Identification and expression of a CHMO from the Pseudomonas aeruginosa strain Pa1242: application to the bioconversion of Cyrene™ into a key precursor (S)-γ-hydroxymethyl-butyrolactone

Louis Mouterde, Julien Couvreur, Maxime Langlait, Fanny Brunois, Florent Allais

Identification and expression of a CHMO from the *Pseudomonas aeruginosa* strain Pa1242: application to the bioconversion of Cyrene™ into a key precursor (*S*)-γ-hydroxymethyl-butyrolactone

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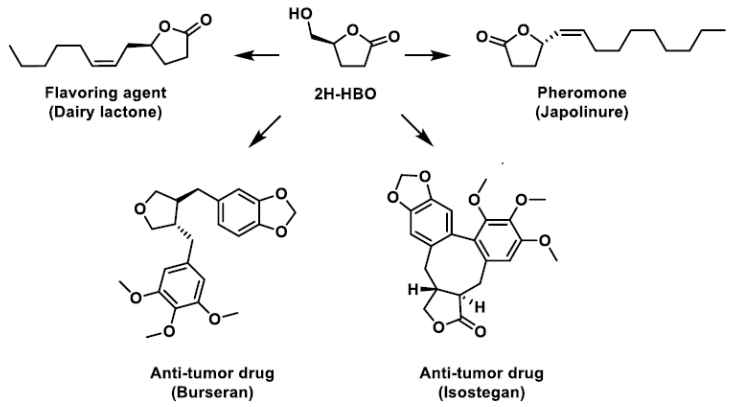
A sequence from the complete genome of *Pseudomonas aeruginosa* strain Pa1242 was identified to code for a potential new Cyclohexanone Monooxygenase (CHMO). The latter was discovered using the Basic Alignment Search Tool (BLAST) with the DNA sequence of the CHMO from *Acinetobacter sp.* NCIB 9871 as parent sequence considering that this enzymes family maintains a relatively high query cover (>95%) and identity percentage (>50%) among many different microorganisms. This was confirmed by the bioconversion of cellulose-based green solvent Cyrene™ into key precursor (*S*)-γ-hydroxymethyl-butyrolactone (**2H-HBO**) using a new strain of E. coli BL21(DE3) containing a plasmid designed for the overexpression of the unprecedent CHMO (pLM7). Besides confirming the CHMO activity of this novel sequence, this bioconversion allows the obtention of **2H-HBO** while maintaining the naturalness of the process. The optimal culture conditions were assessed through a Design of Experiment, and the control of the pH and O_2 level allowed to reach working concentration of 20 g/L of Cyrene™ with total conversion and a productivity rate of 401 ± 36 mg/L/h.

Introduction

The industrial revolution of the 18th century witnessed the emergence of the chemical industry. Relying on fossil resources, it quickly expanded and gave access to a broad variety of petro-based chemicals and materials that lead to many innovations and shaped the world as we know it. Nowadays, 70% and 30% of crude oil is used for energy and chemical industries, respectively.[1] Nevertheless, the increasing scarcity and price of such fossil resource jeopardizes the chemical industry, as well as the energetic and economic balance. Moreover, the processes that were developed often involved toxic reagents and harsh conditions which raised many concerns about environmental damages. In order to overcome these issues, sustainable strategies that use renewable resources, such as plant biomass, and/or green processes, such as biotechnological approaches, were developed. For the latter, the great progresses achieved in the past decades in term of DNA sequencing as well as molecular and synthetic biology allowed research teams to better understand and use the tools provided by Nature to design more sustainable chemical transformations.

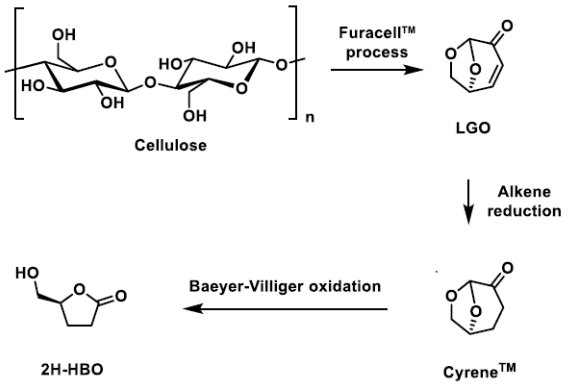
The biodiversity being quite vast and unexplored, new organisms, microorganisms or microorganisms' strains are regularly discovered. In 2017, Espinosa-Camacho *et al.* sequenced the complete genome of *Pseudomonas aeruginosa* strain Pa1242 that was found in children with Bacteremia.[2] These new 7,050,510 base pairs represent a great potential for the discovery of new enzymes. Among the potential enzymes of interest that could be found in this strain, one family of enzymes, that of the cyclohexanone monooxygenase (CHMO), a sub-family of Baeyer-Villiger monooxygenase (BVMO) that are flavin-dependent enzymes catalyzing the Baeyer Villiger oxidation of cyclic ketones to the corresponding lactones, is of particular interest. Various CHMOs are described in the literature, starting with the "original" one discovered by Trudgill *et al.* while studying the metabolism of cyclohexanol in *Acinetobacter sp.* NCIB 9871,[3] followed by the ones from *Nocardia globulera* CL1,[4] or from *Oceanicola batsensis* DSM 15984.[5]

(S)-γ-hydroxymethyl-butyrolactone (aka **2H-HBO**) is a highly valuable and renewable chiral chemical that is a key precursor for the synthesis of many bioactive compounds such as of the flavoring agent dairy lactone (butter, peach or fruity aroma depending on the concentration used),[6, 7] the pheromone of the Japanese beetle *Popillia japonica* (Japolinure),[8] or anti-tumor compounds (Burseran and Isostegane) (Scheme 1).[9]



