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Biotransformations with engineered *E. coli* cells expressing wild-type and mutant Baeyer–Villiger monooxygenases under non-growing conditions

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Abstract

The *Escherichia coli* (*E. coli*) overexpression systems of Baeyer–Villiger monooxygenases (BVMOs), cyclohexanone monooxygenase (CHMO) and cyclopentanone monooxygenase (CPMO) and their mutants derived from directed evolution were used as catalysts in oxidations of six 4-substituted cyclohexanones. The biotransformations were carried out with growing cells (standard screening conditions) and with non-growing cells. The surprising result is that several substrates that give negative results (non-acceptance) under the screening conditions, afford excellent conversions in the transformations under non-growing conditions. The new bioreagents for Baeyer–Villiger oxidations with divergent, high enantioselectivities reported here can be used in scaled-up fermentation under non-growing conditions.

Keywords: Monooxygenases; Oxidations; Non-growing conditions; Enantiopure lactones; CHMO mutants; CPMO mutants

1. Introduction

Enantio and regioselective Baeyer–Villiger oxidations are important reactions in organic synthesis [1] and the development of safe, selective, and environmentally friendly reagents has been pursued through chemical [2,3] and enzymatic [4] methods. Baeyer–Villiger monooxygenases (BVMOs) from bacteria are particularly promising as catalysts as they possess remarkably flexible substrate tolerance often combined with high stereoselectivity [4,5]. Cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 [6] and cyclopentanone monooxygenase (CPMO) from *Comamonas* sp. NCIMB 9872 [7] are two of the enzymes that transform a broad variety of ketones; however, while CHMO is often highly enantioselective [4], CPMO rarely is [8,9]. Recently, both enzymes were evolved to improve enantioselectivity and a number of mutants with desired characteristics were identified [10,11–13]. The most successful mutant identified in the directed evolution of CHMO carried a single mutation F432S; its activity was comparable to that of the WT-CHMO and it showed a dramatic change in enantioselectivity of oxidation of 4-hydroxycyclohexanone (from 9% *R* to 79% *S*).

Despite the fact that these enzymes are highly selective reagents for a broad array of substrates, they have not found widespread acceptance among synthetic chemists because of real and perceived difficulties [14]. It is important to point out, however, that several problems associated with the use of these biocatalysts, such as instability of the isolated enzymes and cofactor (NADPH) recycling, have been solved when these enzymes are overexpressed in hosts such as baker's yeast [15] and *Escherichia coli* (*E. coli*) [16]. The availability of overexpression systems for several BVMOs and their mutants in *E. coli* allows organic chemists to treat them as reagents in organic synthesis. The next hurdle in the development of new reagents is to make them efficient and adaptable to preparative scale. A number of groups addressed the problem of making biocatalytic Baeyer–Villiger reactions practical and chemist "friendly" [15,17–20]. In particular, Walton and Stewart [18] have shown that *E. coli* overexpressing CHMO carry out a model Baeyer–Villiger oxidation under non-growing conditions, with volumetric productivity twenty-fold higher than the corresponding oxidations with growing cells.

Recently, both enzymes were evolved to improve enantioselectivity and a number of mutants with desirable characteristics were identified [10–13]. The most successful mutant found

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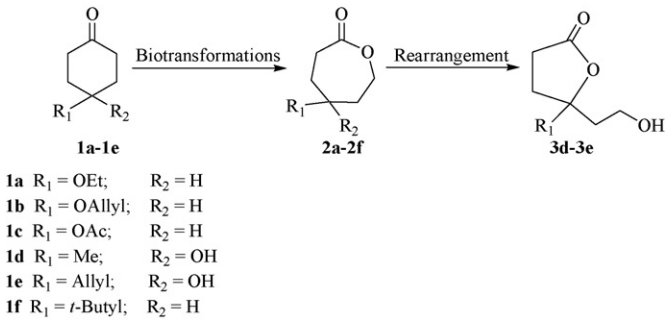
in the directed evolution of CHMO carried a single mutation Phe432Ser; its activity was comparable to that of the WT-CHMO and it showed a dramatic change in enantioselectivity in the oxidation of 4-hydroxycyclohexanone (from 9% *R* to 79% *S*).

Having identified in the screening process the most enantio-improved mutants we scaled-up the production of selected lactones under non-growing conditions. The results were gratifying and interesting since not only was there a dramatic improvement in the conversions for several substrates, but also, in a number of cases where no reaction was detected during the screening with growing cells, the transformation carried out with resting cells gave highly enantioenriched lactones in good yields. The results obtained for the screening of six 4-substituted cyclohexanones using non-growing cells expressing WT-CHMO, WT-CPMO, and their mutants are reported here.



Scheme 1.

