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PRODUCCIÓN BIOCATALÍTICA DE ALCOHOL PERÍLICO UTILIZANDO CÉLULAS COMPLETAS DE *Aspergillus Niger* DSM 821

BIOCATALYTIC PRODUCTION OF PERILLYL ALCOHOL USING WHOLE CELLS OF *Aspergillus niger* DSM 821

PRODUÇÃO DE ÁLCOOL PERÍLICO BIOCATALÍTICA USAR CÉLULAS COMPLETOS *Aspergillus niger* DSM 821

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RESUMEN

En esta investigación se estudió la producción de alcohol perílico (AP) mediante biotransformación del (R)-(+)-limoneno utilizando células completas de Aspergillus niger DSM 821 a escala de laboratorio. Para tal fin se determinó la influencia de diferentes variables sobre la selectividad del microorganismo hacia un producto determinado y el rendimiento de biotransformación. Se evaluó el efecto de la fase del crecimiento del hongo y el efecto inductor del sustrato. De igual forma se incluyeron las siguientes variables: pH, medio de biotransformación y concentración del sustrato. La mayor velocidad específica de crecimiento se alcanzó utilizando Agar Extracto Malta (MEA) como medio de cultivo a 28°C. Además se obtuvieron 246 mg/L de AP, utilizando 12 mM de (R)-(+)- limoneno en caldo Malta y Extracto de Levadura (MYB). Esta producción fue 1,9 y 3,1 veces mayor que la obtenida en caldo de Levadura, Extracto de Malta, Peptona y Glucosa (YMPG) y Caldo de Extracto de Levadura y Glucosa (YG), respectivamente. Las mayores concentraciones de AP (405 mg/L) fueron obtenidas cuando A. niger DMS 821 fue cultivado en medio suplementado con 50 mL de limoneno durante

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la fase exponencial (a las 72 h), en MYB a pH 5,0, 28°C, 300 rpm y 6 días de biotransformación. Adicionalmente se obtuvieron otros subproductos: limoneno-1,2-diol, linalol, carvona, fenil etanol y etil ésteres de ácidos palmitico, oleico y linoleico.

ABSTRACT

In this research, biotransformation of (R)-(+)-limonene for production of perillyl alcohol (POH) at lab scale was studied using whole cells of Aspergillus niger DSM 821. Effects of fungal growth phase, inductive effect of the substrate, pH, type of biotransformation medium and (R)-(+)-limonene concentration on both biotransformation selectivity and yield were evaluated. Using Malt Extract Agar (MEA) as culture medium at 28°C, it was possible to achieve higher specific growth rate of the fungi. Moreover, by using of Malt Yeast Extract Broth (MYB) and 12 mM of (R)-(+)-limonene as substrate in a liquid reaction medium at pH 5,0, it was obtained 246 mg/L of POH. This POH production was 1,9 and 3,1 times higher than yields obtained in liquid medium constituted by Yeast and Malt Extract, Peptone and Glucose (YMPG) and Broth Yeast Extract and Glucose (YG), respectively. Higher concentrations of POH (405 mg/L) were obtained by adding 50 mM of limonene in the exponential phase (at 72 h) of A. niger DSM 821 grown in MYB at pH 5,0, 28°C, 300 rpm and 6 days of biotransformation. Other by-products, such as: limonene-1,2-diol, linalool, carvone, phenyl ethanol and ethyl esters of palmitic, oleic and linoleic acids, were also obtained.

RESUMO

A produção de álcool perílico (POH) por biotransformação de (R)-(+)- limoneno usando células íntegras de Aspergillus niger DSM 821 foi estudada. Foramavaliados os efeitos da fase de crescimento do fungo, o efeitoindutivo do substrato, o pH do meio de biotransformação e a concentração do substrato sobre a seletividade e a produtividade da biotransformação. Usando agar extrato de malte (MEA), como meio de cultura a 28°C, foipossívelobter a maiortaxa específica de crescimento. Alemdissoobtiveram-se 246 g/mL de AP, usando 12 mM de (R)-(+)- limoneno no caldo malta y extrato de levedura (MYB). Esta produçãofoi 1,9 e 3,1 vezesmaior do que a obtidaem caldo de levedura, extrato de malte, peptona e glicose (YMPG) e caldo de extrato de levedura e glicose (YG), respetivamente. As maioresconcentrações de AP (405 mg/L) foramobtidasquando A. niger DMS 821 foi cultivado emmeio suplementado com 50 mL de limoneno durante a fase exponencial (às 72 h), em MYB pH 5,0, 28°C, 300 rpm e 6 dias de biotransformação. Adicionalmente, obtiveram-se outrosprodutos: limoneno-1,2-diol, linalol, carvona, fenil etanol e etil ésteres de ácidos palmítico, oléico e linoléico.

