DOI: 10.1016/j.procbio.2019.01.022

Process Biochemistry 78 (2019) 8–14

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Establishment of culture conditions for bio-transformation of *R*-(+)-limonene to limonene-1,2-diol by *Colletotrichum nymphaeae* CBMAI 0864

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ARTICLE INFO

Keywords:
Aroma
Biphasic system
Fungi
Induction
Scale-up
Terpene

ABSTRACT

Before reaching commercial scale, a biotechnological process must be well characterized in terms of process conditions. Thus, this study aimed to investigate the culture conditions in limonene bio-transformation to limonene-1,2-diol by *Colletotrichum nymphaeae*. The results revealed that this bio-process was aerobic, suggesting that aeration is an important parameter to be considered on a larger scale (e.g. bio-reactors). Moreover, a single feeding of *R*-(+)-limonene (15 $g.L^{-1}$) at the start of the bio-transformation resulted in the highest concentration (4.19 $g.L^{-1}$) and yield (27.9%, w. w^{-1}) of limonene-1,2-diol, indicating that fed-batch operation was not a good choice. In terms of biomass age, 72 h-old biomass performed better when compared to 24 or 48 h-old biomasses. Substrate induction test suggested that this bio-transformation is carried out by non-inducible enzymes. Three successive freeze-thawing processes did not present any observable change in the production, indicating that the biomass could be stored frozen. It was also evidenced that the use of resting cells was as efficient as growing cells, and, therefore, biomass recovery/re-suspension was unnecessary. Although feasible, the bio-transformation in an aqueous-hexadecane biphasic system was not indicated for this process, since lower concentration of diol was obtained. These results are very important to guide scaled-up studies of this bio-transformation process.

1. Introduction

Terpene bio-transformation may be regarded as a biotechnological process aligned to the three pillars of sustainable development, i.e., economic, social and environmental features [1]. Some of the advantages related to this bio-process include the use of by-products as raw materials, mild reaction conditions, high regio- and stereo-selectivity, and the generation of products that might have a "natural" label [2]. Bio-transformed products from this process have applications in various fields, such as food, pharmaceutical, cosmetics, materials, and fuels, which makes terpene biotechnology a flourishing science with many recent achievements [3].

Limonene, for instance, is one of the main substrates employed in terpene bio-transformations [4]. *R*-(+)-Limonene is the main constituent of citrus oil, an agro-industrial by-product with a yearly production of 30,000 tons [5]. One of its bio-transformation products, limonene-1,2-diol, has a significant inhibitory effect on the pro-inflammatory activities of $CD4^+$ and $CD8^+$ T lymphocytes [6], potential anticancer activity [7,8], may be used as flavoring of beverages, chewing gum, gelatins and puddings [9], and also presents insect-attractant properties [10]. The conversion of limonene to limonene-1,2-diol may also be economically attractive. To have an idea, this product is marketed at US$ 11,500/kg (www.molbase.com), while the substrate costs US$ 34/L [1].

So far, the bio-transformation of limonene to its corresponding 1,2-diol has been described for *Gibberella cyanea*, *Diplodia gossypina*, *Corynespora cassiicola* [11], *Phomopsis* sp. [12], *Fusarium oxysporum* [13], *Cladosporium* species [14] and *Aspergillus cellulosae* [15] resulting in concentrations varying from 1.5 to 13.9 $g.L^{-1}$. To better understand and optimize a bio-transformation process, it is first essential to characterize its parameters, including oxygen requirements, resistance to substrate toxicity, biomass (inoculum) age, enzyme induction, among others [13,16,17]. Regarding terpene bio-transformation, the use of biphasic (aqueous-organic) media may be a convenient approach, since this system can protect the microbial cells from the toxic effect of terpenes, as well as reducing volatilization (loss of substrate end product), and possibly increasing yields [18]. In summary, understanding some properties of the bio-catalyst and its relationship with the surrounding

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https://doi.org/10.1016/j.procbio.2019.01.022
Received 27 November 2018; Received in revised form 25 January 2019; Accepted 28 January 2019
Available online 29 January 2019
1359-5113/

environment may help to achieve greater productions just by adjusting some process conditions, besides providing fundamentals for studies on scale-up and product recovery.

Considering that one of the main challenges involving the bio-transformation of terpenes is related to their toxicity towards biological systems, the use of phytopathogens in these processes may be a good choice, since such microorganisms are presumably more resistant to terpenes [19]. In this context, preliminary studies from our group have shown that the phytopathogenic fungi *Colletotrichum nymphaeae* CBMAI 0864 was able to bio-transform *R*-(+)-limonene to limonene-1,2-diol [19]. However, the characteristics of such a process have not been elucidated. Therefore, the main objective of this work was to characterize this bio-transformation in terms of oxygen requirement, substrate feeding regimen, biomass age and cell inducibility, as well as analyzing the influence of biomass freezing and the use of biphasic medium in the production of limonene-1,2-diol by the bio-transformation of *R*-(+)-limonene by *C. nymphaeae*.