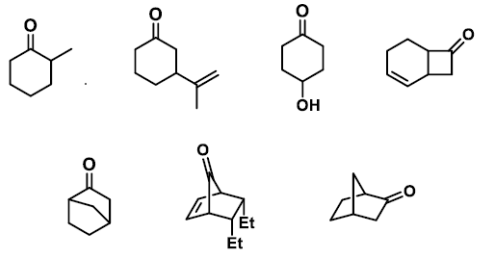
Scheme 1. Some applications of 2H-HBO as a building block

The most sustainable pathway developed to date yielding **2H-HBO** uses Levoglucosenone (**LGO**), a chiral molecule derived from the catalytic fast pyrolysis (CFP) of cellulose, as precursor.[10, 11] The reduction of the alkene moiety of **LGO** gives access to Cyrene™, an efficient REACH-compliant substitute for toxic dipolar aprotic solvents, such as NMP, DMF or sulfolane.[12] Cyrene™ can also be used as substrate to synthesize **2H-HBO** through Baeyer-Villiger oxidation (Scheme 2). While enzymatic approach was successfully used to perform the alkene reduction of **LGO** into Cyrene™, classical organic chemistry remains the only available tool to convert the latter into **2H-HBO**.[13] Koseki *et al.* reported the oxidation using the classic – yet hazardous and toxic – oxidizing agent for such reaction (*m*-CPBA). Later on, the procedure was optimized and greened up by Allais *et al.* by using only 30% aq. H_2O_2 (Scheme 2).[14-18] Nevertheless, despite being efficient in terms of yields and cost, this method can be hazardous to perform, notably at large scale, because of its wayward and strong exothermicity.



Scheme 2. The different applications of Cyrene® as green solvent

In this work, sequence alignment of the CHMO from *Acinetobacter sp.* NCIB 9871 with the complete genome of *Pseudomonas aeruginosa* strain Pa1242 was performed which highlighted a sequence for a potential new CHMO. To check this hypothesis, the latter was expressed in *Escherichia coli* BL21(DE3) strain and its suspected CHMO activity was investigated in an original process converting the bio-based cellulose-derived Cyrene™ to **2H-HBO**. This biotechnological approach would solve the limitation of the current procedures by offering a more sustainable and safer method. Moreover, although CHMOs have shown relatively good activity towards classic cyclic ketones (Scheme 3), it is the very first evidence of their activity towards bicyclic compounds containing an α-ketoacetal moiety.[19-21]



Scheme 3. Examples of cyclic ketones that can be efficiently oxidized by CHMOs

Results and Discussion

Identification of a new CHMO from *Pseudomonas aeruginosa* strain Pa1242. The protein sequence of the CHMO from *Acinetobacter sp.* NCIB 9871 was submitted to the Basic Local Alignment Search Tool (BLAST) using the NIH database on NCBI website. It resulted in 198 hits, all from the bacteria kingdom. The sequence that presented the best alignment score, outside the *Acinetobacter* family, corresponded to a sequence from the *Pseudomonas* family with a query cover of 97% and an identity percentage of 67%, resulting in a query cover/identity percentage ratio very representative of the CHMO family. Indeed, we obtain highly similar results comparing known CHMOs sequences from different bacteria strains (Table 1). Further investigation showed that the DNA sequence corresponding to the predicted NAD(P)/FAD-dependent oxidoreductase was found in the complete genome of *Pseudomonas aeruginosa* strain Pa1242.[2] However, no mention of expressing this enzyme nor its implementation to a biotransformation was ever reported in the literature.

Table 1. Protein sequence alignment results between CHMO from different bacteria strains*

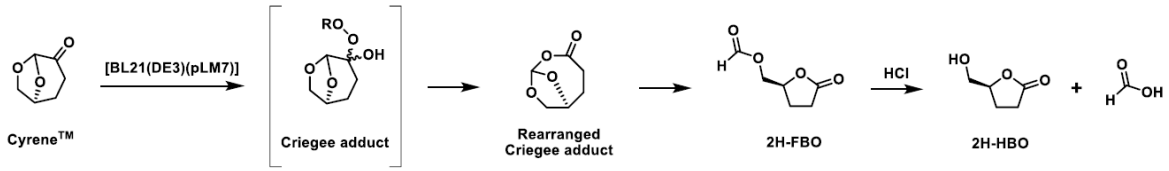
CHMO 1	CHMO 2	Query cover	Identity percentage
Acinetobacter sp. NCIB 9871	*Pseudomonas aeruginosa* strain Pa1242	97%	67%
Acinetobacter sp. NCIB 9871	*Oceanicola batsensis* DSM 15984	97%	63%
Acinetobacter sp. NCIB 9871	*Rhodococcus sp.* strain HI-31	96%	58%
Acinetobacter sp. NCIB 9871	*Arthrobacter sp.* BP2	96%	57%
Oceanicola batsensis DSM 15984	*Rhodococcus sp.* strain HI-31	97%	59%
Oceanicola batsensis DSM 15984	*Arthrobacter sp.* BP2	95%	59%
Rhodococcus sp. strain HI-31	*Arthrobacter sp.* BP2	99%	83%

* Protein sequence alignments were performed using BLAST on NCBI website

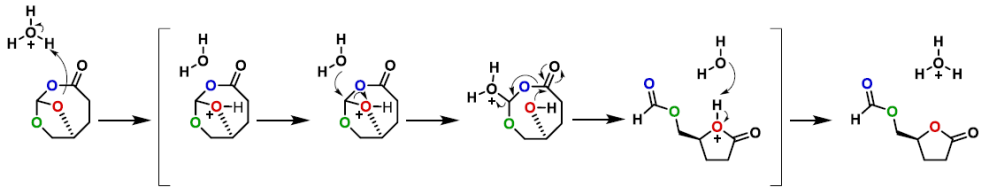
Construction of an *E. coli* overexpression system of the CHMO from *Pseudomonas aeruginosa* strain Pa1242. A plasmid containing the DNA sequence corresponding to the predicted NAD(P)/FAD-dependent oxidoreductase was designed. The *Escherichia coli* expression plasmid pET 22b (+) was used as vector and the sequence inserted between the restriction site NdeI and EcoRV, conferring to the enzyme a *N*-term Histidine tag. In this construct, protein expression is driven by the strong T7 promoter that can be controlled by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture medium. The final construct was synthesized by GenScript®. The latter was used to transform *E. coli* BL21(DE3) and therefore create a new strain ([BL21(DE3)(pLM7)]) that overexpresses the new CHMO at a level of ca. 20% of total protein.[22] It is noteworthy to mention that this level is five-fold higher than other *Escherichia coli* overexpression systems and ten-fold higher than typical expression levels in *Saccharomyces cerevisiae*.