PALABRAS CLAVE:
Biotransformación, Bioconversión, (*R*)-(+)-limoneno, *Aspergillus niger.*

KEYWORDS:
Biotransformation, Bioconversion, (*R*)-(+)-limonene, *Aspergillus niger.*

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INTRODUCTION

Nowadays, industrial application of biocatalysis (e.g. cells or enzymes) has increased its use in organic chemistry, because of their properties of high catalytic activity, specificity and regio/selectivity in mild reaction conditions

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[1]. For these reasons, it has begun to use more biocatalytic process to synthesize and/or biotransform chemical and pharmaceutical compounds [2]. For example, (*R*)-(+)-limonene, the most abundant monoterpene in the essential oil extracted from citrus peels (96%), can be biotransformed for obtaining compounds with high economic added value [3].

In general, monoterpenes possess preserving, antioxidant and anti-carcinogenetic activities [4, 5, 6, 7]. Limonene and some of its oxygenated derivatives (OD), such as: perillyl alcohol (POH), menthol, carveol and carvone, can inhibit the progression of metastatic melanome cells and also destroy existing malignant tumors [8]. Specifically, C-7 limonene, POH and perillic acid (PCOOH) are well recognized as non-toxic agents. They have demonstrated antitumor and therapeutic activities for the treatment of lung, liver, colon, skin, prostate pancreas, and also breast and ovarian cancers [9, 10, 11, 12, 13, 14].

In recent years, the number with cancer risks has been increased in the world. For this reason, several alternatives solutions have been raised. For example, minimal exposures to environmental carcinogens and use of exogenous factors (dietary constituents, supplements or drugs and immunization) have positively impacted in reducing tumor growth in early stages of the carcinogenic process. Therefore, the demand for anti-cancer compounds has greatly increased [15].

The POH with anti-carcinogenetic potential can be extracted from natural sources, such as essential oils of citrus plants, lavender, mint, caraway, cherry and blackberry. However, this process is not economically viable, due to the low content of essential oils (less than 0,1%w/w) [16, 17]. Therefore, the production of POH and its derivatives (aldehyde and acid) at industrial scale is carried out by using chemical catalysis of β-pinene and limonene monoterpenes. In the case of β-pinene, conversion yields are very low. Additionally, chemical synthesis and reaction conditions are not environmentally friendly [18, 19a, 20, 21]. In the case of limonene, terpenoid mixtures may occur. This is because the allylic methylene and methyl carbons in the molecule of limonene can introduce hydroxyl groups and carbonyls nonspecifically. Because of these limitations, use of biocatalysis in monoterpenes oxy-functionalization is a promising methodological strategy, mainly for their additional benefits such as the lower toxicity of the process and inherent chemo and stereoselectivity [1].

Limonene biotransformation has been traditionally carried out by using of whole cells from different microorganisms, such as: Mesophilic fungi *A. niger* [22] and *A. cellulosae* [23]; psycrotrophic fungi *M. minutissina, C. pannorum, M. alpina, P. chrysosgenum, P. cyclopium*and *P. islandicum* [24]; bacteria *P. putida* MTCC 1072 [25] and DSM 12264 [26], *Mycobacterium* HXN-1500 [27] and yeasts *C. parapsilosis* and *Y. lipolytica* [28]. These microorganisms can produce POH and PCOOH by regioselective hydroxylation C-7-limonene methyl group. Applications of bacteria as biocatalysts for production of POH have been also published in some Patents [19b, 29, 30].

On the other hand, genetic engineering techniques have improved the enzymatic activity of biocatalysts for oxy-functionalization of limonene at C-7. By using of *Mycobacterium alkanehydroxylase* genes, co-expressed in *P. putida to* produce the enzyme with a specific activity of 3U/g of dry cells, was possible to hydroxylate C-7-limonene [27]. PCOOH production from limonene has been carried out on a continuous packed bed bioreactor by using of immobilized cells of *P. putida* DSM 12264. Product recovery was carried out *in situ* (ISPR). With this configuration was obtained 31 g of PCOOH/L [26].

