Revista Mexicana de Ingeniería Química
ISSN: 1665-2738
amidiq@xanum.uam.mx
Universidad Autónoma Metropolitana
Unidad Iztapalapa
México

Velasco-Bucheli, R.; Gil, J.H.; García, C.M.; Durango, D.L.
TRANSFORMATION OF CINNAMYL ALCOHOL USING IMMOBILIZED Colletotrichum acutatum CELLS AS CATALYST
Revista Mexicana de Ingeniería Química, vol. 16, núm. 1, 2017, pp. 55-65
Universidad Autónoma Metropolitana Unidad Iztapalapa
Distrito Federal, México

Vol. 16, No. 1 (2017) 55-65
Revista Mexicana de Ingeniería Química

TRANSFORMATION OF CINNAMYL ALCOHOL USING IMMOBILIZED *Colletotrichum acutatum* CELLS AS CATALYST

TRANSFORMACIÓN DE ALCOHOL CINAMÍLICO EMPLEANDO COMO CATALIZADOR CÉLULAS INMOVILIZADAS DE *Colletotrichum acutatum*

R. Velasco-Bucheli[1] *, J.H. Gil[2], C.M. García[3], D.L. Durango[3]

[1] *Universidad Complutense de Madrid, Departamento de Bioquímica y Biología Molecular I, Facultad de Biología, Madrid, España.*

[2] *Universidad Nacional de Colombia-Sede Medellín, Facultad de Ciencias Agrarias, Departamento de Ingeniería Agrícola y Alimentos, Medellín, Colombia.*

[3] *Universidad Nacional de Colombia-Sede Medellín, Facultad de Ciencias, Escuela de Química, Grupo de Química de los Productos Naturales y los Alimentos, Medellín, Colombia.*

Received June 19, 2016; Accepted December 21, 2016

Abstract

The reusability and influence of three supports (calcium alginate, chitosan and polyurethane foam) in the biotransformation of cinnamyl alcohol by the spores and whole cells from the Colombian native fungus *Colletotrichum acutatum* was evaluated. The metabolic products cinnamaldehyde, 3-phenylpropanal, 3-phenyl-1-propanol, 1-phenylpropane-1,3-diol, 3-hydroxy-1-phenyl-1-propanone, hydrocinnamic acid, 2-phenylethanol, 1-phenylethane-1,2-diol, cinnamyl acetate, cinnamic acid and 3-phenyl propyl acetate were detected by GC-MS. The concentration of these products was clearly dependent on support. Thus, the bioprocesses with immobilized spores or whole cells into polyurethane foam were more efficient to obtain 1-phenylpropane-1,3-diol, and allowed reusing the support at least 3 or 4 times without significant loss of activity. However, under the same conditions, alginate and chitosan were inadequate supports for this purpose, and instead, 3-phenyl-1-propanol and cinnamic acid were the major metabolic compounds. Therefore, the biotransformation of cinnamyl alcohol using immobilized *C. acutatum* cells in polyurethane foam can potentially offer a simple and efficient way to obtain 1-phenylpropane-1,3-diol.

Keywords: phytopathogenic fungus, metabolic pathway, immobilization, reuse, 1-phenylpropane-1,3-diol.

Resumen

Se evaluó la reutilización e influencia de tres soportes (alginato de calcio, quitosano, y espuma de poliuretano) en la biotransformación de alcohol cinamílico por esporas y células completas del hongo nativo colombiano *Colletotrichum acutatum*. Se detectaron por CG-EM los productos metabólicos cinamaldehído, 3-fenilpropanal, 3-fenil-1-propanol, 1-fenil-1,3-propanodiol, 3-hidroxi-1-fenil-1-propanona, ácido hidrocinámico, 2-feniletanol, 1-fenil-1,2-etanodiol, acetato de cinamilo, ácido cinámico y acetato de 3-fenilpropanol. La concentración de estos productos fue claramente dependiente del soporte. Así, los bioprocesos con esporas o células completas inmovilizadas en la espuma de poliuretano fueron más eficientes para obtener 1-fenil-1,3-propanodiol, y permitieron su reutilización al menos 3 o 4 veces sin pérdida significativa de la actividad. Sin embargo, bajo las mismas condiciones, alginato y quitosano no fueron soportes adecuados para este propósito, y en su lugar, 3-fenil-1-propanol y ácido cinámico fueron los compuestos metabólicos mayoritarios. Por lo tanto, la biotransformación de alcohol cinamílico usando células de *C. acutatum* inmovilizadas en espuma de poliuretano puede ofrecer potencialmente una vía simple y eficiente para obtener 1-fenil-1,3-propanodiol.

