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BIOCONVERSION OF (+)-NOOTKATONE BY *Botryodiplodia theobromae* USING A MEMBRANE AERATED BIOFILM REACTOR

BIOCONVERSIÓN DE (+)-NOOTKATON POR *Botryodiplodia theobromae* UTILIZANDO UN REACTOR DE BIOPELÍCULA DE MEMBRANA AIREADA

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Abstract

The aim of this work was to evaluate the bioconversion of (+)-valencene to (+)-nootkatone by *B. theobromae* using a membrane aerated biofilm reactor (MABR) in a two liquid phase system with orange essential oil as the organic phase. In the aqueous phase system, a (+)-nootkatone production rate up to 3.98 mg L^{-1} h^{-1} was achieved, obtaining a final product concentration of 398.08 mg L^{-1} with a bioconversion of 62 %. A two liquid phase system, using orange essential oil as the dispersed phase, was also studied and a final (+)-nootkatone concentration of 310.37 mg L^{-1} was achieved in the organic phase, with a bioconversion of 30.5 % and a production rate of 2.46 mg L^{-1} day^{-1}. The lower performance obtained using the two phase system was probably due to mass transfer limitations. The present work is the first report on an MABR for the bioconversion of (+)-valencene to (+)-nootkatone. Further studies on bioconversion products and optimization of biofilm reactor operations are needed to enhance bioconversion.

Keywords: bioconversion, (+)-nootkatone, *Botryodiplodia theobromae*, orange essential oil, membrane aerated biofilm reactor.

Resumen

El objetivo de este trabajo fue evaluar la bioconversión de (+)-valenceno a (+)-nootkaton por *B. theobromae* usando un reactor de biopelícula de membrana aireada (MABR) en un sistema de dos fases líquidas con aceite esencial de naranja como fase orgánica. En el sistema de fase acuosa, se logró una tasa de producción de (+)-nootkaton de hasta 3.98 mg L^{-1} h^{-1}, obteniendo una concentración de producto final de 398.08 mg L^{-1} con una bioconversión de 62 %. También se estudió un sistema de dos fases líquidas, utilizando aceite esencial de naranja como fase dispersa, y se alcanzó una concentración final de (+)-nootkaton en la fase orgánica de 310.37 mg L^{-1}, con una bioconversión de 30.5 % y una tasa de producción de 2.46 mg L^{-1} $día^{-1}$. El menor rendimiento obtenido mediante el sistema de dos fases fue probablemente debido a las limitaciones de transferencia de masa. El presente trabajo es el primer reporte utilizando un MABR para la bioconversión de (+)-valenceno a (+)-nootkaton. Se necesitan estudios adicionales sobre los productos de bioconversión y la optimización de las condiciones de operación del reactor de biopelícula para mejorar la bioconversión.

Palabras clave: bioconversión, (+)-nootkaton, *Botryodiplodia theobromae*, aceite esencial de naranja, reactor de biopelícula de membrana aireada.

1 Introduction

The compound (+)-nootkatone is a sesquiterpenoid which possesses an intense grapefruit-like taste and other valuable properties that are highly appreciated by the fragrance and flavor industries (Ladaniya, 2010). The production of (+)-nootkatone is performed via chemical synthesis, mainly from the sesquiterpene

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(+)-valencene, which is readily available from the orange industry and also through the use of environmentally unfriendly oxidising agents, such as tert-butyl peracetate (Wilson and Shaw, 1978) and tert-butyl hydroperoxide in combination with catalytic metal supported on silica (Salvador and Clark, 2002). However, the resulting (+)-nootkatone produced via chemical synthesis cannot be marketed as a "natural" product and does not satisfy increasing market demands for natural aromatic compounds. In order to meet this demand, many efforts have been focused on the use of biotechnological processes with bacteria, fungi or plants (Fraatz *et al*., 2009).