In this work, biotransformation of (*R*)-(+)-limonene to POH by whole cells of *A. niger* DSM 821 was evaluated. We studied addition of limonene in various stages of growth of the organism to induce limonene biotransformation. Finally, it was evaluated best conditions for POH production, such of pH, culture medium, reaction time and substrate concentration.

METHOD

Chemical and reagents

(*R*)-(+)-limonene (98%) was obtained from Merck (Germany) and (-)-perillyl alcohol from Fluka (Switzerland). All reagents and solvents were of analytical grade and obtained from Merck (Germany).

Microorganism and culture media. *Aspergillus niger* DSM 821 was obtained from German Collection of Microorganisms and Cell Cultures DSMZ (Braunschweig, Germany). The culture mediums PDA (potato dextrose agar), MEA (malt extract agar) and raw materials required to prepare the YGA complex liquid medium (yeast extract 3,0 g/L, malt extract 20 g/L, glucose 20

g/L, bacteriological peptone 1,0 g/L and agar 20 g/L), the YMPG (yeast extract 5,0 g/L, malt extract 10 g/L, glucose 10 g/L and bacteriological peptone 10 g/L), the MYB (yeast extract 3,0 g/L, malt extract 20 g/L, glucose 20 g/L and bacteriological peptone 10 g/L) and the YG (yeast extract 3,0 g/L, malt extract 20 g/L, glucose 20 g/L and bacteriological peptone 1,0 g/L, were purchased from OXOID (England).

Microbial growth kinetics of *A. niger* DSM 821 in solid medium. *A. niger* DSM 821 was grown in PDA, MEA and YGA media at 28°C for 10 days, adding 10 μL of $1*10^7$ spores/mL suspension. Growth kinetics of *A. niger* were determined by monitoring the colony diameter. Radial Growth Rate (RGR, mm/h) was estimated according to the method described by Pirt [31]. The best growth medium for microbial cultures was selected according RGR values and it was used for the maintenance of the strain. All experiments were performed in triplicate.

Evaluation of antifungal effect of substrate. Different concentrations of (*R*)-(+)-limonene (between 0 and 50,0 mM) were added to the MEA medium before gelation. Subsequently, the media were inoculated with 20 μL of spore suspension. Microbial growth was determined by measuring the change in diameter of the colonies after 8 days [31]. Medium without (*R*)-(+)-limonene was used as a negative control. The inhibition growth was calculated using the expression Eq1, where Dc is the average diameter of fungal colony in presence of inhibitor and De is the average diameter of the control. The limonene concentration required to inhibit the growth of *A. niger* up to a maximum of 25% was defined as the Minimum Inhibitory Concentration (MIC) and the concentration that inhibited up to 100% of growth, as the lethal concentration (LC) [32].

$$\text{Inhibition (\%)} = ((Dc - De) / Dc) * 100 \qquad \text{(Eq.1)}$$

Preparation of spore suspension. Spores from *A. niger* DSM 821were harvested from MEA medium a 28°C after 8 days of culture. Subsequently, they were re-suspended in 10 ml of saline 0,85% NaCl and 0,1% Tween 80. The initial concentration of spores in the biotransformation medium was $1*10^7$ spores/mL.

General process for biotransformation of limonene at lab scale. The bioassays were carried out in 22 mL vials fitted with Teflon stoppers, containing 5 mL of sterile liquid medium (YMPG, MYB and YGA) and inoculated with 50 μL of a 10^7 spores/mL suspension. Culture media were pre-incubated at 28°C for 72 h and 300 rpm of stirring using an orbital shaker (Heidolph Vibramax 100). After pre-incubation, (*R*)-(+)-limonene was added at different concentrations according to each experiment. Samples were taken periodically during 6 days. At the same time, a control biomass (spores suspended in the reaction medium without substrate) and control substrate (reaction medium and substrate without suspension of spores) were made to determine acid catalysis reactions. The reaction products and remaining substrate were extracted using ethyl acetate and identified by GC-MS. All experiments were performed in duplicate using independent samples. The reported value is the average of the two measurements.

