Biocatalysis and Biotransformation

ISSN: 1024-2422 (Print) 1029-2446 (Online) Journal homepage: http://www.tandfonline.com/loi/ibab20

Rapid conversion of cyclohexenone, cyclohexanone and cyclohexanol to ε-caprolactone by whole cells of Geotrichum candidum CCT 1205

André L. Patrício Silva, Poliane K. Batista, Augusto D. Filho, Claudionor S. do Nascimento Junior, Julio S. Rebouças & Juliana A. Vale

To cite this article: André L. Patrício Silva, Poliane K. Batista, Augusto D. Filho, Claudionor S. do Nascimento Junior, Julio S. Rebouças & Juliana A. Vale (2017): Rapid conversion of cyclohexenone, cyclohexanone and cyclohexanol to ε-caprolactone by whole cells of Geotrichum candidum CCT 1205, Biocatalysis and Biotransformation, DOI: 10.1080/10242422.2017.1310207

To link to this article: http://dx.doi.org/10.1080/10242422.2017.1310207

Published online: 18 Apr 2017.

BIOCATALYSIS AND BIOTRANSFORMATION, 2017
http://dx.doi.org/10.1080/10242422.2017.1310207

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Rapid conversion of cyclohexenone, cyclohexanone and cyclohexanol to ε-caprolactone by whole cells of *Geotrichum candidum* CCT 1205

André L. Patrício Silva, Poliane K. Batista, Augusto D. Filho, Claudionor S. do Nascimento Junior, Julio S. Rebouças and Juliana A. Vale

Departamento de Química, Universidade Federal da Paraíba, João Pessoa, Brazil

ABSTRACT

ε-Caprolactone (ε-CL) was obtained with excellent conversion and short reaction times from the substrates cyclohexenone, cyclohexanone and cyclohexanol using whole cells of Brazilian *Geotrichum candidum* (CCT 1205). The reactions were monitored over time by gas chromatography, and the intermediates of the one-pot cascade biotransformation involving reductions of C=C and C=O bonds as well as the Baeyer–Villiger oxidation were identified and quantified. The Baeyer–Villiger monooxygenase (BVMO) enzyme was predominant, and all three substrates were completely converted into ε-CL. Furthermore, the whole cells of *Geotrichum candidum* were recycled and reutilized in the biotransformation of cyclohexanone, producing ε-CL at least six consecutive times without a significant loss of activity, reaction yields or product purity.

ARTICLE HISTORY
Received 16 August 2016
Revised 26 December 2016
Accepted 16 February 2017

KEYWORDS
Biotransformation; Baeyer–Villiger monooxygenase; ε-caprolactone; *Geotrichum candidum* and cascade reaction

Introduction

The oxidation of ketones through the Baeyer–Villiger reaction is of extreme importance in chemical synthesis (Punniyamurthy et al. 2005) and industrial processes (Leisch et al. 2011). For example, the industrial oxidation of cyclohexanone produces the ε-caprolactone (ε-CL) monomer, which is used in the manufacture of poly-caprolactone, a polymer that is used in the manufacture of adhesives, dyes and compounds used for environmental protection and medical purposes due to its biocompatibility, non-toxicity and biodegradability (Weissermel and Arpe 2003).

The high interest in this reaction is due to its high chemo-, stereo- and enantioselectivity control (Brink et al. 2004). The most studied chemical method for the oxidation of ketones uses peracids, particularly m-chloroperbenzoic acid (Hussain et al. 2014; Yachnin et al. 2014); however, a search for safer, more selective and environmentally friendly methods is underway. Biotransformation has emerged as an alternative and efficient method for the synthesis of lactones and esters by transformation from different substrates by the Baeyer–Villiger monooxygenases (BVMOs), which are found mainly in several microorganisms (Ratuś et al. 2009). BVMOs are known as flavoenzymes and require NADPH for their activity (Mihovilovic et al. 2006; Cuetos et al. 2012).

Carballeira et al. (2002) achieved good results in a 72-hour reaction by applying growing and immobilized cells of *Geotrichum candidum* NCYC49 for the production of ε-CL from cyclohexanone. In a similar experiment, Mandal et al. (2002) applied whole cells of *Fusarium* sp. and obtained quantitative yields of ε-CL (30% wet weight) after 48 hours of reaction.