Implementation of the Baeyer-Villiger oxidation of Cyrene™ using the engineered *Escherichia coli.* The [BL21(DE3)(pLM7)] *Escherichia coli* strain was cultured in Lysogeny broth (LB) medium (50 mL) in order to produce enough biomass and thus express a sufficient level of CHMO for the biotransformation. At an OD_{600} of 0.9, the overexpression was induced by IPTG, 10 mM Cyrene™ was then added to the culture medium and the total consumption of Cyrene™ was monitored using TLC analysis. However, LB being a nutritionally rich medium in terms of molecular diversity, the analysis of the crude reaction mixture through ^{1}H NMR is ruled out. Moreover, the use of such medium complicates the purification of the final product. In order to overcome these limitations, a switch in culture medium was performed after the first growth. At an OD_{600} of 0.9, cells were centrifuged and resuspended in a M9 minimal media composed of micronutrients and a nitrogen source (NH_4Cl). Overexpression was then induced by IPTG and 10 mM Cyrene™ was added to the culture medium (50 mL). After total consumption of Cyrene™ (TLC), ^{1}H NMR analysis of the crude reaction mixture revealed that Cyrene™ was converted into a mixture of the rearranged Criegee adduct and **2H-FBO**, the formate of **2H-HBO**. Upon acidification of the culture medium, these intermediates were readily hydrolyzed into **2H-HBO** (Scheme 4). It is noteworthy to mention that, after a simple evaporation of the hydrolyzed mixture, ^{1}H NMR revealed a purity of 95% without the need of any other purification step.

The unobvious rearrangement of the rearranged Criegee adduct into **2H-FBO** is water dependent and preferably occurs under acidic conditions. As described in Scheme 4, the oxygen of the orthoformate can be protonated, thus promoting the addition of water onto the activated carbon. The resulting alcohol can then attack the carbonyl of the intermediary compound resulting in the formation of a five membered ring and the opening of the eight membered ring with a loss of water. Finally, the deprotonation of the last intermediary give access to the **2H-FBO** (Scheme 5).



Scheme 4. Bioconversion of Cyrene™ into **2H-HBO**



Scheme 5. Proposed rearrangement mechanism of the rearranged Criegee adduct into **2H-FBO**

The aforementioned protocol was then tested with different concentrations of Cyrene™ (20, 40, 80 and 160 mM) in order to evaluate the maximum working concentration in a non-optimized medium. Full conversion was observed at 20 mM and only partial conversion was observed at 40 mM. However, it has to be noted that partial conversion was also observed at 160 mM which suggests that such concentration is not bactericidal and does not have a negative effect on CHMO overexpression (Figure 1).

Although a working concentration with total conversion of 20 mM is encouraging, data suggested that there was ground for optimization. To further optimize this novel CHMO-catalyzed Baeyer-Villiger oxidation of Cyrene™ into **2H-HBO** while working at higher concentrations, a Design of Experiments (DoE) was performed.

Optimization of the biotransformation. As we hypothesized that the conversion rate was directly linked to the CHMO overexpression, both glucose and IPTG concentrations, as well as the OD_{600} of induction were investigated through a DoE (Table 2). Because the rearranged Criegee adduct was not observed at 160 mM, the concentration of Cyrene™ was fixed at 80 mM (ca. 10 g/L).

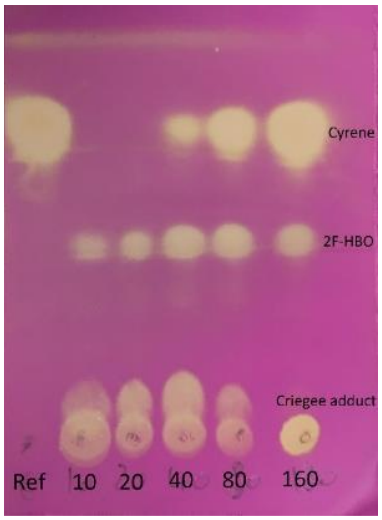


Figure 1. TLC analysis of the CyreneTM conversion at different concentrations 10-160 mM (Ref: CyreneTM) Some applications of **2H-HBO** as a building block

Table 2. Variables and their variation corresponding to defined levels of the DoE.

	Levels		
Variables	-1	0	1
Glucose	4	22	40
IPTG	0.1	0.3	0.5
OD_{600} of induction	2	3	4

To find a relationship between theses variables and response surface, the following second-order polynomial equation was used:

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_i \alpha_j x_i^2 + \sum_{ij} \alpha_{ij} x_i x_j \quad (\text{Eq. } 1)$$

where Y represents the response (Cyrene™ consumption), x_i are the variables, α_0 is a constant and α_i, α_j and α_{ij} are the linear, quadratic and interaction coefficients. Regression coefficients were determined by multiple linear regressions (MLR). The significant parameters in the model were found by analysis of their p-value (<0.05). The model validation was based on the variance for each response, namely, by the analysis of R_2, Q_2, and *lack of fit* (LOF) test. R_2 measures how well the regression model fits the experimental data, Q_2 shows an estimate of the future prediction precision, and LOF assesses whether the models' error is comparable to the replicate error. A Box Behnken design consisting in 15 experiments, including 3 three replications at the central point to evaluate their reproducibility, was used to determine the optimum set of experimental parameters to optimize the Cyrene™ consumption.