Evaluation of the inducible effect from (*R*)-(+)-limonene on biotransformation. To determine the possible inducible effect of (*R*)-(+)-limonene on expression of oxidative enzymes, biotransformation experiments were carried out inoculating the medium YMPG with 50 μL of $1,0*10^7$ spores/mL suspension, obtained from a fungal growth in 20 mL of MEA medium cultivated at 28°C in presence of inducible (0,74 mM of (*R*)-(+)-limonene). Biotransformations reactions were performed during 72 h at 28°C and 300 rpm stirring speed, using 12 mM (*R*)-(+)-limonene (dissolved in 20% ethanol) in the culture medium. Periodically, samples were taken from the reaction medium for monitoring the biotransformation during 6 days and analyzed by GC-MS. Spore suspension grown without inducible was used in a control experiment.

Evaluation of the effect of pH on biotransformation. For these experiments, initial pH of the liquid medium YMPG was adjusted to values of 3,5, 5,0 y 6,5 using 0,1 M citrate-phosphate buffer. Culture media were inoculated with as described above. Subsequently, biotransformation reactions were performed according to the previously described methodology. The experiments were carried out by duplicated.

Growth kinetics of *A. niger* in liquids medium. The growth kinetics of *A. niger* DSM 821 was estimated by quantification of cell concentration by using dry weight cell method [35] during 15 days. Liquid media YMPG (broth malt extract 1%, yeast extract 0,5%, peptone 0,5% and glucose 1%), MYB (broth malt extract 2%, yeast extract 0,3%, peptone 1% and glucose 1%) and YM (broth malt extract 2%, yeast extract 0,3%, peptone 0,1% and glucose 2%) were adjusted at pH 5,0 using 0,1 M citrate-phosphate buffer and inoculated

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as previously described. The specific growth rate (μ) of fungi in each culture medium was determined [31]. The experiments were carried out by duplicated.

Evaluation of the effect of culture medium composition. The effect of the culture media YMPG, MYB and YM of pH 5,0 on the biotransformation of (*R*)-(+)-limonene was studied. The experiments were performed in the liquid media under the conditions described before. The biotransformation experiments were initiated by adding 12 mM (*R*)-(+)-limonene. Microbial kinetics biotransformation was monitored each 48 h for six days. The culture medium with highest bioconversion values was selected to evaluate other biotransformation variables.

Evaluation of the effect of addition of limonene at different growth phases of *A. niger.* To determine the inducing effect of the addition of (*R*)-(+)-limonene at different growth phases of *A. niger* DSM 821, on its biotransformation, the fungi were grown in MYB at pH 5,0 during different times period: 6 h and 1, 3, 5 or 7 days. These times corresponding with all sections of microbial growth curve (the lag, early, medium and final exponential and stationary phases) which were determined previously. When *A. niger* had reached the desired growth phase, the reaction was initiated by the addition of 12 mM (*R*)-(+)-limonene into the culture medium. The biotransformation kinetics was monitored every 72 h for nine days to each growth phase. The growth phase with highest concentration of POH was selected for evaluation of further variables. The tests were carried out by duplicated.

Evaluation of the effect of substrate concentration. The effect of (*R*)-(+)-limonene concentration on biotransformation by *A. niger* DSM 821 was evaluated using different concentrations (between 0 and 100 mM) in the MYB media at pH 5,0 during 9 days. The optimum concentration of substrate was determined according to the selectivity (to a single compound produced) and the concentration of POH produced.

Extraction, identification and quantification of products. DO and remaining substrates formed by biotransformation were extracted two times with ethyl acetate (2 x 2,5 mL) followed by centrifugation at 4000 rpm for 5 min. The organic phase was collected and dried with anhydrous Na_2SO_4 and concentrated by N_2 stream. Subsequently, 3 μL of *n*-tetradecane (*istd*) were added and diluted to 1 mL. Substrate and DO were identified and quantified by GC-MS using Agilent model 6890N (Palo Alto, CA, USA) chromatograph coupled to a mass selective detector Agilent Technologies 5975C with a detector system of electron impact ionization (70 eV) and a quadrupole mass analyzer, operated in full scan mode from 40-400 Dalton (m/z). An automatic injector HP 7683 Series with a split ratio of 1:13 and a DB-WAX capillary column (60 m x 0,25 mm x 0,25 μm) were used. Temperature was programmed from 45°C maintained for 10 minutes, with a heating rate of 3°C/min until 220°C, maintaining this temperature for 30 min. The identification of compounds was performed comparing mass spectra of the samples with spectra from compound library data from the databases ADAMS, NSB 75K, 138K NIST 05 and WILEY available in the data systems G1701BA HP Enhanced Chemstation.