Starting from cyclohexanol as a substrate, ε-CL is obtained by two combined biocatalytic oxidation steps. The first step involves the catalytic alcohol dehydrogenase (ADH) enzyme and provides an intermediary cyclohexanone, while the $NADP^+$ cofactor (oxidized form) is converted to NADPH (reduced form). In a second oxidation reaction, cyclohexanone is then transformed *in situ* to yield ε-CL by nucleophilic oxygenation in the presence of BVMO and NADPH (Mallin et al. 2013).

Staudt et al. (2013), using an ADH from *Lactobacillus kefir* and BVMO from *Acinetobacter calcoaceticus*, with both enzymes recombinantly overexpressed in *E. coli*, showed that the desired product, ε-CL, was obtained with greater than 94% conversion when operating at a cyclohexanol concentration in the range of 20–60 mM.

With enone substrates, reduction of the C=C double bond catalysed by enoate reductase in the presence of reduced nicotinamide cofactor NADPH was

CONTACT Juliana A. Vale julianadqf@yahoo.com.br Departamento de Química, Universidade Federal da Paraíba, João Pessoa, PB 58051-970, Brazil

observed (Paul et al. 2013). Using recombinant cells in the Baeyer–Villiger biooxidation, it was observed that the bioreduction reaction of the double bond of enones occurs prior to oxidation to the corresponding lactone (Mihovilovic et al. 2006).

In this study, the self-sufficient Baeyer–Villiger biotransformation of three substrates was evaluated (cyclohexenone, cyclohexanone and cyclohexanol) for the synthesis of ε-CL using whole cells of *Geotrichum candidum* from the Brazilian tropical culture collection (CCT 1205) as biocatalysts. The advantage of using whole cells is that they make the process less complex than the use of genetically modified biocatalysts, while eliminating the use of synthetic cofactors, which are very expensive. Cascade biotransformations via oxidation and reduction processes were also evaluated by monitoring the time courses on each of the substrates.

Materials and methods

Chemicals

Cyclohexenone, cyclohexanone, cyclohexanol and ε-CL were obtained from Sigma-Aldrich (St. Louis, MO). Glucose, malt extract, yeast extract and peptone were obtained from HIMEDIA (Mumbai, India), and ethyl acetate and other solvents were obtained from TEDIA (Fair Lawn, NJ).

Analytical

Chromatographic analyses were performed with a GC-QP2010-Shimadzu chromatograph equipped with an FID detector using an Rtx-5 fused capillary column (30 m × 0.25 mm ×0.25 mm film thickness) and N_2 as a carrier gas (1 mL/min). The used heating gradient was 60 °C (1 min), 100 °C (35 °C/min), 150 °C (25 °C/min), and 240 °C (45 °C/min).

Microorganisms and culture conditions

Geotrichum candidum CCT 1205, which was used in this study, was acquired from the CCT collection (CCT-Coleção de Cultura Tropical de Pesquisas, Fundação André Toselo, Campinas, Brazil) and grown in an Erlenmeyer flask (500 mL) containing 250 mL of autoclaved yeast extract medium YM (D-glucose 10.0 g L^{-1}), malt extract (3.0 g L^{-1}), yeast extract (3.0 g L^{-1}) and peptone (5.0 g L^{-1}). The inoculum was grown under orbital shaking (200 rpm) for 48 hours. The *Geotrichum candidum* cells were separated from the broth by centrifugation (2500 rpm) for 10 min, and the biomass was washed twice with distilled water to remove residual culture medium.

Reaction methodologies

The washed *Geotrichum candidum* biomass was weighed and placed into an Erlenmeyer flask (125 mL) containing 10 mL of phosphate buffer (pH 6.5), and 10 μL (∼100 μmol) of the described substrates was added. The flasks were incubated at 28 °C and 180 rpm in an orbital shaker. The aliquots of the reaction mixture were extracted with ethyl acetate, and the organic phases were analysed using gas chromatography. Recycling and reuse tests of the *Geotrichum candidum* cells were conducted by filtering the cells and washing them three times with distilled water after each reaction. All experiments were performed in triplicate.