The experimental data of the Box Behnken design was fitted to the second-order polynomial equation (Eq. 1). Analysis of variance shows a good correlation of the second-order polynomial model between the response (conversion) and the significant variables ($p < 0.05$). The p-value of the regression model below 0.05 shows the statistical significance of the polynomial regression. The lack of fit ($p > 0.05$) shows the low replicate errors of the model. Finally, the design gives a very good coefficient of determination ($R_2 = 0.951 > 0.5$) and acceptable coefficient of cross-validation ($Q_2 = 0.719 > 0.5$) that corresponds to a good fit and prediction of the model. The figure 2 shows the coefficients (α_0 as constant, α_i, α_j and α_{ij} of the Eq. 1) of the model for the response. A positive value means a positive influence on the Cyrene™ consumption while a negative value means a negative influence.

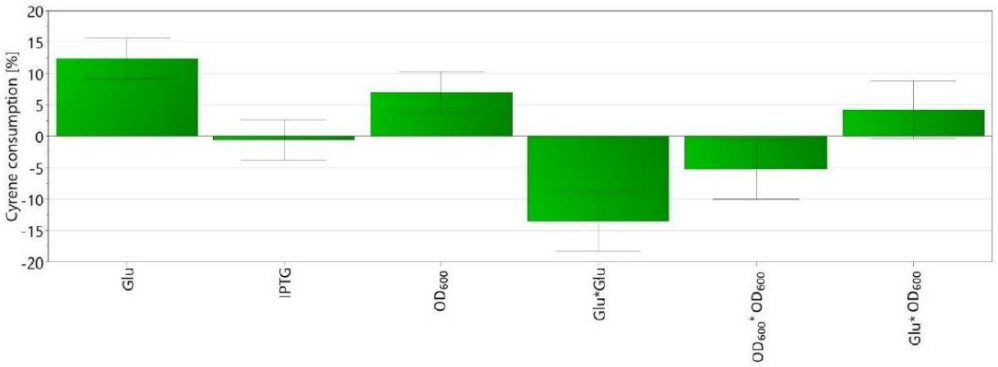


Figure 2. Regression coefficients of the quadratic model, Eq 1

Thereby, results show that the glucose concentration and OD_{600} of induction have a significative positive influence on the Cyrene™ consumption whereas IPTG concentration is not significant effect on the Cyrene™ consumption (p-value> 0.05). The equation of the model (Eq. 2) is as follows:

Cyrene™ consumption (%) = 72.61 – 12.375xGlu + 7xOD_{600} - 13x(Glu*Glu) – 5.326x(OD_{600}* OD_{600}) (Eq. 2)

The analysis of the 4D response contour (Figure 3) allowed the visualization of the influence of each parameter. In the interval considered and taking account of the process cost, the optimal glucose concentration and OD_{600} of inductions are evaluated at 20 g/L and 1.8, respectively. Applying these optimal conditions allowed a Cyrene™ consumption of ~75%.

Cyrene™ consumption being incomplete, another important factor was investigated, the carbon over nitrogen (C/N) ratio. Indeed, this ratio can greatly influence the overexpression of an enzyme and therefore a biotransformation efficiency. Optimal conditions obtained in the DoE were used with different concentration of NH_4Cl from 1 to 7.75 g/L (Table 3). However, no significant effect, either negative or positive, was observed on Cyrene™ consumption.