RESULTS

Growth kinetics of *A.niger* DSM 821 in solid wculture media

Firstly, we evaluated fungal growth of *A. niger* DSM 821 in different culture media (PDA, MEA and YGA). The growth kinetics of *A. niger* was affected by the composition of the media. Best results were obtained in the MEA medium followed by the PDA and YGA media. This may be because the medium MEA has a higher carbon content, different sources of nitrogen and slightly more acidic pH compared with PDA and YGA media.

Highest RGR of *A. niger* DSM 821 was obtained in the MEA media (0,46±0,05) and was 1,1 and 2 times higher than in PDA (0,41 ±0,05) and YGA media (0,23±0,07), respectively. Therefore, MEA medium was selected for periodic maintenance of *A. niger* and obtaining spore solution used in the experiments of biotransformation.

Evaluation of antifungal activity of substrate

To determine the effect of (*R*)-(+)-limonene on the growth of *A. niger* DSM 821, substrate concentration in MEA culture media was varied (between 0 and 50 mM). It was observed that the presence of limonene as an additional source of carbon and energy affects negatively fungal growth decreasing the RGR (table 1). There was a minimum inhibitory concentration (MIC) of 12 mM, while 50 mM limonene or more in the culture medium were lethal for fungal growth (LC). The results are indicating toxicity of (*R*)-(+)-limonene,

Table 1. Antifungal activity of (*R*)-(+)-limonene on *A. niger* DSM 821 cultured in solid MEA medium at 28°C.

Limonene concentration (mM)	Colony diameter (mm)[a]	Inhibition (%)	RGR (mm/h)
0,0	78		0,46±0,02
3,7	76	2,56	0,43±0,04
7,3	75	3,85	0,38±0,02
11,0	72	7,69	0,34±0,04
12,0	58	25,54	0,28±0,02
14,7	55	29,49	0,14±0,01
22,0	52	33,33	0,13±0,02
30,0	46	41,03	0,12±0,01
50,0	0	100,00	0,00±0,00

[a:] 8 days of incubation

probably due to the polarity of this compound, which has a partition coefficient in *n*-octanol-water (Log P_{ow}) of 4,8 [33, 34, 35]. In previous studies, it has been described that terpenes with Log P_{ow} between 1-5 can cause a loss of specific permeability and integrity of the cell membrane [35, 36, 37].

Effect of the inductor (*R*)-(+)-limonene on biotransformation

We evaluated the inductive effect of (*R*)-(+)-limonene in the biotrasnformation of *A. niger* DSM 821 according to Tan and Day [33], using a substrate concentration of 0,74 mM. The experiments in YMPG medium at 12 mM limonene, 28°C and 300 rpm, showed that biotransformation was different when *A. niger* was preexposed to limonene. In the presence of inducer, the oxidation and hydroxylation reactions led to the production of OD such as carvone, *cis/trans*-carveol and limonene1,2-diol (table 2). By contrast, in the absence of inducer, only hydroxylation reactions were performed with POH as main product. Other derivatives were *cis/trans*-carveol, linalool and phenyl ethanol. Under these conditions was produced 129 mg POH/L and it was demonstrated that production of POH by *A. niger* by biotransformation of limonene is not inducible.

Effect of pH on the biotransformation

Biotransformation of (*R*)-(+)-limonene by *A. niger* DSM 821 was performed at pH 3,5, 5,0 and 6,5, in

Table 2. Biotransformation products of (*R*)-(+)-limonene by *A. niger* DSM 821.

Without Inducible		With inducible (100 mg limonene/L)	
Product	**Concentration (mg/L)**	**Product**	**Concentration (mg/L)**
Remainder limonene	1244,20±8,5	Remainder limonene	1502,12± 8,9
Linalool	80,61±2,2	Carvone	60,90± 2,8
Trans-carveol	71,14±1,9		
Cis-carveol	54,26±1,5	limonene 1,2-diol	22,66 ±1,7
Phenyl etanol	55,72±2,0	*Trans*-carveol	21,09± 2,0
Perillyl Alcohol	129,07±2,9	*Cis*-carveol	28,23 ±2,4
Conversion (%)[a]	23,89±0,6	Conversion (%)	8,12 ±0,5
Yield[b] (%)	7,89±0,4		

[a:] Conversion (%): mg products/mg initial substrate*100)