2. Results and discussion

The substrates investigated are shown in Scheme 1, the results of the screening under growing conditions [8,12] (Table 1) are compared with the transformations performed under non-growing conditions (Table 2). The design and construction of CPMO mutants used in this study has been reported previously [11], and the preparation of substrates and characterization of the lactonic products, including their absolute configuration, was described in earlier publications [8,12]. Under non-growing conditions, biotransformations were carried out on 10 mg samples in 125 mL flasks as described in Section 3. The initial scale-up reactions were performed on 100 mg samples of substrates in 500 mL baffled flasks. The reaction progress was monitored by chiral phase GC using the conditions that allow baseline resolution for the enantiomeric lactones which have been established previously [11,12].

A model study by Walton and Stewart [18] of Baeyer–Villiger oxidation of cyclohexanone catalyzed by non-growing *E. coli* cells overexpressing cyclohexanone monooxygenase (CHMO) demonstrated that the volumetric productivity of non-growing cells was an order of magnitude greater than that achieved with growing cells. Our earlier work on the reductions of β-lactams catalyzed by *E. coli* overexpressing yeast reductases [21] showed that improved conversion and shorter reaction time were obtained with non-growing cells. Since that time, we have routinely used non-growing cells in all scaled-up biotransformations. Thus, after the preliminary screening with the growing cells that identified the "useful" mutants, the following scale-ups were carried out with non-growing cells. An interesting development occurred when we decided to re-run

Table 1
Baeyer–Villiger oxidations of 4-substituted cyclohexanones to the corresponding lactones catalyzed by WT-CHMO, WT-CPMO and their mutants under growing conditions [8,12]

No.	Substrates **1a–f** R_1/R_2	WT-CHMO conversion %[a] (ee%)[b]	I-K2-F5 Phe432Ser conversion % (ee%)	WT-CPMO conversion % (ee%)	B1-A10 Phe156Gly/Leu157Phe conversion % (ee%)
1a	OEt/H	NR	NR	100 (37 *R*) (24 h)	77 (95 R) (24 h)
1b	OAllyl/H	NR	NR	100 (53 *S*) (24 h)	23 (>90 *R*) (24 h)
1c	OAc/H	NR	NR	81 (5 *S*) (24 h)	10 (ND) (24 h)
1d	OH/Me	100 (94 *R*) (24 h)	100 (97 *R*) (24 h)	81 (85 *S*) (24 h)	NR
1e	OH/Allyl	100 (27 *R*) (24 h)	100 (97 *R*) (24 h)	92 (44 *S*) (24 h)	NR
1f	*t*-butyl	NR	NR	NR	NR

[a] Conversion monitored by GC.
[b] ee monitored by chiral phase GC.

Table 2
Baeyer–Villiger oxidations of 4-substituted cyclohexanones to the corresponding lactones catalyzed by WT-CHMO, WT-CPMO and their mutants under non-growing conditions

No.	Substrates **1a–f** R_1/R_2	WT-CHMO conversion %[a] (ee%)[b]	I-K2-F5 Phe432Ser conversion % (ee%)	WT-CPMO conversion % (ee%)	B1-A10 Phe156Gly/Leu157Phe conversion % (ee%)
1a	OEt/H	33 (50 *S*) (24 h)	77 (**>99 *S***) (24 h)	100 (35 *R*) (4 h)	100 (**94 *R***) (9 h)
1b	OAllyl/H	24 (71 *S*) (24 h)	87 (**>99 *S***) (24 h)	100 (52 *S*) (4 h)	100 (**85 *R***) (9 h)
1c	OAc/H	32 (79 *S*) (24 h)	91 (**>99 *S***) (24 h)	100 (8 *S*) (4 h)	100 (68 *R*) (11 h)
1d	OH/Me	100 (96 *R*) (8 h)	100 (86 *R*) (12 h)	100 (85 *S*) (10 h)	NR
1e	OH/Allyl	100 (28 *R*) (12 h)	100 (**97 *R***) (8 h)	100 (55 *S*) (8 h)	NR
1f	*t*-butyl	18 (**98 *S***) (24 h)	NR	NR	NR

[a] Conversion monitored by GC.
[b] ee monitored by chiral phase GC.

the biotransformations of 4-*tert*-butylcyclohexanone **1f** with WT-CHMO under non-growing conditions. It has been reported before that **1f** was oxidized to the lactone in a reaction catalyzed by the isolated CHMO enzyme [22]; the conversion was only 17% but the enantiomeric excess was 98%. However, several attempts to duplicate these results with whole-cell catalyzed reactions under log-phase growth conditions failed. Thus, we were pleased to discover that in the course of the reaction with non-growing cells the substrate was accepted, and that the conversion and the enantiomeric excess were comparable to those obtained in the reaction catalyzed by the isolated enzyme. Since the control experiments revealed that the mass of growing cells produced over the duration of the biotransformation (24 h) is comparable with the mass of cells used under the non-growing conditions, it was tempting to conclude that reactions performed with non-growing cells reflected the performance of the isolated enzyme better than the reactions with growing cells.