Results and discussion

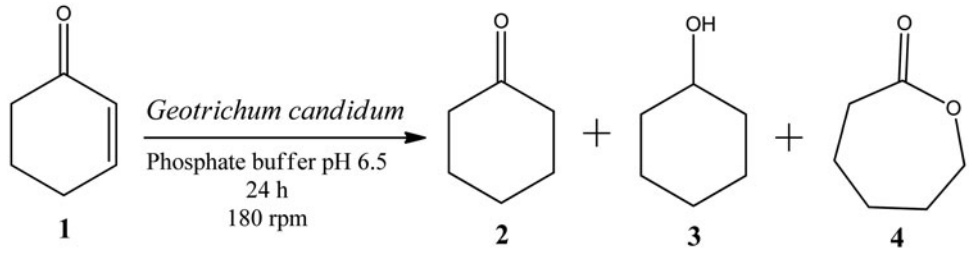
Preliminarily, the biotransformation of cyclohexenone (**1**) was studied to evaluate the cascade reaction through one-pot reduction and oxidation processes. In the initial reactions, two culture media (YM and Sabouraud) were tested based on the wet weight of the cells of *Geotrichum candidum* CCT 1205.

The reactions only achieved substrate conversion to ε-CL in YM culture media. The presence of glucose inhibits oxidation reactions, and only reduction products of the C=C and C=O bonds are obtained. To optimize the results, several tests were carried out with variations of the whole-cell concentrations of *Geotrichum candidum* and the substrate concentration. The reaction progress was monitored using gas chromatography with commercial standards, and the results are shown in Table 1.

As seen in Table 1, the complete biotransformation of cyclohexenone (**1**) is slow at high substrate concentrations (entries 2 and 3), and only ene-reductase (ERED) and ADH enzymatic activities were observed, which predominantly indicates intermediates **2** and **3**. In contrast, when the proportion of whole cells was increased (entry 4), BVMO was significantly more active, and substrate **1** was completely converted to ε-CL (**4**).

The reaction time of 24 hours to evaluate the concentration of product **4** was chosen arbitrarily and is significantly longer than the time required to convert **1** to **4**. To better understand the catalytic activity of the cascade reaction and to evaluate the concentrations of the intermediates as a function of time during the biotransformation, a time course of the reaction

Table 1. Biotransformation of cyclohexenone under various conditions.



Entry[a]	Biomass[b] (g)	Substrate (mM)	**1** (%) cyclohexenone	**2** (%) cyclohexanone	**3** (%) cyclohexanol	**4** (%) ε-CL
1	1.0	3.1	0	67	14	18
2	1.0	10.3	63	33	4	0
3	2.0	10.3	29	62	9	0
4	3.0	10.3	0	0	0	100

[a]The conversion percentage was determined using gas chromatography.
[b]Biomass refers to the wet weight of the cells.