[b:] Yield respect to perillyl alcohol

the YMPG medium. Selectivity of the biotransformation was affected by the different conditions of pH and hydroxylated derivatives were produced. Only at pH 5 was produced POH. At pH 3,5, mainly phenyl ethanol (450 mg/L) was obtained whit a concentration 15 to 100 times higher than linalool and limonene-1,2-diol, respectively (see table 3). It is important to note that POH was the main product with a concentration of 129 mg/L at pH 5,0. Other products were alcohols such as linalool, limonene-1.2-diol and phenyl ethanol. At pH 6,5 the reaction was more selective because only phenyl ethanol was formed and bioconversion of limonene-1,2-diol, was lower.

Effects of the culture medium on the growth of *A. niger* and the biotransformation of limonene

Growth kinetics of *A. niger* DSM 821 in liquid media YMPG, MYB and YG at pH 5,0 (figure 1) showed that the composition of the culture medium influences microbial growth of the fungus. Better results of microbial growth were achieved in the medium MYB. Fungal

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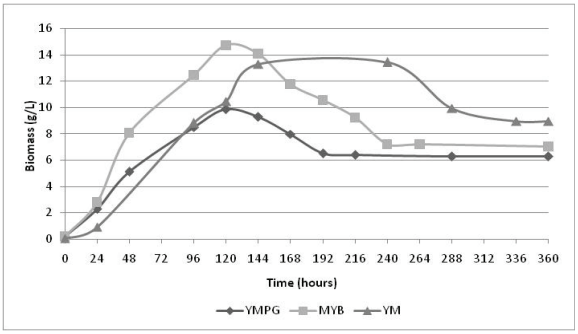
Figure 1. Growth kinetics of *A. niger* DSM 821 in liquid culture media at pH 5,0, 28 °C and 300 rpm.



growth was higher in the medium MYB compared to YMPG and YG. However, specific growth rates (μ) in the three media were relatively similar (0,03 h^{-1} for MYB and YG, and 0,02 h^{-1} for YMPG).

In all experiments, the POH was the main product (table 4). However, in the MYB medium, the concentration of POH was 246 mg/L, which is 1,7 and 3,4 higher than obtained in YMPG and YM media

The highest yield of POH in MYB is possibly due to higher nitrogen content represented in the peptone. In this way, would influence the biomass concentration and induction of oxidative enzymes. Obtained results are very interesting when compared with those reported in the literature. For example, 72,6 mg/L of POH (14,7%) were obtained from biotransformation of 4,93 mM limonene using *A. niger* grown in YMPG [38, 39]. The studies with *M. minutissina* produced 125 mg POH/L in the YMPG media using 58,71 mM of substrate [25], while another strain of *A. niger* BFQU 68 transformed limonene (37 mM) in TSB (tryptone-soya broth) medium to produce POH with a 28,5% of bioconversion [22].

In addition, cells of *A. niger* DSM 821 were able to biotransform (*R*)-(+)-limonene when they were in the exponential phase media (72 h), reaching a maximum concentration of POH of 246 mg/L at 6 days of bioreaction (figure 2). In the final exponential phase (120 h), biotransformation of limonene was directed towards production of *trans*-carveol (100 mg/L) 6 days after adding of substrate. In other phases, e.g. lag, early exponential and stationary, no biotransformation of (*R*)-(+)-limonene into POH as observed.

Effect of the substrate concentration on the biotransformation of (*R*)-(+)-limonene. Substrate concentration affected the selectivity of the reaction and level of bioconversion. Selectivity decreased with increasing concentrations of limonene in the medium (figure 3). However, at 50 mM (*R*)-(+)-limonene, POH production was increased, reaching a concentration of 405 mg/L. Compared with previously results, we could obtain POH production 1,7 times higher than obtained in effects of pH, medium of biotransformation and growth phase of *A. niger*.

The formation of the oxygenated compounds of limonene by means of hydroxylation reactions in different carbons of limonene, oxidation reaction and break cycles it is shown in figure 4.