It appears also, that the frequently encountered problem of substrate acceptance and/or conversion may, in many cases, be linked to the sluggish transport of substrate into the cytosol where the reaction takes place. The apparently more effective transport in the non-growing cells is likely related to the increased membrane permeability which is caused by a high concentration of hydrophobic substrate and product in the fermentation mixture [23].

The above considerations prompted us to re-examine several other "failed" reactions and we were pleased to discover that a number of other substrates, not accepted or poorly accepted during screening with growing cells, were converted to the corresponding lactones efficiently and with high enantiomeric excess (Table 2). The maximum conversion was achieved in several cases in a considerably shorter time. The most striking results were observed in oxidations of 4-ethoxy, 4-allyloxy, and 4-acetoxy cyclohexanones catalyzed by the CHMO mutant Phe432Ser which gave essentially enantiopure (>99% ee) (*S*)-**2a**, (*S*)-**2b**, and (*S*)-**2c** with excellent conversions within 24 h. Under the same reaction conditions, CPMO mutant Phe156Gly/Leu157Phe provides an access to their (*R*) antipodes in good to excellent yields.

The oxidations of ketones **1a**-**c** are coherent with the results obtained for the *tert*-butyl substrate **1f**. Thus, 4-ethoxy cyclohexanone **1a** is not converted by growing cells, it is converted by the non-growing cells, but the conversion is only 33% after 24 h. On the other hand, the same substrate is transformed by WT-CPMO under both growing and non-growing conditions. In the latter case, however, the transformation under the non-growing conditions gives 100% conversion after only 4 h. These results suggest that **1a** is a poor substrate for the WT-CHMO; in addition, its transport through membrane is slow. The combined effect is that under growing conditions no lactone is formed within 24 h. When the reaction is performed with non-growing cells, **1a** is transported through a more permeable membrane more efficiently, and the product accumulates slowly. These rationalizations are supported by several control experiments that monitor cell-biomass used in these biotransformations. Thus, we found for all wild-type and mutant strains studied that the 24 h fermentations using growing cells produced cell-biomass than was very close to the bio-mass of the non-growing cells used in our experiments. This suggests that the total quantity of the enzyme available under the growing over 24 h period is comparable to the quantity supplied under non-growing conditions.

In conclusion, the experiments described here underscore the advantages of the biotransformations with the non-growing cells, the reactions are faster, cleaner (no metabolites), and better suited for scale-up work in fermenters in agreement with the conclusions of Walton and Stewart [18]. Furthermore, they demonstrate that substrate-acceptance profiling frequently carried out with growing cells may fail to identify organisms and/or mutants that are suitable for the conversion of a substrate used in screening. Finally, overexpression systems for the mutants tested here are both highly active and enantioselective; overexpressed in easy to handle organisms they enrich a repertoire of divergent bioreagents for Baeyer–Villiger reactions.

3. Experimental

The synthesis and charaterization of all compounds used in this study have been published previously [8,11,12]. Chemical oxidations with *m*-chloroperbenzoic acid were performed on all substrates prior to biotransformations to establish appropriate conditions for the GC resolution of all lactones.

3.1. Biotransformations under growing conditions with E. coli/CHMO and E. coli/CHMO mutants

The *E. coli* strain BL21(DE3)(pMM4) (or JM109(DE3)(pET-22b)) was streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until the colonies were 1–2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and shaken at 37 °C, 250 rpm, overnight. A 250 μL aliquot of pre-culture was used to inoculate 25 mL of LB-Ampicillin medium supplemented with 1.5 mL of 20% glucose in a 125 mL baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm, until OD_{600} was approximately 0.3–0.4. IPTG stock solution (200 mg/mL) was added (0.1 μL/mL medium) and the flask was shaken for another 30 min at 24 °C. Then 10 mg substrate was added along with cyclodextrin were necessary to alleviate solubility or toxicity problems. The culture was shaken at 24 °C, 250 rpm, and monitored by GC analysis until reaction was complete.