Bioconversion plays an increasingly important role in organic synthesis for the production of fine chemicals, such as the synthesis of chiral precursors (Faber, 2004). However, an important number of substrates and products of bioconversion are poorly soluble in water, the medium in which most biocatalysts have optimal biological performance. Additionally, the substrate and product above critical concentrations are known to inhibit biocatalytic activity (D´Arrigo *et al*., 2000; Held *et al*., 2000). Therefore, a significant obstacle to the application of bioconversion in industry is the limiting loading of the substrate and product allowed in the reactor. Partitioning bioreactors overcome this limitation since favorable partitioning coefficients of substrates and products toward the organic phase allow the use of high concentrations in this phase, while maintaining low concentrations in the aqueous phase. However, there might be some drawbacks; for example, the intensive mixing of the aqueous and organic phases can cause strong emulsions that make downstream separation a practical constraint. The presence of the organic solvent might inhibit or even stop the biocatalyst activity (D´Arrigo *et al*., 2000; Held *et al*., 2000; Dutra-Molino *et al*., 2014).

MABRs have been applied for the degradation of volatile contaminants in waste water (Qureshi *et al*., 2005; Judd, 2008) but have not yet been used in oxidative bioconversion. MABR was found to be a suitable method to decrease the loss of volatile substances during a transformation process (Casey *et al*., 1999; Lilly and Woodley, 1996). Onken and Berger (1999) studied the use of an MABR for the biotransformation of citronellol by the basidiomycete *Cystoderma carcharias*; production rates up to 150 mg of citronellol L^{-1} day^{-1} were reached and led to a product concentration of 866 mg L^{-1} with a conversion rate of 52%. The total loss of the added volatile substrate via the exhaust air was 4.5% when this aeration method was used. In addition, González-Brambila and López-Isunza (2007) reduced oxygen transfer limitations across the biofilm supplying oxygen from inside the membrane and simultaneous sparing of air to the residual water, thus improving MABR performance.

The aim of this work was to evaluate the bioconversion of (+)-valencene to (+)-nootkatone by *B. theobromae* using an MABR. The bioconversion was tested in both a single liquid phase system and a two liquid phase system using orange essential oil as the organic phase.

2 Materials and methods

2.1 Chemicals

(+)-Valencene (CAS 75-05-6) and (+)-nootkatone (CAS 93-78-5) with a purity of >70% and >85%, respectively, were purchased from Fluka (Switzerland) and used as standards. Ethyl acetate (99.5%) was purchased from Quimex (México). Analytical grade orange essential oil (*Citrus aurantium*, var. Amara) was purchased from Cosmopolita Drugstore (Mexico, DF) and used as the organic dispersed phase to carry out bioconversion studies.

2.2 Microorganism

Botryodiplodia theobromae 1368 from the Instituto Nacional de Investigaciones Fundamentales de la Agricultura Tropical (La Habana, Cuba) isolated from Cuban *Citrus cinensis* Osbeck cv Valencia, was used. The strain was stored on dextrose potato agar slants at 4°C and subcultured every month at 30°C for 7 days. Spores were harvested with 5.0 mL of Tween-80 (0.01% v/v) and were used as inoculum (1×10^6 spores mL^{-1}).

2.3 Culture media

Culture medium with the following basal salt composition was used (in g L^{-1}): sucrose, 50; $NaNO_3$, 7.5; KH_2PO_4, 2.0; KCI, 0.3; $MgSO_4.7H_2O$, 0.6; $FeSO_4.7H_2O$, 0.6; $ZnSO_4.7H_2O$, 0.03; $MnSO_4.7H_2O$, 0.003; $CuSO_4.7H_2O$, 0.003; $Na_2MoO_4.2H_2O$, 0.003; yeast extract, 1.0 (Eng *et al*., 1998). After autoclaving, the initial pH was adjusted to 5.5-5.6 with 2 M HCl.

2.4 Suspended biomass determination

Samples of a known volume were dried until a constant weight in metal plates which had been previously weighed after being left in an oven at 90°C overnight. All analyses were performed in triplicate.

2.5 Sucrose consumption

The sucrose consumption of *B. theobromae* was estimated by analyzing the sucrose concentration in the medium using the technique described by Miller *et al*. (1960). Analyses were performed in triplicate.