Higher concentrations of limonene (> 50 mM) produced hydroxylated derivatives of limonene such as linalool, *trans*/*cis*-menth-2,8-dien-1-ol, *cis*/*trans*-carveol, carvone, perillyl aldehyde, limonene-1,2-diol, limonene 1,2 epoxide, phenyl ethanol, and ethyl esters of palmitic, oleic, linoleic and stearic (peak 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, respectively), as it is shown in figure 5. These ethyl esters may be formed by the action of oxygenases synthesized by *A. niger* and subsequent reaction using as co-solvent. Menéndez *et al.,* have reported for the rmation biotransforma-

Table 3. Effect of pH on the biotransformation of (*R*)-(+)-limonene using whole cells of *A. niger* DSM 821.

Concentration of biotransformation products (mg/L)								
pH 3,5			pH 5,0				pH 6,5	
Linalool	Limonene-1,2-diol	Phenyl ethanol	Perillyl alcohol	Linalool	Limonene-1,2 diol	Phenyl ethanol	Limonene-1,2, diol	Phenyl ethanol
4,5±0,3	30,8±2,3	450,7±18,2	128,6±2,9	18,4±2,6	35,1±3,1	66,9±3,7	30,7±1,9	85,23±,0
Conversion (%)		29,7±1,3	Conversion (%) Yield[a] (%)			15,2±0,8 7,44±0,7	Conversión (%)	7,1±0,3

Conversión (%): mg product/mg initial substrate*100; [a]: yield respect to perillyl alcohol (referred to the initial concentration of limonene).

Table 4. Metabolites produced of the biotransformation of limonene by *A. niger* DSM 821 at pH 5,0.

Biotransformation products	YMPG		MYB		YG	
	Conversión (%)	Cocentration (mg/L)	Conversión (%)	Cocentration (mg/L)	Conversión (%)	Cocentration (mg/L)
Linalool	1,1±0,2	10,7±2,6	0,3±0,1	16,6±2,1	3,7±0,2	74,5±3,4
Trans-varveol	1,0±0,1	18,4±2,6	4,2±0,2	71,8±3,4	*NDb*	*NDb*
PNIa	1,3±0,2	28,4±1,9	5,6±0,2	94,5±3,9	1,0±0,1	20,4±1,9
Perillyl alcohol	9,0±0,2	143,3±2,9	13,0±0,1	246,4±5,6	3,6±0,2	73,0±2,8
Phenyl ethanol	5,4±0,2	60,4±3,7	1,4±0,2	54,3±2,1	4,2±0,1	45,6±2,3
Limonene-1,2-diol	2,4±0,1	68,4±5,8	1,2±0,1	71,1±2,7	0,5±0,1	10,9±1,1
Total	**19,5**±1,0	**349,6**±16,8	**21,7**±0,8	**554,7±15,8**	**13,0**±0,6	**234,3**±11,3

aPNI: no identified product; b: no detected; c: percentage referred to the initial concentration of limonene

Figure 2. Effect of addition of (*R*)-(+)-limonene at different growth phases on biotransformation by *A. niger* in MYB medium at pH 5,0, 28°C and 300 rpm.

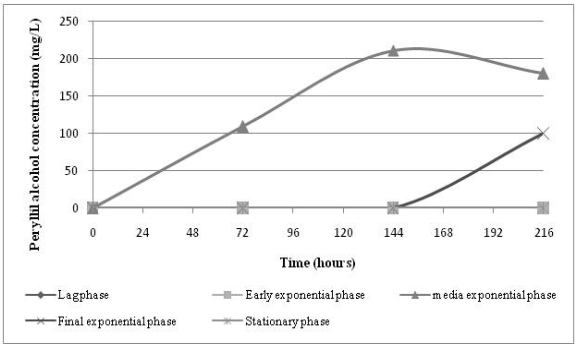
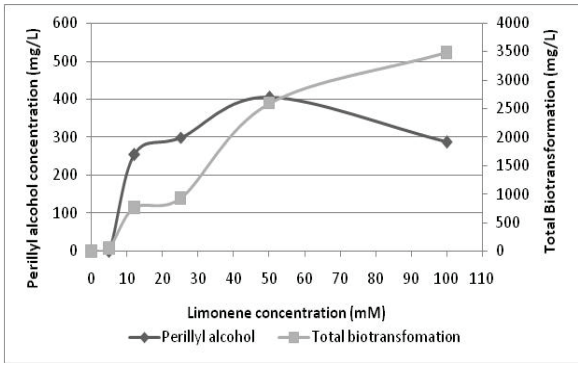


Figure 3. Production of perillyl alcohol for biotransformation of (*R*)-(+)-limonene by *A. niger*.



tion of limonene with *A. niger* BFQU 68, the production of short chain fatty acids (propanoic, isobutyric and isopentane) in absence of co-solvents [22]. Production of ethyl esters of higher fatty acids during terpene biotransformation processes has not been reported to date. In a recent work, esterification of POH with fatty acids catalyzed by lipases was carried out. The presence of ethyl esters of higher fatty acids in the natural extract obtained from the biotransformation of (*R*)-(+)- limonene by *A. niger* DSM 821 probably can broad its spectrum of application [34].