Palabras clave: hongo fitopatógeno, ruta metabólica, inmovilización, reutilización, 1-fenil-1,3-propanodiol.

1 Introduction

Phytopathogen fungi of the genus *Colletotrichum* are known for their abilities to transform natural compounds. In a previous report, we have described the biotransformation of cinnamyl alcohol (A) using free whole cells of *Colletotrichum acutatum* as biocatalyst (Velasco *et al*., 2010). The substrate was mainly converted to 3-phenyl-1-propanol (B), 3-phenyl propyl acetate (C), hydrocinnamic acid (D),

* *Corresponding author. E-mail*: rodrigov@ucm.es; Tel. +34-91-394-41-50, Fax +34-91-394-46-72

2-phenylethanol (E), 1-phenyl-1,2-ethanediol (F), and 1-phenylpropane-1,3-diol (G). We have also shown that the relative abundance of metabolic compounds was dependent not only on the biotransformation time, but also on the type of culture medium used. Interestingly, some of the metabolic compounds from the biotransformation of cinnamyl alcohol by *C. acutatum* have been widely used in the food, beverage, cosmetic, chemical and pharmaceutical sector as flavor and fragrance (Sun *et al*., 2016; Tian *et al*., 2015; Velasco *et al*., 2015; Behan *et al*., 1996; Savina *et al*., 1999; Sendovski *et al*., 2010). Furthermore, the compound 1-phenylpropane-1,3-diol is an important intermediate in the synthesis of therapeutic agents for the treatment of psychiatric disorders (depression, anxiety, alcoholism) and metabolic problems (obesity and bulimia), such as fluoxetine and norfluoxetina (Kumar *et al*., 2004). Also 1-phenylpropane-1,3-diol has been suggested as starting material to obtain tolterodine, a drug for the treatment of urinary incontinence (De Castro and Rhee, 2008). Here we describe the biotransformation of cinnamyl alcohol by immobilized spores and whole cells of *Colletotrichum acutatum*, and the reusability of some supports (*i.e*., calcium alginate, chitosan and polyurethane foam) under repeated batch biotransformation conditions as well. A special attention was paid to the production of 1-phenylpropane-1,3-diol.

2 Materials and methods

2.1 Analytical methods

Gas chromatography was developed on a Hewlett-Packard 6890 (Agilent Technologies) gas chromatograph coupled with a mass selective detector-quadrupole type (HP 5973 MSD). A HP-5 fused silica capillary column (30 m x 0.25 mm, 0.25 μm film thickness) was used. The chromatographic conditions were: column temperature, 50-250°C at 10°C/min, keeping it for five minutes; injector temperature, 150°C; detector temperature, 280°C; carrier gas, N_2 at 1 mL/min. The relative composition of each constituent was determined from the average area of the peaks. Metabolic products were identified by comparison of their mass spectra with those of reference substances and by comparison with the NIST 2002 Mass Spectral Library. The cells incorporation and morphology of beads was examined using a stereoscopic microscope Nikon SMZ1000. The spores count was achieved by using a microscope Leica CME 1349521X.

2.2 Biological and chemical materials

Dr. Afanador-Kafuri (Afanador-Kafuri *et al*., 2003) kindly donated the strain of *C. acutatum*. The microorganism was conserved and freshly subcultured on PDA before use. Fungal spores were stored in 20% (w/v) glycerol at -20°C. Cinnamyl alcohol (A) of analytical grade and chitosan were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Bacto Agar was obtained from Becton, Dickinson and Co. (Franklin Lakes, NJ, USA). Calcium chloride dihydrate and anhydrous alpha-D(+)-glucose were acquired from Merck KGaA (Darmstadt, Germany) and Acros Organics (Fair Lawn, NJ, USA), respectively. Alginic acid (sodium salt) of very low viscosity was from Alfa Aesar (Ward Hill, MA, USA). The polyurethane foam (PUF) employed was Scotch-Brite 3M.