2. Materials and methods

2.1. Micro-organisms and chemicals

The micro-organism *C. nymphaeae* strain CBMAI 0864 was donated by the Brazilian Collection of Environmental and Industrial Microorganisms (www.cpqba.unicamp.br/colecoes/cbmai.html) (Unicamp, Paulínia, SP, Brazil). *n*-Decane, hexadecane, *R*-(+)-limonene (99% purity), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH, ≥97%, CAS: 606-68-8) and riboflavin 5′-adenosine diphosphate disodium salt hydrate (FAD, disodium salt, ≥97%, CAS: 84366-81-4) were acquired from Sigma-Aldrich (www.sigmaaldrich.com) and all solvents presented analytical grade.

2.2. Biomass production and bio-transformation procedure

For biomass production, a piece of agar (~1.5 cm^2) containing a pre-grown culture (30 °C for 72 h) in yeast and malt (YM) agar (10 $g.L^{-1}$ glucose; 5 $g.L^{-1}$ peptone; 3 $g.L^{-1}$ yeast extract; 3 $g.L^{-1}$ malt extract; 20 $g.L^{-1}$ agar, pH ~ 6.7) was transferred to a 125-mL Erlenmeyer filled with 50 mL of YM Broth (the same medium described above, without agar addition). The material was homogenized under sterile conditions with an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until there was complete disruption of solid matter. After incubation for 7 h at 150 rpm at 30 °C, the biomass was recovered by vacuum filtration using a Buchner funnel and paper filter Whatman #1. During biomass production, the dry mass was monitored by gravimetry and glucose was quantified by employing the dinitrosalicylic acid (DNS) method [20].

The biomass obtained as described above (0.33 g dry weight) was recovered and re-suspended (under aseptic conditions) in 50 mL of phosphate buffer pH 7.0 20 μmol.L^{-1} (resting cells, final concentration of 6.6 $g.L^{-1}$ dry weight) added with 20 $g.L^{-1}$ *R*-(+)-limonene. The bio-transformation was carried out in triplicate at 30 °C and 150 rpm [21]. S were collected at 0, 48, 96, 144 and 192 h to detect and quantify the products formed (Section 2.8). Alternatively, an assay was carried out with growing cells by adding the substrate to the growth medium after 72 h incubation. In this case, biomass recovering (filtering) and re-suspending in buffer (resting cells) was unnecessary.

The bio-transformation using crude enzymatic extract was done following the method proposed by van der Werf et al. [27], with adaptations. Briefly, the biomass re-suspended in 50 mM potassium phosphate buffer (pH 7.0) (obtained as described above) was sonicated (QSonica Q700 sonicator, 75% amplitude for 5 min at 120 W/40 J) and filtered through a 0.45 mm filter to obtain the so-called crude enzyme extract. The reaction mixtures contained 10 mM NADH and 5 μM flavin adenine dinucleotide (FAD) in 2 mL of crude enzyme extract. The reaction was initiated by adding 1 μL of a limonene-acetone mixture (1:2), and the vials were placed on a horizontal shaker (300 rpm/30 °C) for 2 h. The reaction mixture was extracted with the same volume of ethyl acetate and this organic extract was analyzed in GC as described in Section 2.8. A control experiment was done under the same conditions, replacing the crude enzyme extract with a 50 mM potassium phosphate buffer (pH 7.0).

2.3. Fed batch bio-transformation

To evaluate the influence of sequential substrate feeding, the bio-transformation was carried out employing single feeding and fed-batch techniques. Thus, after re-suspending the biomass to get resting cells (Section 2.2), four substrate feeding schemes were applied: (i) 20 $g.L^{-1}$ at time 0 h (single feeding); (ii) 15 $g.L^{-1}$ at time 0 h (single feeding); (iii) 10 $g.L^{-1}$ at time 0 h and 10 $g.L^{-1}$ at 96 h (fed-batch); (iv) 5 $g.L^{-1}$ at time 0 h, 5 $g.L^{-1}$ at 48 h and 5 $g.L^{-1}$ at 96 h (fed-batch) [22]. Except for limonene feeding, the same bio-transformation conditions described in Section 2.2 were used.

2.4. Bio-transformation in anaerobiose

To run the bio-transformation in anaerobic conditions, the re-suspended biomass (resting cells; see Section 2.2) was transferred to a conical flask with two inlets (one at the base and another at the neck) plugged with a rubber stopper. Before initiating bio-transformation, after each sampling, the medium was flushed (bubbling N_2 for 5 min) from the base inlet to remove oxygen from the system [23]. The other process conditions may be seen in Section 2.2.