Finally, it is noteworthy that this work has allowed to reach a concentration of POH of 405 mg/L, equivalent to the maximum amount of this oxygenated limonene derivatives obtained by biotransformation processes using fungi.

CONCLUSIONS

Biotransformation of (*R*)-(+)-limonene by *A. niger* DSM 821 was directed mainly towards the production of perillyl alcohol, reaching a maximum concentration of 405 mg/L, using the following conditions: the fungus in the exponential phase (72 h), no prior exposure to limonene, MYB adjusted to pH 5,0 (using citrate-phosphate buffer 0,1 M), substrate concentration of 50 mM, 28°C and 300 rpm. In these experimental conditions, had the highest concentration of POH reported to date. *A. niger* DSM 821 also showed the ability to hydroxylate limonene at a carbon center than C-7 leading to terpenoids, *cis*/*trans*-carveol, limonene1,2-diol, linalool and phenyl ethanol. Additionally the biotransformation *in vivo* gives rise to ethyl esters of higher fatty acids, palmitic, oleic and linoleic.

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Figure 4. Biotransformation of (*R*)-(+)-limonene (50 mM) products by *A. niger* DSM 821.

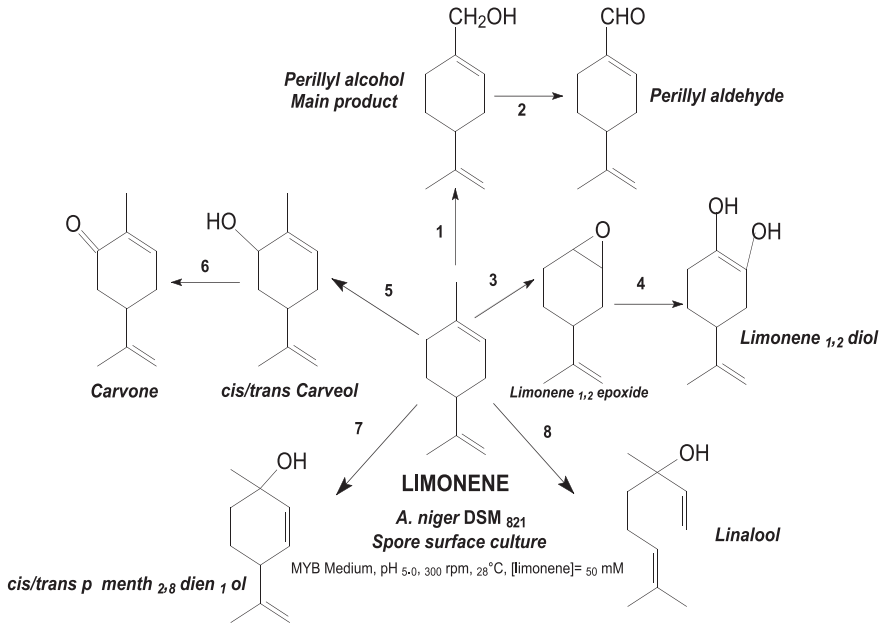
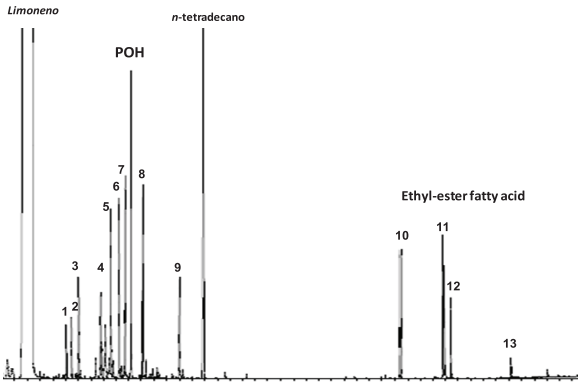


Figure 5. Chromatogram of (*R*)-(+)-limonene ([> 50 mM]) biotransformation by *A. niger* DSM 821.



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