2.6 Cell viability

Cell viability during growth and the bioconversion process was determined by measuring the concentration of living cells using the methylene blue technique, as described by Bonora and Mares (1982). Analyses were performed in triplicate.

2.7 Membrane aerated biofilm reactor

The MABR used was previously described by González-Brambila and López-Isunza (2007). The Pyrex tube-bioreactor had the following dimensions: 200 mL volume, 300 mm height, 30 mm outside diameter, and 2 mm thickness. An oxygen permeable membrane of silicon rubber (290 mm length, 1.5 mm inner radius, and 0.3 mm thick) was used. The membrane tube was placed inside the glass bioreactor. One side of the tube was connected to the air supply valve, and the other side was partially blocked to force oxygen to flow through the membrane wall. The system operated continuously at 30°C and pH 5.5. Atmospheric air was filtered through a sterile 0.22 μm filter and was pumped through the membrane at a flow rate of 0.135 L min^{-1}. The culture medium (140 mL) was fed upward with a hydraulic retention time (HRT) of 0.185 h.

2.8 Bioconversion processes

The MABR was inoculated using 1× 10^6 spores of *B. theobromae* mL^{-1}. After 7 days of growth, a biofilm had formed outside of the membrane. Then, bioconversion studies were conducted by: (a) adding a concentrated solution of (+)-valencene to reach a final concentration of 1.0 g L^{-1} or (b) dropping a total volume of 135 mL of orange essential oil through the MABR during the bioconversion process (Figure 1). In order to determine the effect of adding valencene and other components of orange essential oil to the culture medium, a control without the addition of (+)-valencene was included using 50 g of sucrose L^{-1}, which was performed in duplicate. Every day, 2 mL of sample was taken and centrifuged at 5000 rpm for 10 min. Sesquiterpene extraction was carried out using 500 μL of sample and 500 μL of ethyl acetate. The mixture was vortexed twice for 20 s in a vortex, and settled until phase separation occurred, then 1 μL of the ethyl acetate phase was injected to a gas chromatograph (Girhard *et al*., 2009; Kaspera *et al*., 2005).

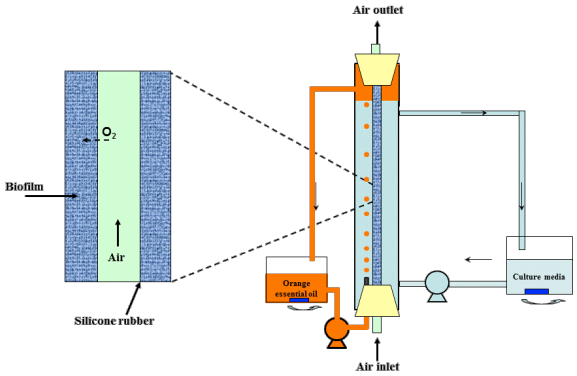

Fig. 1. Diagram of the membrane aerated biofilm reactor for the bioconversion of (+)-valencene to (+)-nootkatone by *B. theobromae*.

2.9 Partition coefficients determination

Partition coefficients of (+)-valencene and (+)-nootkatone were determined by mixing equals volumes of a phosphate buffer (0.1 M) adjusted to pH 5.5 containing 3.2 g of valencene L^{-1} and orange essential oil containing 3.2 g of nootkatone L^{-1} in a total volume of 6 mL. The mixture was kept under mechanical agitation for 6 h at 30 °C to reach thermodynamic equilibrium then the phases were separated by centrifugation. Sesquiterpenes extraction was carried out by adding 500 μL of ethyl acetate in both phases. Mixtures were vortexed twice for 20 s in a vortex, and settled until phase separation occurred, after that 1 μL of the ethyl phase was injected to Gas Chromatograph (GC) to determine (+)-valencene and (+)-nootkatone concentrations. Later partition coefficients (K_P) were determined. The partition coefficient was calculated as the ratio of the concentration of solute in the organic phase divided by the concentration of the solute in the aqueous phase at equilibrium.