2.5. Influence of substrate induction and biomass age

For testing the effect of different biomass (inoculum) ages, independent bio-transformations were carried out with resting cells (biomasses recovered and re-suspended after growing, according to the conditions indicated in Section 2.2) at three different times: 24 h (mid log phase), 48 h (late log/early stationary phase), and 72 h (stationary phase). For the substrate induction test, the experiment was repeated with the addition of 2 $g.L^{-1}$ of *R*-(+)-limonene after achieving 2/3 of the biomass growth time, i.e., at 16, 32, and 48 h before recovering the biomasses grown for 24, 48, and 72 h, respectively (based on [24]). The conditions for biomass recovery and bio-transformation may be seen in Section 2.2.

2.6. Influence of biomass freezing

The influence of the freeze–thawing process on bio-transformation performance was evaluated by treating the biomass obtained (Section 2.2) as follows: (i) a single freeze-thawing process and (ii) three successive cycles of freeze-thawing. A freeze-thawing cycle considered freezing the biomass recovered by filtration (Section 2.2) for 4 h followed by thawing for 30 min at 30 °C [16]. The treated biomasses were re-suspended (resting cells) and used in bio-transformation procedures, under the same conditions as described in Section 2.2.

2.7. Bio-transformation in biphasic medium

For the biphasic bio-transformation system, the biomass produced following the protocol described in Section 2.2 (0.33 g dry weight) was re-suspended in a 125-mL Erlenmeyer filled with 40 mL of aqueous phase (consisting of phosphate buffer 20 μmol.L^{-1} pH 7.0) (resting cells) and 10 mL of organic phase (hexadecane). To this mixture, 2 g of *R*-(+)-limonene was added to reach a concentration of 50 $g.L^{-1}$ based on the aqueous phase [13,25]. The other process conditions may be found in Section 2.2. Every 48 h samples of the aqueous and organic phases were collected for monitoring substrate consumption and limonene-1,2-diol production. The aqueous phase was extracted (according to Section 2.8) before being analyzed, while the organic phase

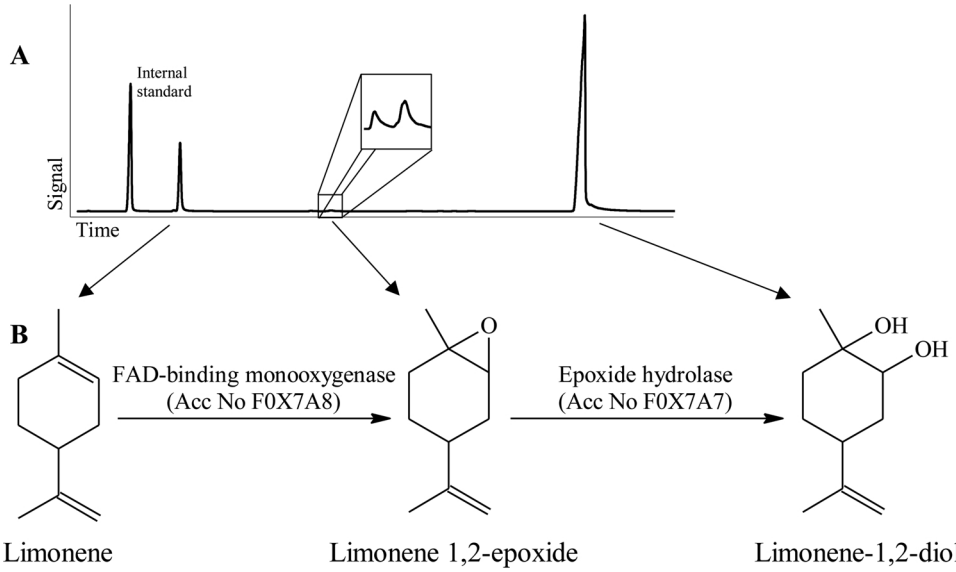

Fig. 1. Example of a chromatogram of limonene bio-transformation by *C. nymphaeae* (A). Proposed metabolic pathway of limonene degradation reported for *Grosmannia clavigera* [28], which is supposedly the same for *C. nymphaeae* (B).

could be directly injected into the gas chromatograph.