2.3 Isolation of C. acutatum spores

Petri dishes cultivated with the fungus for 8 days on Potato Dextrose Agar (PDA) were washed with distilled water (2x2 mL). The suspension was filtered on Whatman No. 1 filter paper. Then, the filtrate was centrifuged at 10000 rpm for 5 min and the supernatant was eliminated; this procedure was repeated two times. The spores (precipitate) were recollected, suspended in 20% (w/v) glycerol, and stored at −20°C until used; the colony-forming units (CFU) were counted in a Neubauer chamber by microscopy after staining with lactophenol blue. The solution was homogenized at room temperature and used in the immobilization and biotransformation processes.

2.4 Immobilization of fungal aggregated mycelium and spores into PUF

The whole microorganism and the spores (1×10^5 CFU/mL) of *C. acutatum* were inoculated in PUF with density of 82 mg/cm^3 according to the manufacturer. The foam was cut in parallelepipeds ($1.0 \times 1.0 \times 0.7$ cm^3), washed threefold with abundant distilled water under heating and agitation, and sterilized in separate flasks (Wanga *et al*., 2013; Mulla *et al*., 2012; Siddiqui *et al*., 2009). Each 100 mL Erlenmeyer flask was supplied with five cubes of PUF in 25.0 mL of Potato Dextrose Broth (PDB). Then, the fungus (spores or whole microorganism) was added into the medium. It was grown and fixed to the support during

7 days (preculture). All immobilization processes were carried out under sterile conditions. A control without inoculums was performed.

2.4.1 Immobilization of spores into PUF

The spore solution was added in the medium with the support (5 mL_{CFU}/250 mL_{broth}). The foam with the fungus attached was removed by filtration, and the cubes were washed with sufficient water.

2.4.2 Immobilization of whole microorganism into PUF

The fungus previously cultivated on PDA (1 month of age) was added as inoculums. Thus, the content of a Petri dish (15 cm in diameter) was employed for each 1 L of culture medium. Similarly, the foams with the fungus were removed by filtration, and the cubes were washed with sufficient water. Erlenmeyer flasks were shaken at 120 rpm and room temperature for 168 h.

2.5 Immobilization within calcium alginate beads

The immobilization was done as described earlier by Romano *et al*. (2006) with some modifications. Briefly, sodium alginate was dissolved in distilled water at 50°C under constant agitation. Solutions of sodium alginate and $CaCl_2$ 0.1 M were sterilized before use, and then allowed to reach room temperature. 5 mL of spore solution (1×10^5 CFU/mL) was suspended into the alginate up to 60 mL of solution 3.0% w/w alginate concentration. The resulting suspension was degassed and dripped through a needle into 10 mL of iced $CaCl_2$ solution. After hardening at 4°C for 3 h, the beads were separated from the $CaCl_2$ solution by vacuum filtration. They were washed on a filter with abundant water to remove excess of calcium ions and un-entrapped cells, and the beads were used directly in the biotransformation, or stored at 4°C in sterile distilled water.

2.6 Immobilization within chitosan beads

The immobilization was carried out as described previously by Barreto *et al*. (2010) with some modifications. Briefly, chitosan was dissolved in acetic acid 1.0%, and the solution was adjusted to pH 6.0. Solutions of chitosan and 8.0% NaOH were sterilized before use. 5 mL of spore suspension (1×10^5 CFU/mL) was transferred to 60 mL of 4.0% w/w chitosan solution. Then, the mixture was degassed and extruded dropwise through a sterilized syringe into an 8.0% NaOH solution for coagulation and formation of the beads. After 3 min at room temperature, the beads were filtered, washed with water and used directly in the biotransformation, or stored at 4°C in sterile distilled water.

2.7 Biotransformation process

Previous studies have shown that *C. acutatum* efficiently transformed cinnamyl alcohol (A) at 40 mg/L after 15 days (Velasco *et al*., 2010). Accordingly, the supports containing the immobilized fungus were washed with distilled water, filtered and used to inoculate 250 mL PDB culture medium containing the substrate at the same concentration described above. The cultivation was carried out by stirring (reciprocating shaker, 120 rpm) at room temperature. The calcium alginate and chitosan beads prepared previously were distributed uniformly into five Erlenmeyer flasks. In the immobilization with foam, 10 cubes of PUF were added into each flask. After incubation, the broth was filtered and the supports were removed, washed with abundant water, and reused for another reaction cycle (15 days) employing identical experimental conditions as described above. In order to test the reusability of immobilized *C. acutatum*, the supports were used repeatedly in six consecutive batches.