2.10 Sesquiterpenes analysis

The concentrations of (+)-valencene and (+)-nootkatone were determined by gas chromatography (GC-FID) using a Perkin Elmer Auto System XL gas chromatograph equipped with a flame ionization detector (Cyclosil-B capillary column; 30 m × 0.32 mm × 0.25 μm; J & W Scientific). The temperatures of the injector and detector were constant at 250°C and 270°C, respectively. 1 μL of sample was automatically injected with a split ratio of 1:4, using helium gas as the carrier. The column temperature was initially set to 120°C for 4 min then ramped up to 250°C at a rate of 10°C per min and maintained at 250°C for 5 min, using the technique reported by Girhard *et al*. (2009). Analyses were performed in triplicate. GC/MS spectra were obtained using a GC/MS-HP6890 (Agilent) gas chromatograph equipped with an HP-5 column (30 m × 250 μm × 0.25 μm, Agilent). The same conditions of separation as the GC-FID analysis were used. Compounds in the samples were identified by comparison of the mass spectra to those of pure compounds.

3 Results and discussion

3.1 Sesquiterpenes in orange essential oil (Citrus aurantium, var. amara)

The orange essential oil used in this work was analysed by GC coupled to mass spectrometry. The concentrations of (+)-valencene and (+)-nootkatone were determined to be 3.2 g L^{-1} and 0.94 mg L^{-1}, respectively. The (+)-valencene concentration obtained was similar to that reported by Del Rio *et al*. (1991) at 2.99 g L^{-1} for the same variety of orange. Depending on the harvest of the fruit and its specific variety, the concentration of sesquiterpenes varies for different species of the *Citrus* genus (Espina *et al*., 2010; Elston and Rouseff 2005). The highest concentration reported for (+)-valencene was 6.0 g L^{-1} for the species *C. sinensis* (Sharon-Asa *et al*., 2003). In orange essential oil, there are a large number of compounds used in the food and beverage industry as antimicrobial agents. Limonene, a terpene with high antimicrobial activity, is the compound in greatest abundance (Mustafa *et al*., 2012).

3.2 Membrane aerated biofilm reactor during bioconversion in a monophasic system

To investigate the effect of (+)-valencene on the whole bioconversion process in the MABR, an aqueous phase system was studied. The MABR was inoculated with 1× 10^6 spores suspended in 240 mL of mineral medium with 50 g of sucrose L^{-1} as the carbon source. The biofilm was allowed to develop for 7 days at 30ºC. However, suspended biomass was observed, reaching a maximum biomass concentration of 23.5 g L^{-1} after approximately 90% sucrose consumption. At this point, a stock solution of valencene was added to reach an initial (+)-valencene concentration of 1.0 g L^{-1} in the MABR to initiate the bioconversion process. The reactor was operated with a recycle rate of 12.61 mL min^{-1} and an overall HRT of 0.185 h, and the ascending liquid velocity parallel to the membrane was ~0.04 cm s^{-1}. The bioconversion of (+)-valencene was measured by GC. The kinetics of cell growth, sucrose consumption, and cell viability during the bioconversion process are plotted in Figure 2. It was observed that cell viability decreased linearly to roughly 30% between 8 and 12 days. Final cell viability might indicate that (+)-valencene or an intermediary may induce the inhibition of (+)-nootkatone bioconversion.

The kinetics of (+)-valencene consumption and (+)-nootkatone production during the bioconversion process are plotted in Figure 3. After 96 h, a concentration of 398.08 mg L^{-1} of (+)-nootkatone was reached, thus converting 0.526 g L^{-1} of valencene (i.e. bioconversion of 62 %). It was observed that the initial (+)-valencene concentration (1.0 g L^{-1}) decreased in about 50% after 96 h of bioconversion at 30ºC.