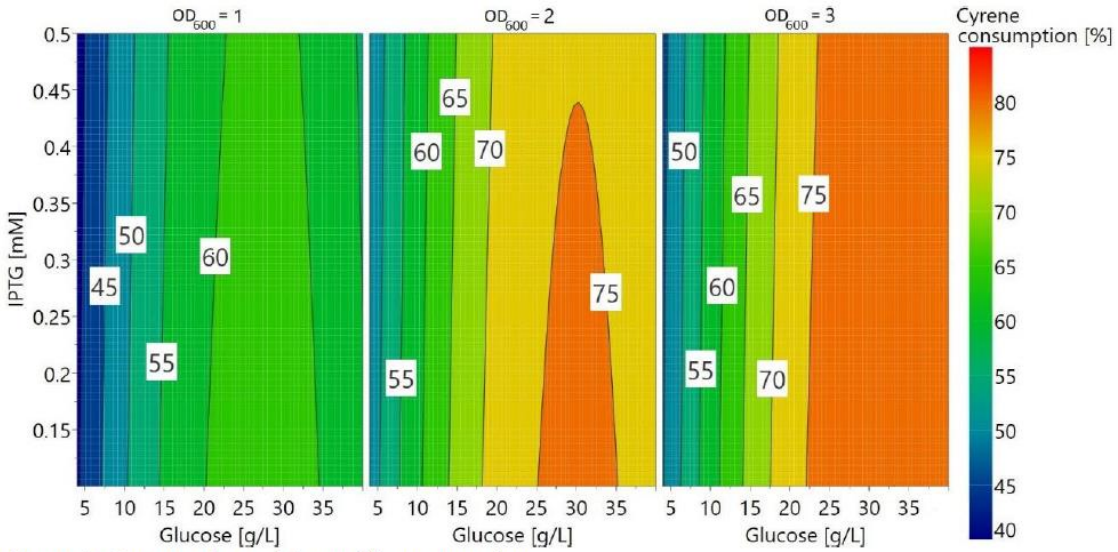


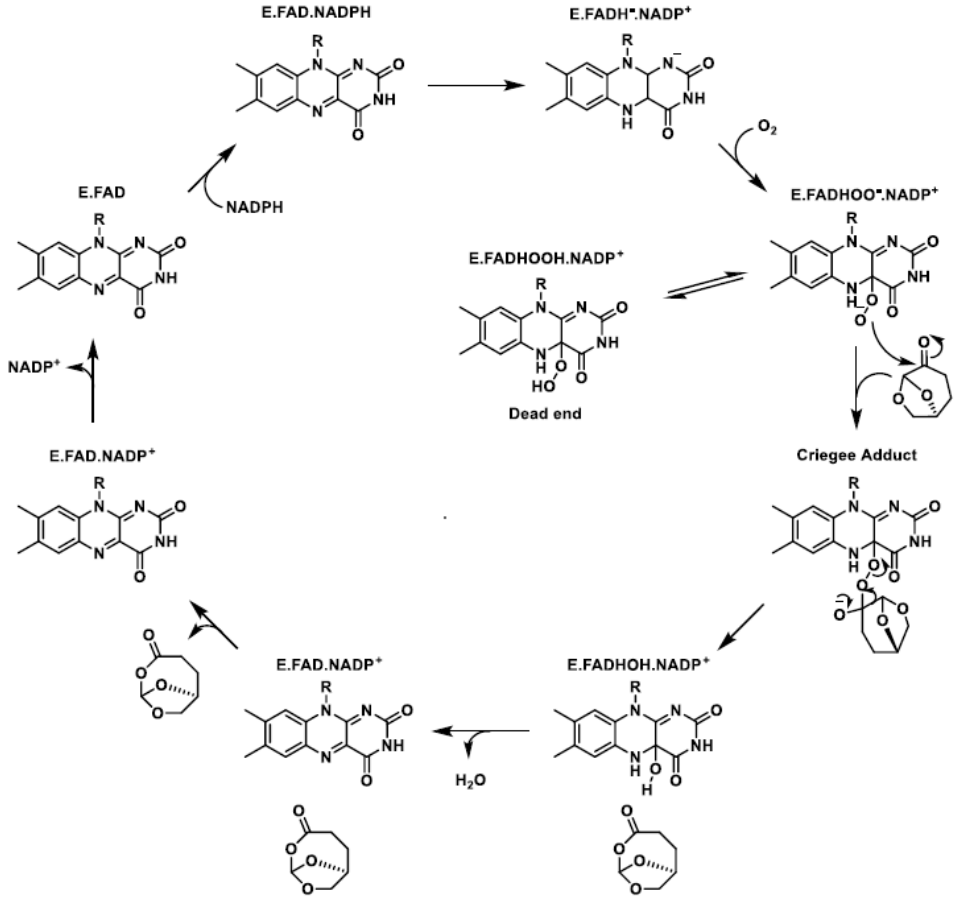
Figure 3. Contour plots of Cyrene™ consumption

Table 3. Effect of the variation of the C/N ratio on Cyrene™ consumption*

Entry	NH_4Cl (g/L)	Cyrene consumption (%)
1	1	77
2	3.25	75
3	5.5	76
4	7.75	73
5	10	73

*Concentration of Glucose and OD_{600} of induction were set at 20 g/L and 2, respectively

Implementation of optimized conditions and kinetic monitoring. After defining the optimal conditions to maximize Cyrene™ consumption, the kinetic of the reaction and the influence of oxygen on the latter were assessed. Indeed, the mechanism of the CHMO-catalyzed Baeyer-Villiger oxidation involves the activation of the oxygen by the prosthetic flavin adenine dinucleotide (FADH) in the active site (Scheme 6). Therefore, its level in the biotransformation medium should greatly influence the rate of the reaction.



Scheme 6. Mechanism of the conversion of Cyrene™ to the corresponding rearranged Criegee adduct in the active site of a CHMO

To verify this hypothesis, the biotransformation was carried in baffled and unbaffled flasks. Because a plateau was observed in the last hours of the experiment using baffled flasks, the productivity of each system was calculated on the first 8 hours. When the biotransformation was carried out in an unbaffled flask, Cyrene™ consumption initial rate was 368 ± 16 mg/L/h, when it was 494 ± 18 mg/L/h in a baffled flask (Figure 4). However, it is worthy to notice that the final concentrations of Cyrene™ were quite similar in both set of experiments (*c.a.* 2.5 g/L). Full conversion was observed when carrying the biotransformation at a concentration of 40 mM of Cyrene™ with an isolated yield of 90%.

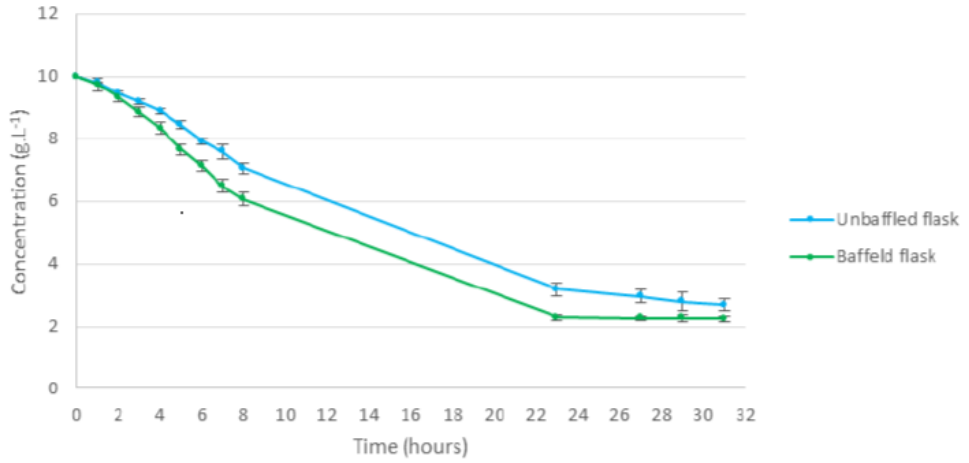


Figure 4. Influence of the oxygen level on the kinetic of Cyrene™ consumption. Results are average of two independent assays performed in duplicate, and error bars correspond to standard deviations.