2.8 Extraction and identification of products

The culture medium from every cycle was saturated with NaCl, refrigerated, filtered and extracted with CH_2Cl_2 (3×2 L). The organic extract was dried over anhydrous Na_2SO_4 and concentrated in vacuum, and the crude extract was chromatographed by GC-MS. The metabolic compounds 3-phenyl-1-propanol (B), 3-phenyl propyl acetate (C), hydrocinnamic acid (D), 2-phenylethanol (E), 1-phenylethane-1,2-diol (F), 1-phenylpropane-1,3-diol (G), cinnamaldehyde (H), 3-phenylpropanal (I), 3-hydroxy-1-phenyl-1-propanone (J), cinnamyl acetate (K), and cinnamic acid (L) were detected and identified by comparison of mass spectra and GC retention times with those of authentic references standards, and by comparison with the NIST 2002 Mass Spectral Library (Velasco *et al*., 2010; Velasco *et al*., 2012).

3 Results and discussion

The use of immobilized whole cells as a catalyst offers different advantages compared to free whole cells. The immobilization may extend the life of biocatalyst and facilitate its recovery and reuse. Also, the separation of products from immobilized cells is easier than that from suspended cells (Arroyo *et al*., 2017). In a previous work, we found that free whole cells of *C. acutatum* was able to transform cinnamyl alcohol (A) to 3-phenyl-1-propanol (B), 3-phenyl propyl acetate (C), hydrocinnamic acid (D), 2-phenylethanol (E), 1-phenylethane-1,2-diol (F), and 1-phenylpropane-1,3-diol (G) (Velasco *et al*., 2010). In addition, it was found that the culture medium causes differences in the chemical yield; thus, the diol (G) reached maximum levels of almost 90, 20, and 8% when media PDB, Czapek-Dox, and Sabouraud were used, respectively. In view of these results, PDB was chosen as culture medium to be used on the biotransformation of cinnamyl alcohol by immobilized cells of *C. acutatum*.

As immobilization matrixes, two natural polymers (alginate and chitosan), and one synthetic polymer (polyurethane) were employed. Under these experimental conditions, in addition to the products (A) and (G), five other products were observed by GC-MS. Their mass spectra and retention times were similar to those of authentic samples or those reported by the NIST library, for cinnamaldehyde (H), 3-phenylpropanal (I), 3-hydroxy-1-phenyl-1-propanone (J), cinnamyl acetate (K), and cinnamic acid (L). Also, in order to evaluate the reusability of immobilized whole cells in these matrixes, a repeated batch system (six cycles) was studied. Each cycle of batch production was of 15 days duration. It is important to mention that (B), (C), (E), (I) and (K) have been recognized as important aromas. On the other hand, (F) and (G) have been used as building blocks for organic synthesis, and their functionality are found in a number of pharmaceutically important compounds. It is noteworthy to mention that most of previous reports about the synthesis of 1-penylpropane-1,3-diol from the corresponding epoxy alcohol or allylic alcohol, or even allylbenzene required a reaction highly regioselective that use expensive raw materials (Klein *et al*., 1971; Fronza *et al*., 1991). Due to the increasing stringent environmental constraint, nowadays a new synthetic method is necessary.

3.1 Repeated biotransformation of (A) with immobilized whole microorganism in PUF

C. acutatum was found to grow abundantly on PUF cubes after seven days of incubation. Visual observations showed that the surface of PUF was completely covered by fungal mycelia. A thin biofilm of mycelia around the foam with a dark orange-pink pigmentation was observed. According to stereoscope observations, the biomass was detected inside of the foam after preculture, and simultaneously covered practically all the surface of the cube, which imply that biomass growth occurred during the bioprocess. Usually, PUF has been considered an adequate support for the immobilization of fungi due to its porosity, which enhances the available surface area and protects the microorganism against the detrimental effect of shear forces (Asther *et al*., 1990). However, visual observations showed that the mycelia outside of each cube increases progressively along the six batches and the presence of free mycelial mass adhered to flask surface after five days, indicating that some hyphal fragments were broken off. Despite the amount of hyphal fragments was negligible, these cells could enhanced the amount of enzymes available to transform the substrate. Unfortunately, free cells were not quantified.