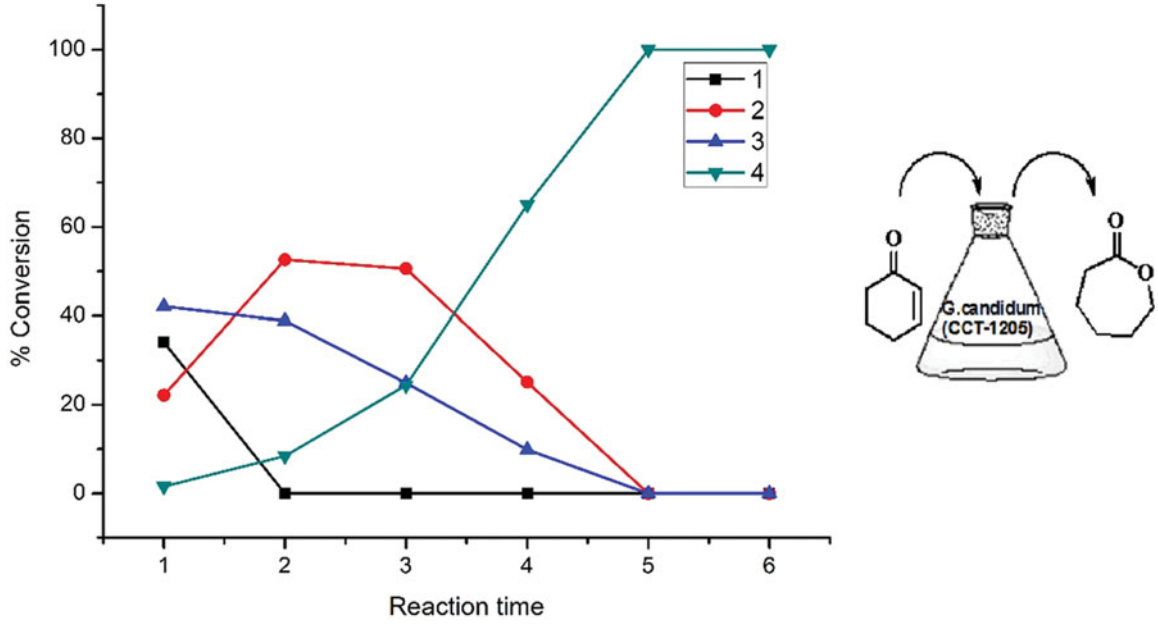


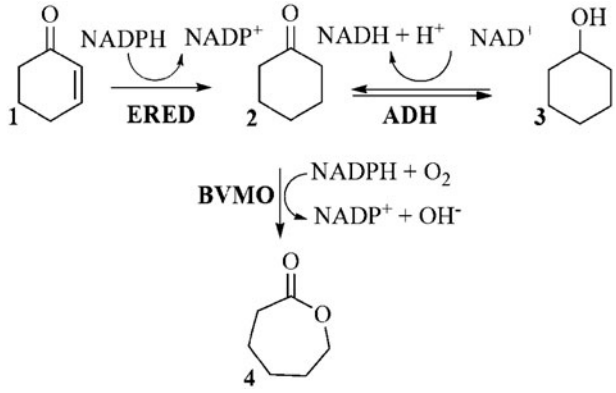
Figure 1. Reaction time course of the biotransformation of cyclohexenone. Conditions: 3 g of whole cell biomass, 10 μL of substrate, 10 mL of pH 6.5 phosphate buffer, six hour reaction time, and agitation at 180 rpm. Conversion of intermediates and product was monitored by gas chromatography.

using entry 4, but with a last reaction time of six hours, was determined using gas chromatography to monitor aliquots extracted from the reaction medium every hour (Figure 1).

As seen in Figure 1, first, the C=C enone endocyclic bond was reduced by ERED enzyme, and then the C=O bond was reduced by ADH enzyme (Scheme 1). After two hours of reaction, substrate **1** was completely consumed, and ε-CL (**4**) concentrations increased due to the subsequent oxidation of intermediates **2** and **3** by ADH and BVMO, respectively. Throughout the reaction, the concentration of product **4** increased as the concentration of intermediates **2** and **3** decreased, and after five hours of reaction, only the desired product ε-CL (**4**) was obtained, with quantitative yield. The kinetic profile shows that the conversion of **1** to **4** was extremely fast, requiring only five hours of reaction time. Our results corroborate the results of Carballeira et al. (2002), which showed that the production of caprolactone involves an equilibrium between intermediates **2** and **3**. The yields obtained using the one-pot cascade biotransformation described here are much higher than those obtained by Mihovilovic et al. (2006), who obtained only 52% ε-CL conversion in 48 hours using BVMO expressed in *E. coli*.

To better understand the equilibrium between intermediates **2** and **3** during the conversion of ε-CL,

the biotransformation from cyclohexanone (**2**) and cyclohexanol (**3**) as substrates using *Geotrichum candidum* cells was studied. First, the best conditions for the biotransformation of **2** to **4** were determined. This reaction occurred extremely quickly when only 1 g of whole cell biomass and 10 μL of the substrate were used, with a quantitative yield of ε-CL in just four hours of reaction time. These results were much better than those reported by Carballeira et al. (2002), who obtained ε-CL (**4**) with quantitative yield in 72 hours using a strain of immobilized *Geotrichum candidum* NCYC49 in various matrices. The kinetic profile of this biotransformation was determined using gas chromatography by monitoring aliquots extracted from the reaction medium every 30 min (Figure 2).

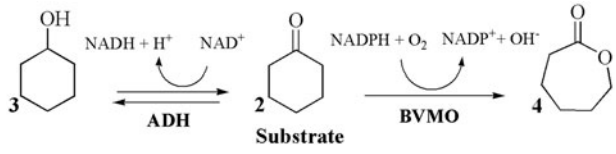


Scheme 1. Microbial oxidation and reduction of cyclohexenone. The product of the Baeyer–Villiger oxidation is caprolactone.