The formation of a plateau in the last hours of the bioconversion at 80 mM in the baffled flask set of experiments suggests that an equilibrium was reached or that another factor hindered Cyrene™ oxidation. The pH of the culture was measured to be about 4 after 30 hours of incubation which leads to the death of [BL21(DE3)(pLM7)] cells and the denaturation of the CHMO. This value can be explained by the natural acidification of the culture media induced by the *E. coli* strain which will favorize the rearrangement of the rearranged Criegee adduct into **2H-FBO** and therefore the hydrolysis of the latter yielding **2H-HBO** and formic acid. This hypothesis was verified by carrying out the bioconversion in a fermenter with a bioconversion media volume of 1.15 L to be able to control the pH (regulation at 7.0) and validate the scalability of the process. Doing so, it was shown that the totality of the Cyrene™ was oxidized in 26 hours (Figure 5). It has to be noted that the pO_2 of the culture media was also regulated at 30% which appeared to be a good compromise to maintain a sufficient level of oxygen in the culture media for the good completion of the reaction and to limit foaming induced by the bubbling of O_2. The bioconversion was also carried out at an initial concentration of Cyrene of 20 g/L and total consumption was also observed in 52 hours. The Cyrene™ consumption rate was calculated to be 401 ± 36 mg/L/h.

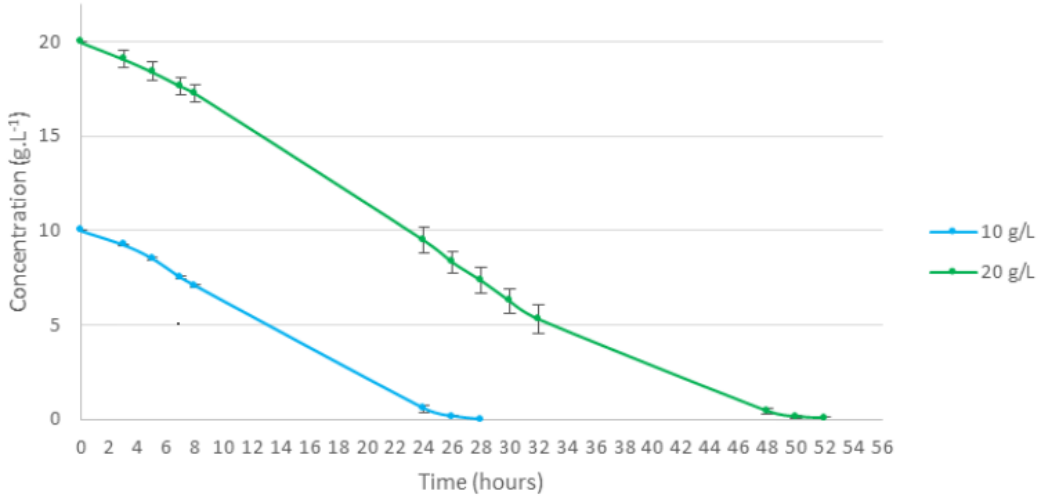


Figure 5. Cyrene™ consumption under pH regulation. Results are average of two independent assays performed in duplicate, and error bars correspond to standard deviations.

Green metrics: E factor and atom economy. The greenness of the process was evaluated using three different factors. The environmental factor (E factor, equation 1), expressed as kg of waster/kg of product, allows the evaluation the amount of waste generated during a reaction. The smaller the E factor is, the greener the reaction. Moreover, a simplified E factor (sEF, equation 2) which excludes solvents was also determined. Such

simplification was used to compare the transformation itself since biotransformations are usually carried out at significantly lower concentration than classical organic synthesis. Finally, the atom economy (AE, equation 3) was used to calculate how many atoms from the reagents actually end up in the desired product. These three factors can be calculated as follows:

Equation 1. $$E\ factor = \frac{\sum m(reagents) + \sum m(solvents) - m(product)}{m(desired\ product)}$$

Equation 2. $$sEF = \frac{\sum m(reagents) - m(desired\ product)}{m(desired\ product)}$$

Equation 3. $$AE = \frac{MW(desired\ product)\ x\ 100}{\sum MW(reagents)}$$

The data provided by Koseki *et al.*, Flourat *et al.*, Bonneau *et al.* and the data reported in this manuscript, allowed us to calculate these three metrics normalized by considering that the reactions were carried out on 5 g of Cyrene™ (Table 4). These calculations highlighted the greenness of the process described in this manuscript. Indeed, the atom economy of the Baeyer Villiger oxidation is the best in the whole cell system (N.B.: Glucose and
IPTG were not included in the calculation as their purpose is to feed the cells and to induce the overexpression of the CHMO, respectively). However, the E factor shows the limitations of biotransformation processes with a value 16.75 times higher than the chemical process described by Bonneau *et al*. That said, when one looks at the sEF value (i.e., the one excluding the solvents), the whole cells process is the greenest one. In addition to these values, it has to be noted that the whole cell process allows the access to a natural pathway towards 2H-HBO, which is not the case for the other processes. Such a "natural process" can be a major advantage that is more and more sought in industrial sectors such as cosmetic or food industries.

Table4. Calculation of the E-factor, sEF and AE for the different processes

Entry	Procedure	E-factor	sEF	AE (%)
1	Koseki *et al.*	18.07	1.57	41.1
2	Flourat *et al.*	26.65	10.47	58.4
3	Bonneau *et al.*	3.82	2.01	71.6
4	This procedure	63.95	1.46	90.6