2.8. Analytical conditions

Ethyl acetate (1:1, v:v) was used to extract (vortex for 40 s) the products in the broth. The organic layer was collected, dried with a pinch of Na_2SO_4 and injected (1 µL, split mode 1:10) into a HP-7890 gas chromatograph (Agilent Technologies) (GC–FID) using a HP-5 column (30 m x 0.25 mm, 0.25 µm film thickness) and a flame ionization detector. The carrier gas (1.0×10^{-3}L. min^{-1}) was helium, and the oven temperature presented the following program: 80 °C for 3 min, heating up to 200 °C at a rate of 20 °C min^{-1}, and 200 °C for more 4 min A temperature of 250 °C was used for both the injector and detector [16]. The quantification of substrate and products was done using internal calibration (*n*-decane as internal standard) prepared with known concentrations of *R*-(+)-limonene in heat-inactivated (autoclaved) biomass re-suspended in phosphate buffer (6.6 g.L^{-1} dry weight), to consider the matrix effect.

Volatile compounds were identified using a HP-7890 gas chromatograph, a HP-5975C mass spectrometer detector (Agilent Technologies) and a HP-5MS column (the same dimensions as described above). The mass spectrometer transfer line was kept at 250 °C. The impact energy was 70 eV and the mass-to-charge ratio (*m/z*) was in the 50–500 range. The other analytical conditions (carrier gas, flow rate, oven and injector temperatures) were the same as above. The compounds were identified through the comparison of the obtained mass NIST library or with the mass spectra of commercial standards, considering 90% similarity.

2.9. Statistical analysis

Three independent assays were run for each experiment. The software Statistica® 10 (www.statsoft.com) was used to run the analysis of variance (ANOVA) and multiple comparison test (Tukey) with a significance level of 5%.

3. Results and discussion

3.1. Sampling and abiotic experiments

Since biomass suspension is highly heterogeneous and limonene is poorly soluble in aqueous systems, medium sampling should be done carefully. This means that the flasks should be manually mixed for 10 s before getting a sample (at least 1 mL) as representative as possible (e.g. minimal phase separation). Considering this procedure, our control experiment showed an average standard deviation of 1 g.L^{-1} considering four independent replicates (different flasks). Control experiments carried out with heat-inactivated (autoclaved) biomass revealed no auto-oxidation products when limonene was used as substrate, while only trace amounts of limonene-1,2-diol were generated in the case of limonene-1,2-epoxide being used as substrate at an initial concentration of 0.5 g.L^{-1} (data not shown).

3.2. Bio-transformation in anaerobiose

We did not detect any products in the bio-transformation under anaerobic conditions. This is corroborated by the characteristics of the limonene degradation pathway via limonene-1,2-diol, which is oxygen-dependent [13,26]. In *Rhodococcus erythropolis*, this pathway begins with the action of an FAD- and NADH-dependent limonene-1,2-monooxygenase, which oxidizes limonene to form limonene-1,2-epoxide, following its conversion to the corresponding diol by a co-factor-independent epoxide hydrolase [27]. However, although minor amounts of limonene epoxide were detected in the aerobic process, our previous study suggested that these enzymes were not present in *Colletotrichum* genomes [19]; instead, there were protein sequences in *C. nymphaeae* genome displaying significant (e-value < -49) identity with an FAD-binding mono-oxygenase (Acc No F0 × 7A8) and an epoxide hydrolase (Acc No F0 × 7A7) described in *Grosmannia clavigera* [28], indicating that both fungi may have a similar limonene bio-transformation pathway (Fig. 1). The dependency on co-factors in the present process was also reinforced by two observations: i) the absence of products when bio-transformation was carried out with crude enzymatic extract obtained according to the method proposed by Molina et al. [13] and ii) by the detection of 212.15 ± 24.7 mg.L^{-1} of limonene-1,2-epoxide (compared to 56.2 ± 12.7 mg.L^{-1} for the control experiment) after 2 h in the experiment with crude enzymatic extract and NADH and FAD (data not shown).

Analogous to the results presented here, the strain *Fusarium oxysporum* 152B showed an aerobic and co-factor-dependent bio-transformation of *S*-(−)-limonene to limonene-1,2-diol [13], whereas the bio-transformation of *R*-(+)-limonene to α-terpineol by this strain was anaerobic and co-factor-independent [23]. In the case of *Pseudomonas fluorescens* NCIMB 11671, two pathways were observed for *R*-

Table 1
Production of limonene-1,2-diol with different *R*-(+)-limonene feeding regimens using *C. nymphaeae* previously grown in YM broth for 72 h, according to Fig. S1. Values are presented as average ± standard deviation. Different letters indicate statistically significant difference ($p < 0.05$).

