMM, the designer cells were subjected to CBFM reduction at 40 g $L^{-1}$ in 0.5 mL phosphate buffer with $NADP^+$ and glucose, giving 67% conversion and excellent enantioselectivity of >99% ee. The limited conversion was ascribed to the formation of gluconic acid causing a drop in pH. Therefore, a feedback-controlled addition of 2 M $Na_2CO_3$ solution was conducted to maintain an initial pH of 6.5. As a result, the whole-cell-catalyzed reduction of CBFM at 200 g $L^{-1}$ in 10 mL aqueous buffer system containing 1 mM $NADP^+$ afforded (*R*)-CMM with full conversion (Table 1, entry 1) even though the CBFM solubility limit was exceeded and a second phase was formed. Then the whole-cell reduction proceeded in the absence of external $NADP^+$ and, interestingly, the conversion reached 93% within 3 h (Table 1, entry 2), suggesting that the constructed enzyme-coupled system was efficient to recycle intracellular NADPH for CBFM reduction.

Next, strategies for improving the productivity were adopted, including reduction of the reaction temperature, increase of the substrate loading and introduction of an organic phase. When the reaction temperature was lowered to 25 °C from 30 °C, in spite of the slightly depressed initial reaction rate, 200 g $L^{-1}$ CBFM was stoichiometrically converted into optically pure (*R*)-CMM after 4 h (Table 1, entry 3). The substrate load was then increased to 400 g $L^{-1}$, resulting in 96% conversion (Table 1, entry 4). Nevertheless, the lower temperature of 20 °C enabled the complete conversion, and, more excitingly, a higher substrate load of 500 g $L^{-1}$ still gave a conversion of higher than 99% (Table 1, entries 5 and 6). It was speculated that the lower temperature might reinforce intramolecular hydrogen bonds and reduce the enzyme mobility, thus leading to a suppression of the protein denaturation.[13] The reaction temperature and substrate concentration had no influence on the enantioselectivity of the bioreaction and the enantiomeric excess of the product was higher than 99% ee in all cases.

Bioreduction of CBFM in an organic solvent-buffer biphasic system was subsequently investigated. Ethyl caprylate, dibutyl phthalate, and toluene were selected as the second phase because their biocompatibilities to YtbE and GDH were exhibited in preliminary experiments of the enzyme stabilities in different organic solvents (Fig. S2). As shown in Table 2, when asymmetric reduction of CBFM at 400 g $L^{-1}$ was carried out in an ethyl caprylate-buffer biphasic system at 25 °C, a nearly complete (99%) conversion was achieved. The best biocompatibility of ethyl caprylate to YtbE accounted for this highest conversion (Fig. S2). It seems that ethyl caprylate acted as a reservoir for the toxic substrate and product, thus regulating the 'toxicant' concentration around the biocatalyst. However, in view of the high boiling point of ethyl caprylate, the aqueous medium seems optimal, which will contribute to the development of green and sustainable synthetic processes.

Finally, the biotransformation was scaled up by ten-folds with a CBFM concentration of 500 g $L^{-1}$ at 20 °C to confirm this whole-cell-catalyzed process. As a result, both the conversion and ee values were higher than 99% after 13 h (Table 1, entry 7). After normal workup, 44 g of (*R*)-CMM was obtained with an isolated yield of 88%, corresponding to a space-time-yield of 812 g $L^{-1}$ $d^{-1}$. As far as we know, this is the first report of YtbE used as a biocatalyst for highly efficient and stereoselective reduction of CBFM to (*R*)-CMM. To work as an attractive alternative to traditional chemical catalysts, biocatalysts must tolerate a high substrate concentration (typically, >100 g $L^{-1}$) for preparative application.[16] As one can see from Table 3, compared to other reported biocatalysts for CBFM reduction, the recombinant *E. coli* cells coexpressing YtbE and GDH offered significantly higher substrate loading and abandon of external cofactor, making it more competitive and promising.