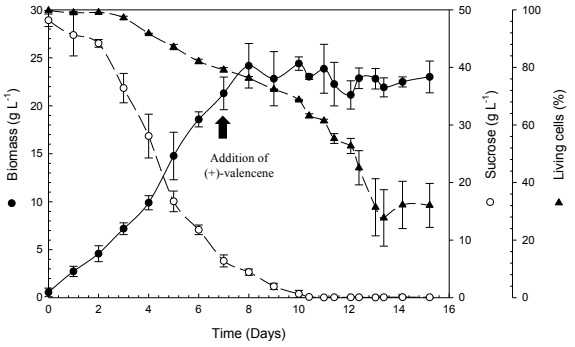

Fig. 2. Kinetics of suspended biomass, sucrose consumption, and cell viability in the aqueous phase system. 1.0 g L^{-1} of (+)-valencene was added to initiate the bioconversion process.

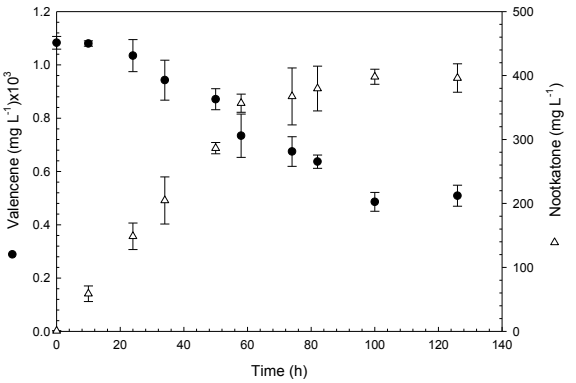

Fig. 3. Bioconversion of (+)-valencene to (+)-nootkatone in an aqueous phase system using *B. theobromae*. Zero time corresponds to 7 days of growth.

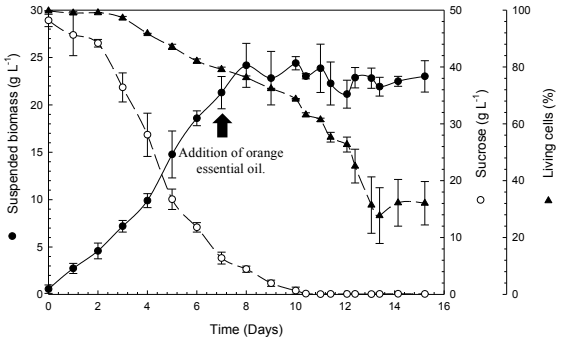

Fig. 4. Kinetics of suspended biomass, sucrose consumption, and cell viability in the biphasic system. Orange essential oil was added to initiate the bioconversion process.

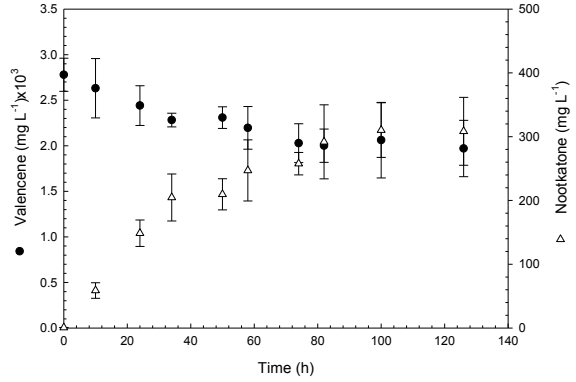

Fig. 5. Bioconversion of (+)-valencene to (+)-nootkatone in a biphasic system using *B. theobromae*. Zero time corresponds to 7 days of growth.

After this period, no further changes in (+)-valencene concentration were observed. However, after 56 h, no statistically significant difference from the (+)-nootkatone produced by *B. theobromae* was observed. The production of (+)-nootkatone from (+)-valencene has been reported by Furusawa *et al.* (2005) using *Botryosphaeria dothidea* and *Botryodiplodia theobromae*, i.e. fungal cells. The authors reported that the biotransformation yielded 93% and 72%, respectively, of enantiomerically pure (+)-nootkatone. In this system, NADP(H), the reduced form of the cofactor, was used to provide the reaction reduction potential and is regenerated by the cells.