Substrate	TM [2]	Lim-diol [3]	Lim remaining [4]	$Y_{p/s}$ [5]	Pr_p [6]
feeding [1]	(h)	($g.L^{-1}$)	($g.L^{-1}$)	(%)	($mg.L^{-1}.h^{-1}$)
20(0)	192	4.15 ± 0.14 [a]	2.99 ± 0.160 [a]	24.4 ± 1.1 [b]	21.6 ± 0.7 [a]
15(0)	192	4.19 ± 0.16 [a]	0.01 ± 0.004 [c]	27.9 ± 1.0 [a]	21.8 ± 0.8 [a]
10(0) **10**(96)	192	4.11 ± 0.13 [a]	2.35 ± 0.200 [b]	23.3 ± 0.8 [b]	21.4 ± 0.7 [a]
5(0) **5**(48) **5**(96)	192	3.82 ± 0.13 [b]	0.01 ± 0.001 [c]	25.5 ± 0.9 [b]	19.9 ± 0.7 [a]

[1] Substrate concentration ($g.L^{-1}$) in bold and the time of substrate feeding (hours) in brackets.
[2] Duration for achieving the maximal limonene-1,2-diol concentration.
[3] Maximal limonene-1,2-diol concentration (after 192 h).
[4] Limonene concentration remaining in the medium (after 192 h).
[5] Yield of limonene-1,2-diol per substrate consumed (%, w. w^{-1}).
[6] Productivity of limonene-1,2-diol.

(+)-limonene: one aerobic and co-factor-dependent producing limonene-1,2-diol and a second, anaerobic and co-factor-independent, producing α-terpineol [26].

3.3. Fed-batch bio-transformation

The use of feeding strategies aims at obtaining higher yields and productivity in bio-processes, being considered a proper technique when applying terpenes with toxic effects on the microbial strain used [29]. Table 1 presents the effects of different feeding regimens on the parameters of limonene-1,2-diol production by *C. nymphaeae*. As may be observed, regardless of the feeding strategy adopted, the highest limonene-1,2-diol concentration was achieved at 192 h (Supplementary Fig. 1). After this period, the concentrations of limonene-1,2-diol were in the range of 4.11–4.19 $g.L^{-1}$, with no statistical differences among them, except for the fed-batch strategy with three sequential feedings of 5 $g.L^{-1}$, which presented significant lower (c.a. 8%) limonene-1,2-diol production. No statistical differences were evidenced for productivity, but in terms of yield, the highest value (27.9 ± 1.0%) was obtained for the single feed of 15 $g.L^{-1}$, while the others did not differ statistically. Therefore, we considered single substrate addition of 15 $g.L^{-1}$ as the most adequate for this process. A fed-batch system would not be able to improve the bio-transformation parameters. Other authors have reported that the use of fed-batch increased the yield in the terpenes bio-transformation, as observed for the α-terpineol production via limonene bio-transformation by *Penicillium digitatum* [22]. Other authors have successfully used a sequential substrate feeding, together with a closed gas loop bio-reactor, to overcome substrate toxicity and to increase yield of monoterpene bio-transformations using *Penicillium digitatum* DSM 62840 [30].

It is known that part of limonene might be partially metabolized to CO_2, since this pathway for limonene (Fig. 1) is reported as an energy producing route [26]. However, we believe that this is not significant in the present process due to two main observations: firstly, the bio-transformation of limonene-1,2-diol has not presented any products (data not shown); and secondly, the yield of limonene-1,2-diol (the ratio between the amount of diol produced and the amount of limonene consumed, not considering substrate volatilization) was close to 100%, since limonene loss by volatilization was reasonably high (there is 4.6 ± 1.3 $g.L^{-1}$ left after 192 h when using an initial concentration of 20 $g.L^{-1}$) and, therefore, only a minor part of it was available for the microorganism.

3.4. Influence of substrate induction and biomass age

The limonene-1,2-diol production kinetics using *C. nymphaeae* biomass with three different ages (24, 48 and 72 h) is presented in Fig. 2. This figure also indicates the effect of substrate induction experiments, i.e., involving the addition of limonene in the stage of biomass production. There were no statistical differences in productivity and in the maximal limone-1,2-diol concentration when using induced 72 h-old biomass or non-induced biomass of 72 or 48 h-old (Supplementary Table 1). The 48 h-old induced biomass presented a statistically lower production and productivity of limonene1,2-diol when comparing to the former experiments. As for the 24 h-old non-induced biomass, both maximal limonene-1,2-diol concentration and productivity were about half of the observed for 72 h-old or 48 h-old non-induced biomass, but approximately two times higher than the values obtained for 24 h-old induced biomass (Supplementary Table 1). However, as the biomass dry weight doubled between 24 h (3.3 $g.L^{-1}$) and 48 h of growth (˜6.4 $g.L^{-1}$) and remained constant in the following 24 h of growth (˜6.4 $g.L^{-1}$ after 72 h) (Fig. 2), the yield of diol per biomass ($Y_{P/X}$) has not shown statistical differences among the experiments with non-induced biomass (Supplementary Table 1).