3.2. Non-growing biotransformations conditions with E. coli/CHMO, E. coli/CHMO mutants

The *E. coli* strains BL21(DE3)(pMM4), JM109(DE3)(pET-22b(+)), or BL21(DE3)(pET-22b(+)) expressing cyclohexanone or cyclopentanone monooxygenase (WT or mutant) were streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until colonies were from 1 to 2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and shaken at 37 °C, 250 rpm, overnight. This culture was used to inoculate 500 mL of LB-Ampicillin supplemented with 4 g/L of glucose in a 1 L baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm

until OD_{600} was approximately 0.3–0.4. IPTG stock solution (200 mg/mL) was added to a concentration of 0.1 mM and the flask was shaken at 30 °C until the cells entered the stationary phase of growth (detectable when change in OD_{600} increased by less then 0.5 AU over a 30 min time period). The cells were then harvested by centrifugation, and the cell pellet was re-suspended in 125 mL of a nitrogen-free minimal salts medium. For the biotransformation reactions, 25 mL of the re-suspended cell culture in M9 minimal medium was pippetted into a 125 mL baffled Erlenmeyer flask. To this was added 10 mg of substrate (1 equivalent of hydroxypropyl β-cyclodextrin was added in the cases of **1a**, **1b**, and **1f** to alleviate solubility problems) and the reaction was shaken at 24 °C, 250 rpm and monitored by GC analysis until reaction was complete, with 20% glucose added as required.

3.3. Cell-biomass formation under growing conditions with E. coli/CHMO, E. coli/CHMO mutant, E. coli/CPMO, and E. coli/CPMO mutant

The *E. coli* strain expressing one of the proteins listed in the title was streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until the colonies were 1–2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and shaken at 37 °C, 250 rpm, overnight. A 250 μL aliquot of pre-culture was used to inoculate 25 mL of an LB-Ampicillin medium reaction culture supplemented with 20% glucose in a 125 mL baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm, until OD_{600} was approximately 0.3–0.4. IPTG stock solution (200 mg/mL) was added (0.1 μL/mL medium) and the flask was shaken for 24 h. After 24 h the cells were removed from the medium via centrifugation, the supernatant was discarded and the cell pellet was re-suspended in 3 mL of water. The re-suspended cell-biomass was transferred to pre-weight 1.7 mL micro centrifuge tubes. The cells were then spun at 8500 rpm for 10 min, the supernatant was removed, and the tubes containing the isolated cell were weighted and their biomass recorded. The results obtained for the growing cells are given in Table 3.

3.4. Cell-biomass formation under non-growing conditions with E. coli/CHMO, E. coli/CHMO mutant, E. coli/CPMO, and E. coli/CPMO mutant

The *E. coli* strain expressing one of the proteins listed in the title was streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until the colonies were 1–2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask and shaken at 37 °C, 250 rpm, overnight. This culture was used to inoculate 500 mL of LB-Ampicillin supplemented with 4 g/L of glucose in a 1 L baffled Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm until OD_{600} was approximately 0.3–0.4. IPTG stock solution (200 mg/mL) was added to a concentration of 0.1 mM and the flask was shaken at 30 °C until the cells entered the stationary phase of growth (detectable when change in OD_{600} increased by less then 0.5 AU over a 30 min time period). The cells were then harvested by centrifugation, and the cell pellet was re-suspended in 125 mL of a nitrogen-free minimal salts medium. For the normalization experiments, 25 mL of the re-suspended cell culture in M9 minimal medium was pipetted into a 125 mL baffled Erlenmeyer flask, and the cells were shaken at 24 °C, 250 rpm until a predetermined length of time for each biotransformation had elapsed (indicated in Table 2). The cells were removed from the reaction culture via centrifugation, the supernatant was discarded and the cell pellet was re-suspended in 3 mL of water. The re-suspended cell-biomass was transferred to pre-weight 1.7 mL micro centrifuge tubes. The cells were then spun at 8500 rpm for 10 min, the supernatant was removed, and the tubes containing the isolated cell were weighted and their biomass recorded. The results obtained for the non-growing cells are shown in Table 4.

Table 3
Cell-biomass formation under growing conditions

BVMO variant	Biomass (mg)			Average biomass (mg)
	1	2	3	
WT-CHMO	192.9	191.9	192.5	192.4
1-K2-F5	213.8	203.6	208.4	208.6
WT-CPMO	188.3	185.1	186.0	186.5
B1 A10	169.1	165.6	168.3	167.7

All normalization reactions carried out for 24 h.

Table 4
Cell-biomass formation under non-growing conditions

BVMO variant (transformation time)	Biomass (mg)			Average biomass (mg)
	1	2	3	
WT-CHMO (24 h)	216.1	230.8	211.2	219.4
1-K2-F5 (24 h)	213.2	196.2	203.1	204.2
WT-CPMO (4 h)	172.4	172.6	171.9	172.3
B1 A10 (9 h)	167.0	164.6	165.4	165.7

The biomass of the cells used under non-growing conditions is very close to the biomass of the corresponding cells produced under growing conditions. The only exception is in the case of WT-CHMO where the quantity formed under growing conditions is consistently slightly smaller.

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