The GC analysis of the biotransformation of (A) by the immobilized whole microorganism showed the formation of eight metabolites. As can be seen in Figure 1, the substrate was transformed in more than 75% for each cycle. The major metabolites detected in the process were (G) and (B); both were found in each one of the cycles performed. In the case of (G), the higher levels were observed during the first four batches (59, 81, 49 and 70%, respectively), whereas (B) was the major metabolite in the fifth and sixth batch (68 and 38%). In the latter two cycles, (G) achieved only a 20% relative abundance. It was also found that the increase in the relative abundance of (B) coincides with the decline of (G). According to the metabolic pathway for the biotransformation of (A) by *C. acutatum*, the metabolite (G) could be obtained from the hydroxylation of the benzylic carbon in (B) (Velasco *et al*., 2010). The reduction in the level of (G) along with the increase of (B) during the fifth and sixth batch could suggest a reduction in the ability to oxidize the side chain. The loss of activity of immobilized cells in the repeated batch production of (G) was probably due to the excessive aggregation of mycelia in multiple layers around the cubes.

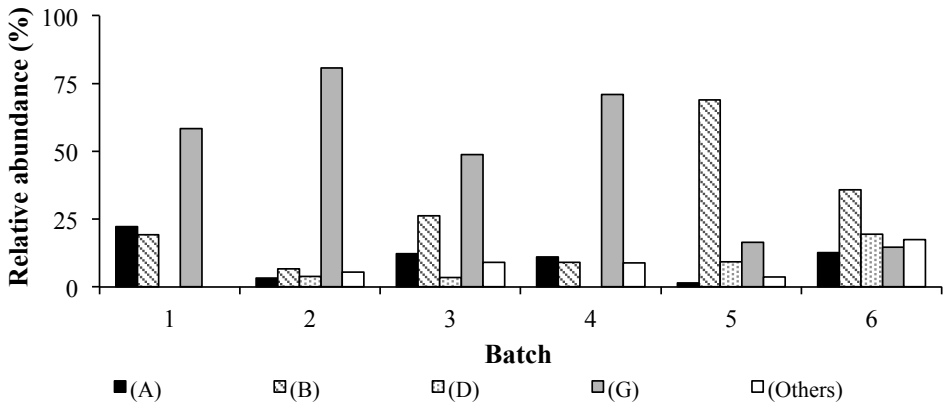

Fig. 1. Time-course experiments for the biotransformation of (A) by whole cells of *C. acutatum* supported in PUF. Cinnamyl alcohol (A); 3-phenyl-1-propanol (B); hydrocinnamic acid (D); 1-phenylpropane-1,3-diol (G); others (3-phenyl propyl acetate (C), 1-phenylethane-1,2-diol (F), cinnamaldehyde (H), 3-hydroxy-1-phenyl-1-propanone (J), cinnamyl acetate (K)).

This affects the mass transfer and generates restrictions to the interaction between mycelium and culture medium, limiting it to the periphery of the aggregate mycelia, and reducing the contact surface. In addition, the successive agglomeration of layers retards the transport of mass into the support, avoiding the diffusion of oxygen and other nutrients, which causes loss of activity (Prasad *et al*., 2006).

It is noteworthy that some minor metabolites absent in the biotransformation using free whole cells (Velasco *et al*., 2010), such as (H) and (J), were detected with immobilized *C. acutatum* under similar conditions (room temperature, 120 rpm). In latter, the accumulation of aldehyde (H) in the culture medium is probably due to the mass transfer limitations resulting from the immobilization on PUF cubes. Low accumulation of aldehydes in the biotransformation process has been associated with the high cellular toxicity of these compounds (Sanromán *et al*., 1995). However, the immobilization and mass transfer limitations could protect the microorganism from the direct interaction with (H), allowing its accumulation in the culture medium.

3.2 Repeated biotransformation of (A) with immobilized spores in PUF

The spore suspension of the fungal strain *C. acutatum* was successfully absorbed on the PUF. The effect of different amounts of spores on the biotransformation of (A) was studied (*data not shown*). It was found that 1×10^5 CFU/mL is sufficient to produce the initial biomass to carry out the process with the fungus immobilized. Also, although no more cells were attached in the PUF after 5 days (almost constant weight), we decided to consider 7 days of fungal growth to perform the inoculums (Velasco *et al*., 2010). Figure 2 shows that the use of spores in PUF proved to be an appropriate technique to obtain (G), at least during the first three batches (75, 49 and 71%, respectively). Then, its abundance was rapidly diminished so that after the third batch (G) was not detected anymore. Under these conditions, the relative abundance of (G) reached levels lower than immobilized whole cells in PUF (81% during the second bath) and free cells (about 90%) (Velasco *et al*., 2010). Again, some minor metabolites, (*e.g*. cinnamyl acetate (K)) were detected, which was not the case when using free cells (Velasco *et al*., 2010). Metabolite (K) appeared in all the cycles under the conditions used in the present experiment. It had been only found when the substrate of the fungus was (L) (Velasco *et al*., 2012).