In all cases, substrate induction did not result in increased limonene-1,2-diol production – in fact, as mentioned earlier, the production profile was equal or lower when compared to the non-induced biomass, indicating possible toxic effects of limonene during biomass growth. The toxicity of terpenes, in particular limonene, is known and some microorganisms have mechanisms to adapt to and resist solvents and terpenes in general [3]. However, early contact with the terpene, without the proper formation of biomass, may have potentialized the toxic effect of limonene.

Substrate induction experiments have already been reported in terpene bio-transformations as a strategy to increase product concentration. For instance, a substrate induction procedure before the *R*-(+)-limonene bio-transformation by *Penicillium digitatum* NRRL 1202 resulted in a 4-fold increase in α-terpineol production [17]. Moreover, this bio-transformation was only evidenced in the early- to mid-log phases of fungal growth [17]. Another *Penicillium* sp. strain was also sensitive to substrate induction, which resulted in an increased production of rose oxide via citronellol bio-transformation. The production of α-terpineol via bio-transformation of *R*-(+)-limonene by *P. digitatum* DSM 62840 could also be increased by 14.58% if 1.47 mM (equivalent to 0.2 $g.L^{-1}$) of substrate were added to the biomass growth medium [31]. On the other hand, it was observed that the induction caused a decrease in carvone and α-terpineol production by *Aspergillus* sp. and *Penicillium* sp. in a limonene bio-transformation process [24]. As for *F. oxysporum* 152b, the bio-transformations of both *S*-(–)- and *R*-(+)-limonene to, respectively, limonene-1,2-diol [13] and α-terpineol [23] were non-inducible, as was evidenced in this study for the bio-transformation of *R*-(+)-limonene to limonene-1,2-diol by *C. nymphaeae*.

3.5. Influence of biomass freezing

The bio-transformation with freeze-thawed cells was performed to evaluate the possibility of maintaining bio-catalytic activity of the biomass after storage at −18 °C. Fig. 3 shows the accumulation of

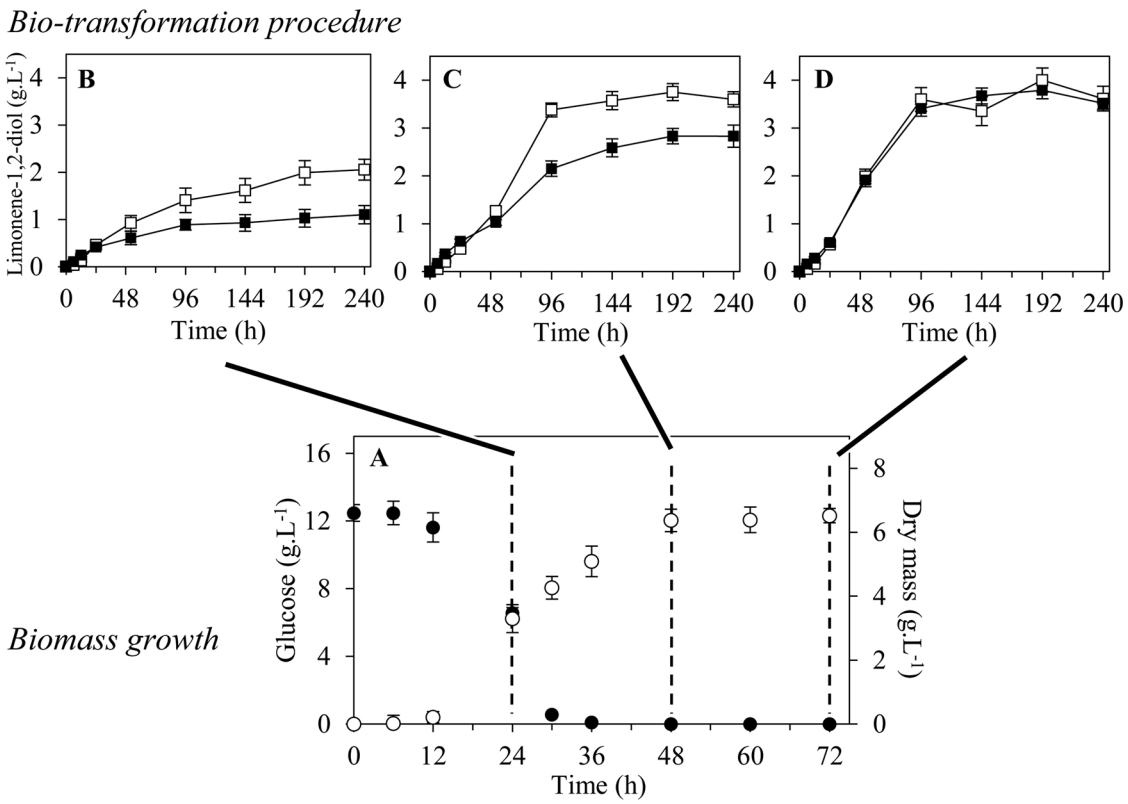

Fig. 2. Glucose consumption (●) and *C. nymphaeae* biomass growth (○) at 30 °C and 150 rpm in YM broth (A) and *R*-(+)-limonene bio-transformation for limonene-1,2-diol production with (■) or without (□) substrate induction using *C. nymphaeae* biomass with different ages, i.e. previously grown for 24 (B), 48 (C), and 72 (D) hours. The dashed lines indicate the moment of biomass recovery. Error bars are the standard deviation of a triplicate.