3.3 Membrane aerated biofilm reactor during bioconversion in a biphasic system

Bioconversion experiments were conducted in a two phase system using orange essential oil as a source of (+)-valencene for bioconversion to (+)-nootkatone. First, the biofilm was allowed to develop for 7 days at 30ºC using 50 g of sucrose L^{-1} as the carbon source. Then, orange essential oil was added. Figure 4 shows the results of the growth kinetics, sucrose concentration and cell viability during the bioconversion process. At 10 days, approximately 90% sucrose consumption and a suspended biomass concentration of 23.25 g L^{-1} were observed. After the addition of orange essential oil, cell viability decreased linearly between 8 and 12 days.

(+)-Valencene and (+)-nootkatone concentrations in the organic phase during the bioconversion process are plotted in Figure 5. At 80 h, a concentration of 310.37 ± 13.4 mg L^{-1} of (+)-nootkatone and an approximate consumption of 0.716 g L^{-1} of (+)-valencene were observed (i.e. bioconversion of 30.5%). During the bioconversion process in the partitioning bioreactor, the (+)-valencene present in orange essential oil was transferred by partition (K_p = 6.37) to the aqueous phase based on the equilibrium and the metabolic demand of the cells. Also, the (+)-nootkatone produced in the aqueous phase was mainly transferred by partition (K_p = 22.5) into the organic phase for *in situ* extraction. Because of the organic phase was dropped using a syringe into the aqueous phase and drops ascended because of a lower density, two main drawbacks were observed: low mass transfer interfacial areas and hydrodynamic constraints. Consequently limited mass transfer should be expected (Lizardi-Jiménez and Gutiérrez-Rojas, 2011). Therefore, the relatively low nootkatone concentration in the organic phase was probably due to substrate mass transfer limitations. On the other

hand, lipophilic compounds, such as terpenoids, that preferentially dissolve in the lipophilic membrane systems of fungal cells (Abraham *et al*. 1997) might influence the bioconversion. Terpenoids, as well as other lipophilic compounds such as polycyclic aromatic hydrocarbons, alkanes and phenols, induce changes in membrane properties and thus cause toxic effects. Fungi counteract these effects by co-metabolizing the poorly water-soluble compounds into water-soluble ones or to carbon dioxide and water, using easily metabolizable substrates (e.g. sucrose) as the main source of energy. The enzyme systems involved in these detoxification processes are comparable to those of other eukaryotic cells, e.g. mammals (Wackett and Gibson, 1982). In the first step, catalyzed by cytochrome P450 mono-oxygenases, the molecule is oxyfunctionalized and, in the second step, hydrolysis (e.g. by epoxide hydrolases) or conjugation (e.g. by glutathione-S-transferases or UDP-glucuronosyltransferases) leads to water-soluble products, which are excreted into the medium.

The bioreactor experiments were performed in a partially blocked-end mode to provide transmembrane flow to diminish blocking of membrane pores by growth of the fungus or by extra-cellular products which provoke a partial blocking of the membrane pores, causing decreased oxygen transfer. Air flow rate used (0.135 L min^{-1}) was similar to those reported (0.2 L min^{-1}) by González-Brambila and López-Isunza (2007). The authors described a detailed process analysis of oxygen mass transfer characteristics across the biofilm using silicone membranes. They showed that dissolved oxygen across the biofilm might be not enough to support the bioreaction. Despite of these limitations, the aeration of fungal cultures in bioreactors using hydrophobic microporous membranes offers attractive perspectives for bioconversions of volatile sesquiterpenes substrates. Further work should focus on the long-term supply of sufficient amounts of oxygen through the aeration membrane to make this approach more applicable to the bioconversion of lipophilic compounds by submerged fungal cultures. This method of regioselective synthesis was performed naturally by a microbial pathway converting the product of commercial interest, with better versatility, so this method may be used as an alternative in the industrial production of nootkatone.

Conclusions

In a biphasic system using *B. theobromae*, a (+)-nootkatone concentration of 310.37 mg L^{-1} was recovered in the organic phase. The addition of a second liquid phase (orange essential oil) presents a potential application for the production and in situ extraction of the sesquiterpene (+)-nootkatone. However, further bioconversion studies and optimization of MABR operation are needed to enhance this process.

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