As seen in Figure 2, intermediate **3** was observed within 30 min by ADH activity, and after only one hour, product **4** was observed by BVMO activity. Similar to Carballeira et al. (2002), an equilibrium was observed between substrate **2** and intermediate **3** during the first hours of the biotransformation reaction. Oxidation occurred simultaneously through ADH and BVMO enzymatic activity in the reaction medium (Scheme 2). ε-Caprolactone (**4**) was formed rapidly in quantitative yield within four hours.

In the chemical industry, ε-CL is synthesized via the oxidation of cyclohexanone; however, recent studies have shown that ε-CL can also be produced via the bio-oxidation of cyclohexanol. For example, Mallin et al. (2013) used PDH and CHMO, which are expressed in *E. coli*.

The biotransformation of cyclohexanol to ε-CL using whole cells of *Geotrichum candidum* (CCT 1205) is extremely rapid, and only 1 g of biomass and 10 μL of substrate are needed for the exclusive formation of ε-CL in just four hours. The course of this reaction was also determined and is shown in Figure 3.



Scheme 2. Microbial oxidation and reduction of cyclohexanone. The product of the Baeyer–Villiger oxidation is caprolactone.

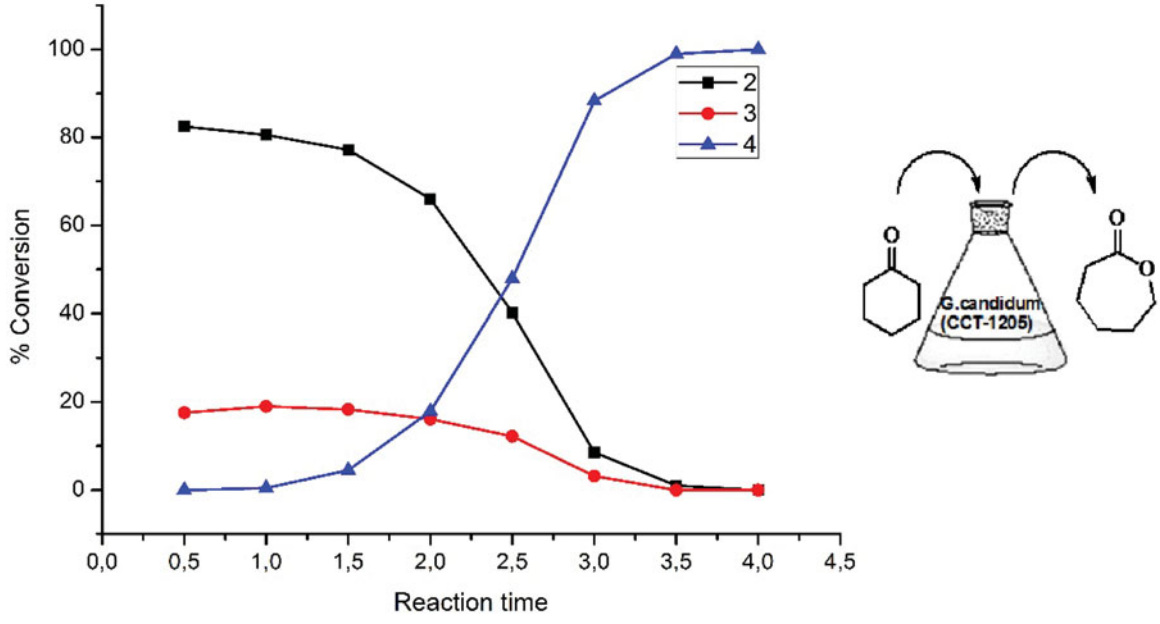


Figure 2. Reaction time course of the biotransformation of cyclohexanone. Conditions: 1 g of whole cell biomass, 10 μL of substrate, 10 mL of pH 6.5 phosphate buffer, four hour reaction time, and agitation at 180 rpm. Conversion of intermediates and product was monitored by gas chromatography.