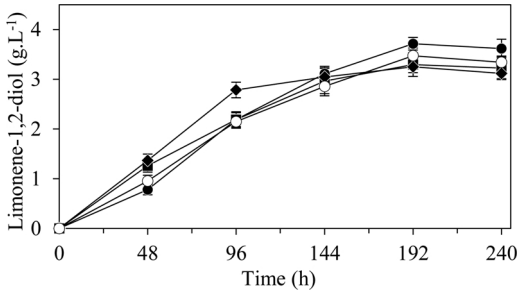

Fig. 3. Production of limonene-1,2-diol from bio-transformation of *R*-(+)-limonene by *C. nymphaeae* biomass previously treated with a single (■) or three successive freeze-thawing cycles (♦), in addition to control biomass without treatment (●) and non-re-suspended biomass (bio-transformation taking place in the growth medium) (○). Error bars are the standard deviation of a triplicate.

limonene-1,2-diol using biomasses submitted to freeze–thawing treatments. As can be noticed, none of the treatments revealed significant differences in limonene-1,2-diol production (Supplementary table 2). In the case of *F. oxysporum*, a freeze-thawing process accelerated the bio-transformation of limonene to α-terpineol (i.e., increased α-terpineol productivity) [16]. This behavior was explained by the semi-permeabilization effect of this treatment, which could release part of the intracellular enzymes and improve the solute transfer rate through the cell membrane [16,32]. However, it is important to recall that limonene itself, and its epoxide as well, have a membrane permeabilization effect, including on fungal spores [33], which make useless any additional treatment. Moreover, the cell permeabilization procedure is only applicable to co-factor-independent processes, such as the above-mentioned bio-transformation of limonene to α-terpineol. The co-factor-dependent bio-transformation of this study, in turn, would not occur with cell-free enzymes with no co-factor-regenerating systems. However, as observed for *F. oxysporum*, *C. nymphaeae* biomass could be stocked in frozen form before being used for limonene bio-transformation, and this is very useful from a practical point of view.

3.6. Influence of biomass re-suspension

Terpene bio-transformation processes are usually carried out with resting cells, in media containing terpene as the only source of carbon and energy. Thus, it is usually necessary to recover the biomass and re suspend it in mineral media or buffers [34]. Considering that after 72 h of cell growth glucose was no longer present in the medium (Fig. 2), we decided to add limonene after this period to check the bio-transformation kinetics using this approach. As may be noticed in Fig. 3 and Supplementary Table 2, there was no significant differences in the production of limonene-1,2-diol regarding the control process (resting cells). Biomass filtration may eliminate remaining compounds from the growth medium, which can interfere in the bio-availability of substrates [35]. However, in this study, we conclude that there was no need for this additional unit operation, thus simplifying the process. Nevertheless, it is important to note that bio-transformation in a complex medium can substantially increase the complexity and costs of a further downstream process. Therefore, a cost benefit analysis should be considered before running this bio-transformation with or without biomass filtration and re-suspension (resting cells).

It is important to emphasize that the use of additional carbon sources (other than the terpene substrate) in the bio-transformation medium can increase the conversion rate [17]. Using different operation regimens (batch, repeated batch and continuous fed bio-conversion of limonene bio-transformation to α-terpineol by immobilized *Penicillium digitatum* NRRL 1202), Tan and Day [36] observed that the yield decreased in the second and third cycles of the repeated-batch process, while regenerating the media with nutrients media after the third cycle helped to improve the yields.

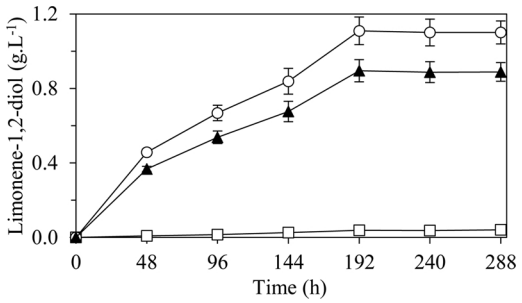

Fig. 4. Accumulation of limonene-1,2-diol in aqueous phase (○), organic phase (□, hexadecane) and in the complete system (aqueous + organic phases) (▲) during bio-transformation in biphasic medium of *R*-(+)-limonene by *C. nymphaeae* previously grown in YM broth for 72 h. Error bars are the standard deviation of a triplicate.