In conclusion, optically pure methyl (*R*)-*o*-chloromandelate [(*R*)-CMM], an important chiral building block for clopidogrel, was obtained via the asymmetric reduction of CBFM with recombinant *E. coli* coexpressing a versatile aldo-keto reductase (YtbE) and

**Table 1**
Asymmetric reduction of CBFM with recombinant *E. coli*[a]

| Entry | CBFM (g) | [CBFM] (g $L^{-1}$) | *T* (°C) | $NADP^+$ (mM) | Time (h) | Conv.[b] (%) | ee[d] (%) |
|---|---|---|---|---|---|---|---|
| 1 | 2 | 200 | 30 | 1 | 3 | >99 | >99 |
| 2 | 2 | 200 | 30 | 0 | 3 | 93 | >99 |
| 3 | 2 | 200 | 25 | 0 | 4 | >99 | >99 |
| 4 | 4 | 400 | 25 | 0 | 10 | 96 | >99 |
| 5 | 4 | 400 | 20 | 0 | 12 | >99 | >99 |
| 6 | 5 | 500 | 20 | 0 | 13 | >99 | >99 |
| 7[e] | 50 | 500 | 20 | 0 | 13 | >99 (88)[c] | >99 |

[a] Reaction conditions: CBFM (quantity and concentration indicated above), lyophilized cells of recombinant *E. coli* (YtbE/GDH) (0.5 g), glucose (1.5 equiv), $NADP^+$ (concentration indicated above), phosphate buffer (100 mM, pH 6.5, 10 mL).
[b] Determined by GC analysis.
[c] Isolated yield of (*R*)-CMM in parentheses.
[d] Determined by HPLC [Chiralcel OD-H column, hexane/*i*PrOH (97:3, v/v)].
[e] 5 g lyophilized cells of recombinant *E. coli* (YtbE/GDH) in 100 mL phosphate buffer.

**Table 2**
Asymmetric reduction of CBFM in biphasic systems[a]

| Entry | Organic solvent (50%, v/v) | Conv. (%) | ee (%) |
|---|---|---|---|
| 1 | None | 96 | >99 |
| 2 | Ethyl caprylate | 99 | >99 |
| 3 | Dibutyl phthalate | 95 | >99 |
| 4 | Toluene | 87 | >99 |

[a] Reaction conditions: CBFM (4 g), lyophilized cells of recombinant *E. coli* (YtbE/GDH) (0.5 g), glucose (1.5 equiv), phosphate buffer (100 mM, pH 6.5, 10 mL), organic solvent (10 mL), 25 °C for 22 h.

**Table 3**
Comparison between recombinant *E. coli* (YtbE-GDH) and other reported biocatalysts for CBFM reduction

| Biocatalyst | CBFM (g) | [CBFM] ($g\,L^{-1}$) | $NAD(P)^+$ (mM) | Time (h) | Conv. (%) | ee (%) |
|---|---|---|---|---|---|---|
| *E. coli* (YtbE/GDH)[a] | 50.0 | 500 | 0 | 13 | >99 (88)[e] | >99 |
| *E. coli* (SCR/GDH)[b] | 19.9 | 199 | 1.2 | 24 | 98 (76)[e] | >99 |
| S. cerevisiae[c] | 0.1 | 16.7 | 0 | 12 | 100 | 96 |
| TtADH/TaGDH[d] | 0.1 | 5 | 1.0 | 24 | 100 (62)[e] | 95 |

[a] Lyophilized cells of recombinant *E. coli* (YtbE/GDH) ($50\,g\,L^{-1}$).
[b] Wet cells of recombinant *E. coli* (SCR/GDH) ($200\,g\,L^{-1}$).[13]
[c] Dried cells of *S. cerevisiae* ($83\,g\,L^{-1}$).[14]
[d] Carbonyl reductase from *Thermus thermophilus* ($0.125\,g\,L^{-1}$) and GDH from *Thermoplasma acidophilum* ($0.0275\,g\,L^{-1}$).[15]
[e] Isolated yield of (*R*)-CMM in parentheses.

a GDH. Excellent productivity as high as $812\,g\,L^{-1}\,d^{-1}$ was achieved at 20 °C on a 50-gram scale (2.5 M) without external addition of expensive cofactor, representing a huge cut in the cost and a good potential for industrial application. This study not only further broadened the biocatalytic applications of the useful reductase but also established an efficient and cost-effective process for direct synthesis of enantiopure clopidogrel intermediate. Further work including optimization of the reaction for large scale manufacture and integration of the bioreduction with chemical steps for the synthesis of (*S*)-clopidogrel are currently underway.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.06.097.

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