Conclusion

A new CHMO from *Pseudomonas aeruginosa* strain Pa1242 was identified *via* the Basic Alignment Search Tool (BLAST) using the DNA sequence coding for the CHMO from *Acinetobacter sp*. NCIB 9871 as parent sequence. This identification is the result of a correlation found between the query cover and the identity percentage among CHMO sequences published in the literature. It has in fact been observed that many known CHMOs have very similar length (query cover > 95%) and that they are sharing at least an identity percentage of 50%. It was therefore hypothesized that sequences with unknown activity that presented such characteristics had a high potential to be CHMOs sequences. Such sequence was found in the complete genome of *Pseudomonas aeruginosa* strain Pa1242 (Query cover and identity percentage of 97% and 67% respectively). A plasmid was constructed with the corresponding DNA sequence and transformed into *E. coli* BL21(DE3) in order to verify the hypothesis. This was confirmed by the successful and unprecedent bioconversion of Cyrene™ into the corresponding rearranged Criegee adduct by the new strain [BL21(DE3)(pLM7)].
It was shown that the rearranged Criegee adduct can then be transformed into **2H-FBO**, then **2H-HBO**, by the addition of HCl to the media. The optimal culture conditions were assessed through a Design of Experiments and determined to be of 20 g/L and 1.8 for the glucose concentration and the OD_{600} of inductions, respectively, giving a maximum conversion rate of Cyrene™ into a mixture of the rearranged Criegee adduct, **2H-FBO** and **2H-HBO** of ~75%. It was later determined that full conversion was not obtained due to the natural acidification of the culture media from the metabolism of the *E. coli* strain that induced a snowball effect with the rearrangement of the rearranged Criegee adduct into **2H-FBO** that is then hydrolyzed into **2H-HBO** and formic acid. Although this acidification represents an important limitation for the scale up of the bioconversion, it shows that the conversion of the rearranged Criegee adduct into **2H-HBO** is possible without affecting the naturalness of the process which makes it an important step forward towards natural enantiomerically pure dairy lactone. In order

to scale up the bioconversion by reaching working concentration higher than 2.5 g/L, the latter was carried out into a fermenter in order to monitor and adjust the pH over time. It was therefore possible to reach a 20 g/L working concentration with a productivity rate of 401 ± 36 mg/L/h. The scale up of the bioconversion did not affect significantly the productivity but allowed us to triple the working concentration, which is not obvious considering that both the substrate, intermediaries and the product a not naturally occurring products and could have been toxic for the *E coli* strain (Figure 6). Indeed, examples in the literature show the necessity of important metabolic modifications on *E. coli* production strains to reach such concentration, even for naturally occurring products.[23-26]

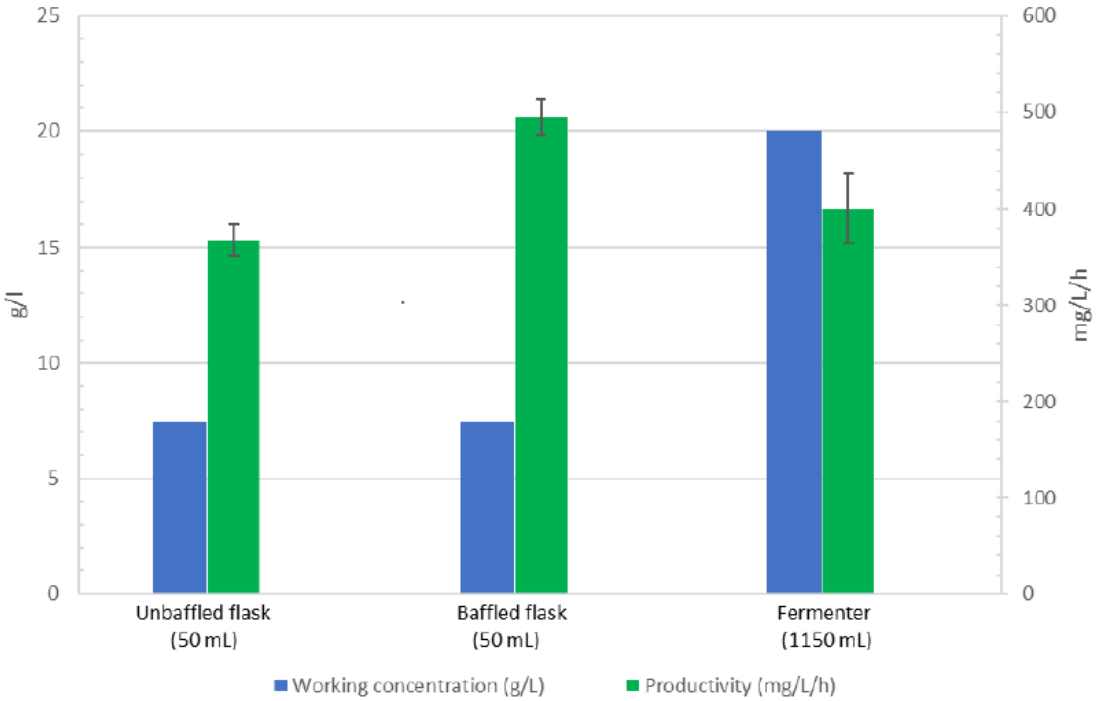


Figure 6. Working concentration and productivity of Cyrene™ consumption on different operating systems (Unbaffled flask, baffled flask and fermenter).

Acknowledgements

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Author contributions

FA and LMMM: conceived the research. LMMM: supervised the research. LMMM: performed the bioinformatic work, designed the DNA plasmid and created the new strain [BL21(DE3)(pLM7)]. LMMM: implemented the Baeyer-Villiger oxidation of CyreneTM using the engineered Escherichia coli strain, designed and performed the DoE for the optimization of the bioconversion conditions. LMMM and JC: performed the scale up of the bioconversion from flask to fermenter scale. FB and MMJL: performed the HPLC analyses. LMMM: provided the technical guidelines. LMMM: reviewed the results. LMMM: wrote and drafted the article. FA and LMMM: reviewed and approved the article.

Experimental Section

General. Evaporations were conducted under reduced pressure at temperatures below 40 °C unless otherwise noted. TLC analysis were performed on silicagel 60 F_{254} from Merck using ethyl acetate as mobile phase unless otherwise noted. ^{1}H NMR spectra were recorded at 300 MHz at 25 °C in the indicated solvent and referenced to residual protons ($CDCl_3$, 7.26 ppm). ^{13}C NMR spectra were recorded at 75 MHz at 25 °C in the indicated solvent and referenced to residual protons ($CDCl_3$, 77.16 ppm). Cyrene™ was a generous gift from Circa Group Ltd, Australia.