3.7. Bio-transformation in biphasic medium

The use of biphasic systems aims at reducing the toxic effect of substrates and products on the biomass during bio-transformation processes, besides aiding product recovery and purification, and also decreasing the losses of substrate or products by volatilization [37]. In terms of limonene loss, our abiotic experiments in an aqueous system indicated that after 192 h only c.a. 5 $g.L^{-1}$ limonene is left (c.a 75% loss). Therefore, we tested the bio-transformation of limonene to limonene-1,2-diol by *C. nymphaeae* in a biphasic system using hexadecane as an organic solvent. This solvent was chosen due to its biocompatibility: it has a log P value of 9.6, which is c.a. two times higher than the minimum limit for bio-compatible solvents [37]. Since limonene solubility in water is low (13.8 $mg.L^{-1}$) and higher in this solvent [38], limonene distributes more to the organic layer (lower toxicity towards the microorganism). In order to increase the solubility of limonene in aqueous media, other authors have preferred the use of co-solvents in the bio-transformation system [17,22].

In Fig. 4, it can be observed that bio-transformation was feasible in this system, but limonene-1,2-diol production was c.a. 75% lower than in the former experiments (Fig. 2 and Fig. 3). The decrease in diol production in this case might be related to the lower substrate transfer rate, since limonene has higher solubility in the organic phase. Moreover, most of the limonene-1,2-diol produced accumulated mainly in the aqueous phase (> 99.0% in mass), suggesting that this system could not avoid a possible toxic effect associated with this product. The Log P value calculated for limonene-1,2-diol and water are, respectively, 1.5 and -0.5, while *R*-(+)-limonene and hexadecane have a Log P of 3.4 and 8.3, respectively [39]. These values and the predicted high water solubility of diol (14.6 $g.L^{-1}$ at 25 °C, www.vcclab.org/lab/alogps/) justify the higher affinity of this product for the aqueous phase.

As mentioned before, this bio-transformation is aerobic, so oxygen is critical and some biphasic processes have oxygen limitation. Even if hexadecane is considered an "oxygen vector" [40], with good solubility to this gas, gas exchange can be compromised, which slows down this process. Therefore, the use of biphasic systems may be limited in aerobic processes. In the (aerobic) bio-transformation of limonene by *Mortierella minutissima*, for instance, a sharp drop in perillyl alcohol production was evidenced when using hexadecane in the medium [41]. But in oxygen-independent bio-transformations, as is the case with *Sphinghobium* sp., this system can be very useful by inhibiting "competing" aerobic pathways of limonene degradation [23].

4. Conclusions

The characterization of a given bio-process is essential to understand the requirements and assess its applicability on a larger scale. This study suggests that, as an aerobic process, aeration would be an important parameter when conducting the bio-transformation of limonene to its corresponding 1,2-diol by *C. nymphaeae* in bio-reactors. Another finding indicates that batch operation would be more suited for such bio-transformation, since a single addition of 15 $g.L^{-1}$ substrate at zero time yielded better results than batch-fed experiments. Moreover, we showed that the biomass to be used in the bio-transformation trials was ideally grown for 72 h with no limonene addition and that it could pass through three freeze-thawing cycles without significant loss in bio-transformation activity. On the other hand, the results obtained indicated that biomass recovery followed by its re-suspension in a mineral medium (bio-transformation with resting cells) was unnecessary, suggesting that bio-transformation could be done with growing cells. Thus, the results presented here will guide the application of such bio-processes in bio-reactors, which is already being carried out by our team.

Declarations of interest

None.

Funding

This study was funded by the National Council of Technological and Scientific Development (CNPq) (grant numbers 473981/2012-2 and 400411/2016-4); the São Paulo Research Foundation (FAPESP) (grant number 2016/21619-7) and the Coordination for the Improvement of Higher Education Personnel (CAPES) (scholarship, A. Sales).

Funding sources had no involvement in study design; collection, analysis and interpretation of data; writing of the report; and the decision to submit the article for publication.

Acknowledgments

The authors acknowledge the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI-CPQBA) for the strain; National Council of Technological and Scientific Development (CNPq) (scholarship for R. Moreira, grant numbers 473981/2012-2 and 400411/2016-4); São Paulo Research Foundation (FAPESP) (grant number 2016/21619-7); Coordination for the Improvement of Higher Education Personnel (CAPES) (scholarship for A. Sales and process number 23038.000795/2018-61); and Espaço da Escrita – Coordenadoria Geral da Universidade (UNICAMP) for the language services provided.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2019.01.022.

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