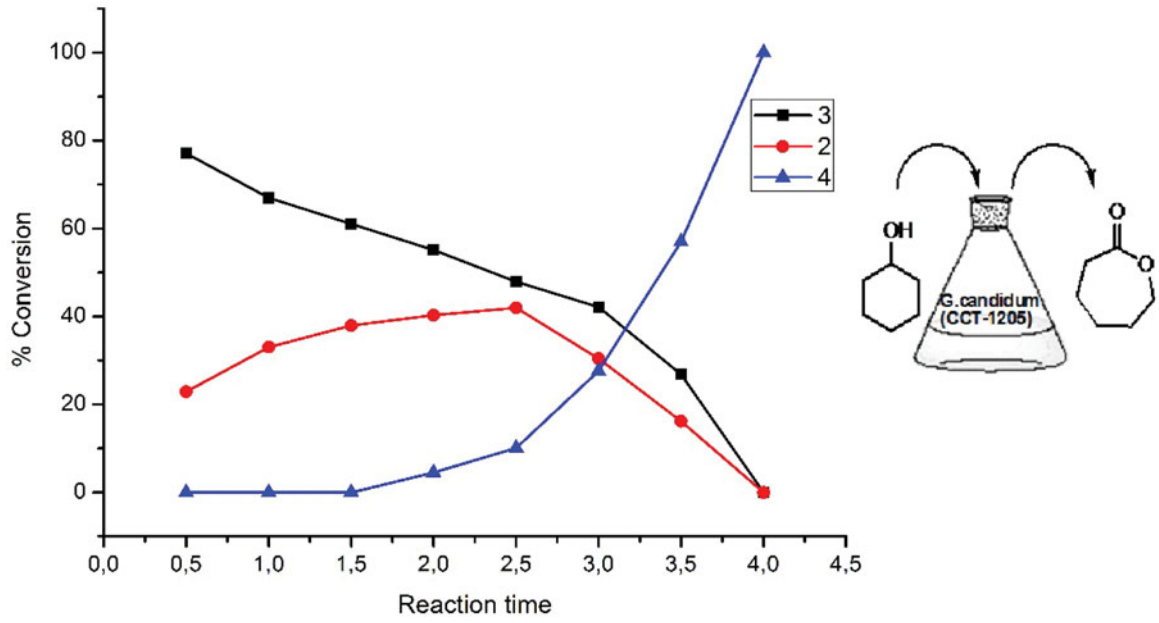
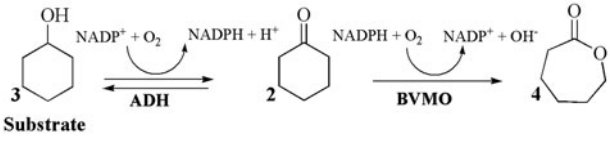


Figure 3. Reaction time course of the biotransformation of cyclohexanol. Conditions: 1 g of whole cell biomass, 10 μL of substrate, 10 mL of pH 6.5 phosphate buffer, four hour reaction time, and agitation at 180 rpm. Conversion was determined using gas chromatography.



Scheme 3. Microbial oxidation of cyclohexanol. The product of the Baeyer–Villiger oxidation is caprolactone.

As seen in Figure 3, the ADH enzyme activity occurs rapidly, forming intermediate **2**. The conversion of intermediate **2** is slow, and an equilibrium between substrate **3** and intermediate **2** is also observed here; this is the rate-limiting step of the overall reaction. After two hours, a second oxidation due to the catalytic activity of BVMO results in the appearance of ε-CL (Scheme 3). The formation of ε-CL (**4**) occurs rapidly and in quantitative yield within 4 hours (Figure 3).

The values obtained for the limit of detection (LOD) and limit of quantification (LOQ) were determined from the parameters of the calibration curve of caprolactone using GC-FID according to the equations **LOD =3SD/b** and **LOQ =10SD/b**, where **SD** is the standard deviation of the intercept and **b** is the slope of the regression line. The results (LOD =0.39) and (LOQ =1.17) showed the method to be sensitive at the low concentrations in all experiments performed in this work.

Due to the rapid conversion of the three substrates into ε-CL (**4**), the *Geotrichum candidum* cells could be recycled and reused for the biotransformation of substrate **2**. After simply washing the cells with buffer, six cycles of reuse were repeated. ε-Caprolactone **(4)** was obtained at quantitative yield in the four first cycles, at 96% yield in the fifth cycle, and at 88% in the sixth cycle. Thus, although a slight decrease in enzymatic conversion occurs, the catalytic process remains very active even after six cycles of reusing the free cells.