HPLC/MS method. The HPLC analyses were performed on a Dionex Ultimate 3000 (Dionex Corporation, USA) system equipped with a VWD 3400 UV/VIS detector and RI 101 (Shodex) refractive index detector. Chromatograms were recorded and processed with Chromeleon 6.8 Software. The components were separated by an Aminex HPX-87H (9 µm, 300 x 7.8 mm; BioRad) column heated at 50 °C. The analysis was

performed in isocratic mode with sulphuric acid at 4 mM during 30 min at 0.5 mL/min. The sample injection volume was 20 µL. The UV acquisition was carried out at 210 nm. The refractive index detector was set on positive polarity. Glucose, Cyrene™, rearranged Criegee adduct, **2H-FBO** and **2H-HBO** were observed with a retention time of 11.12, 17.45, 21.93, 22.95, 22.48 min, respectively, with RI detector.

Identification of the CHMO from *Pseudomonas aeruginosa* strain Pa1242 amino acid sequence. The amino-acids sequence of the CHMO from *Acinetobacter sp.* NCIB 9871 was submitted to the Basic Local Alignment Search Tool (BLAST) of the NCBI website (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi). Sequences presenting an identity percentage and query coverage respectively greater than 59% and 95% were selected for further investigation. A sequence of a predicted NAD(P)/FAD-dependent oxidoreductase from *Pseudomonas aeruginosa* strain Pa1242 was selected.

Creating an overexpression plasmid for the CHMO from *Pseudomonas aeruginosa* strain Pa1242. The DNA sequence corresponding to the predicted NAD(P)/FAD-dependent oxidoreductase from *Pseudomonas aeruginosa* strain Pa1242 was inserted into a pET-22b(+) vector in between the NdeI and EcoRV restriction sites. The resulting construct was synthesized by the company GenScript® and name pLM7. The plasmid pLM7 was used to transform *E. coli* BL21(DE3) cells with selection for ampicillin resistance (50 µg/mL) on LB plates and the new strain was designated [BL21(DE3)(pLM7)].

Negative control for the biotransformation of Cyrene™ to 2H-HBO in LB medium. A single colony of BL21(DE3) was used to inoculate a 2 mL portion of liquid LB medium containing 50 µg/mL ampicillin and the culture was grown overnight at 37 °C and 220 rpm. An aliquot (500 µL) was diluted into 50 mL of the same medium in a 150 mL baffled flask. The culture was grown at 37 °C with shaking at 250 rpm until reaching an OD_{600} of 0.9. The desired protein was overexpressed by adding sterile isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and Cyrene™ was added to the culture at a final concentration of 10 mM. The culture was incubated for an additional 24 hours at 30 °C and the absence of reaction was confirmed by TLC and HPLC.

Method for the biotransformation of Cyrene™ to 2H-HBO in LB medium in flasks. A single colony of [BL21(DE3)(pLM7)] was used to inoculate a 2 mL portion of liquid LB medium containing 50 µg/mL ampicillin and the culture was grown overnight at 37 °C and 220 rpm. An aliquot (500 µL) was diluted into 50 mL of the same medium in a 150 mL baffled flask. The culture was grown at 37 °C with shaking at 250 rpm until reaching an OD_{600} of 0.9. The desired protein was overexpressed by adding sterile isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and Cyrene™ was added to the culture at a final concentration of 10 mM. The culture was incubated for an additional 24 hours at 30 °C and the completion of the reaction was confirmed by TLC.

Method for the biotransformation of Cyrene™ to 2H-HBO in M9 minimal media in flasks. A single colony of [BL21(DE3)(pLM7)] was used to inoculate a 2 mL portion of liquid LB medium containing 50 µg/mL ampicillin and the culture was grown overnight at 37 °C and 220 rpm. An aliquot (500 µL) was diluted into 50 mL of the same medium in a 150 mL baffled flask. The culture was grown at 37 °C with shaking at 220 rpm until reaching an OD_{600} of 1.8, 2, 3 or 4. The cells were harvested by centrifugation, the LB discarded and the cells pellet resuspended in M9 minimal media with different glucose concentrations (4, 20, 22 or 40 g/L). The desired protein was overexpressed by adding sterile isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1, 0.3 or 0.5 mM and Cyrene™ was added to the culture at a final concentration of 80 mM. The culture was incubated for an additional 24 hours at 30 °C and the advancement of the reaction was followed by HPLC. The pH was measured to be 4.5 at the end of the reaction (24 hours) due to the formation of acids by *E. coli* and the release of formic acid by 2H-FBO.

Method for the biotransformation of Cyrene™ to 2H-HBO in M9 minimal media in fermenters. A single colony of [BL21(DE3)(pLM7)] was used to inoculate a 10 mL portion of liquid LB medium containing 50 µg/mL ampicillin and the culture was grown overnight at 37 °C and 220 rpm. The culture was diluted into 1 L of the same medium in a 2 L baffled flask. The culture was grown at 37 °C with shaking at 220 rpm until reaching an OD_{600} of 2.07, cells were harvested by centrifugation, the LB discarded and the cells pellet resuspended in 1.15 L of M9 minimal media (OD_{600} = 1.8) with a glucose concentration of 20 g/L. The desired protein was overexpressed by adding sterile isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM and Cyrene™ was added to the culture at a final concentration of 80 or 160 mM. The culture was incubated for an additional 28 to 52 hours at 30 °C and the advancement of the reaction was followed by HPLC.

Method for the hydrolysis of the rearranged Criegee adduct into 2H-HBO. Culture media was centrifuged, the cell pellet discarded, the supernatant concentrated (C = 0.5 M relatively to Cyrene™) and acidified by addition of concentrated HCl until pH 2. The resulting mixture was kept under magnetic stirring at 60 °C for 16 hours, then

dried under vacuum. The resulting solid was triturated with ethyl acetate (50 mL) and the resulting liquid fraction was dried under vacuum and the crude product analyzed by ^{1}H NMR with a 90% yield.

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