Conclusions

The use of whole *Geotrichum candidum* cells obtained from the Brazilian tropical culture collection (CCT 1205) as biocatalysts efficiently biotransformed the substrates cyclohexenone (**1**), cyclohexanone (**2**) and cyclohexanol (**3**) into ε-CL (**4**) in quantitative yields over short reaction times. In the biotransformation cascade reaction of cyclohexanone, ERED activity catalysing the first step was observed, followed by ADH and BVMO catalysing the next steps. In the bio-oxidation of cyclohexanone (**2**), an equilibrium catalysed by ADH was clearly observed between species **2** and **3**, followed by BVMO enzymatic catalysis generating ε-CL in quantitative yield in just four hours. Similarly, when using cyclohexanol (**3**) as a substrate, an equilibrium between species **2** and **3** catalysed by ADH was also observed, again followed by BVMO enzymatic activity, providing similar results. These results represent the best results to date in terms of experimental simplicity, reaction time and ε-CL (**4**) yields obtained using whole cells.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding

The authors thank CAPES and CNPq for financial support.

References

Brink GJT, Arends IWCE, Sheldon RA. 2004. The Baeyer–Villiger reaction: new developments toward greener procedures. Chem Rev 104:4105–4124.

Carballeira JD, Álvarez E, Sinisterra JV. 2002. Biotransformation of cyclohexanone using immobilized *Geotrichum candidum* NCYC49: factors affecting the selectivity of the process. J Mol Catal B: Enzym 28:25–32.

Cuetos A, Rioz-Martínez A, Valenzuela ML, Lavandera I, de Gonzalo G, Carriedo GA, Gotor V. 2012. Immobilized redox enzymatic catalysts: Baeyer–Villiger monooxygenases supported on polyphosphazenes. J Mol Catal B: Enzym 74:178–183.

Hussain H, Al-Harrasi A, Green IR, Abbas IAG, Rehman NU. 2014. meta-Chloroperbenzoic acid (mCPBA): a versatile reagent in organic synthesis. RSC Adv 4:12882–12917.

Leisch H, Morley K, Lau PC. 2011. Baeyer–Villiger monooxygenases: more than just green chemistry. Chem Rev 111:4165–4222.

Mallin H, Wulf H, Bornscheuer UT. 2013. A self-sufficient Baeyer–Villiger biocatalysis system for the synthesis of ε-caprolactone from cyclohexanol. Enzyme Microb Technol 53:283–287.

Mandal D, Ahmad A, Khan MI, Kumar R. 2002. Biocatalytic transformation of cyclohexanone by *Fusarium* sp. J Mol Catal A: Chem 181:237–241.

Mihovilovic MD, Snajdrova R, Grötzl B. 2006. Microbial Baeyer–Villiger oxidation of 4, 4-disubstituted cyclohexan- and cyclohexenones by recombinant whole-cells expressing monooxygenases of bacterial origin. J Mol Catal B: Enzym 39:135–140.

Paul CE, Gargiulo S, Opperman DJ, Lavandera I, Gotor-Fernández V, Gotor V, Taglieber A, Arends IWCE, Hollmann F. 2013. Mimicking nature: synthetic nicotinamide cofactors for C = C bioreduction using enoate reductases. Org Lett 15:180–183.

Punniyamurthy T, Velusamy S, Iqbal J. 2005. Recent advances in transition metal catalyzed oxidation of organic substrates with molecular oxygen. Chem Rev 105:2329–2364.

Ratuś B, Gładkowski W, Wawrzeńczyk C. 2009. Lactones 32: new aspects of the application of Fusarium strains to production of alkylsubstituted ε-lactones. Enzyme Microb Technol 45:156–163.

Staudt S, Bornscheuer UT, Menyes U, Hummel W, Gröger H. 2013. Direct biocatalytic one-pot-transformation of cyclohexanol with molecular oxygen into ε-caprolactone. Enzyme Microb Technol 53:288–292.

Weissermel K, Arpe HJ. 2003. Industrial organic chemistry. 4th ed. Weinheim: Wiley-VCH.

Yachnin BJ, McEvoy MB, MacCuish RJD, Morley KL, Lau PCK, Berghuis AM. 2014. Lactone-bound structures of cyclohexanone monooxygenase provide insight into the stereochemistry of catalysis. ACS Chem Biol 9:2843–2851.