It is remarkable that although the chemical profiles during the six batches were different, the fungus never lost its ability to modify (A). The strong variation between the third and fourth batches could indicate a reaccommodation of the fungus to new reaction conditions, where diffusion into the support was not as accessible as it was at the beginning (Mahmoud and Rehm, 1987; Carballeira *et al*., 2009), and quite possibly the amount of biomass was not suitable for the bioprocess (Carballeira *et al*., 2009; Sanromán *et al*., 1995; Mahmoud and Rehm, 1987). Similar to the previous case (whole microorganism and PUF), the amount of cells attached to the foam increased considerably during the experiments, which enlarged noticeably the size of the cube.

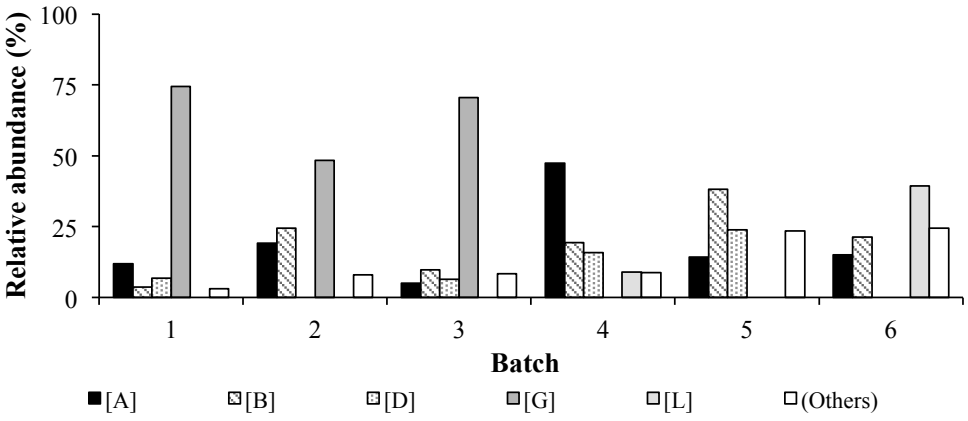

Fig. 2. Time-course experiments for the biotransformation of (A) by spores of *C. acutatum* supported in PUF. Cinnamyl alcohol (A); 3-phenyl-1-propanol (B); hydrocinnamic acid (D); 1-phenylpropane-1,3-diol (G); cinnamic acid (L); others (3-phenyl propyl acetate (C), 1-phenylethane-1,2-diol (F), cinnamaldehyde (H), 3-hydroxy-1-phenyl-1-propanone (J), cinnamyl acetate (K)).

According to the stereoscope observations (*data not shown*), the fungus showed affinity for the PUF at all assessments (spores and whole microorganism). Variations in the chemical profiles of immobilized whole cells and spores in PUF, and free cells may also result from differences in morphology, which seriously modifies the productivity, enzyme availability, or both (Sanromán *et al*., 1995; Orozco-Santos *et al*., 2004). Unlike processes of immobilization, the free fungus grows as dispersed cells and filaments (hyphae) in a dense mass of interwoven mycelium (Sanromán *et al*., 1995). Therefore, in the biomass aggregation, cell-to-cell interactions and the signaling are qualitatively dissimilar. Taking in account the metabolites found in the immobilization processes (H, J, K, L), a metabolic pathway for the biotransformation of (A) by *C. acutatum* was proposed (Figure 3).

According to Figure 3, (A) was converted to metabolite (K) through acetylation. The metabolite (K) had been obtained through the biotransformation of (L) by *C. acutatum* (Velasco *et al*., 2012). It had been found that free cells of this fungus were able to reduce (L) to (H), then to the alcohol (A) and subsequently to the acetyl ester (K) (Velasco *et al*., 2012). The reduction reactions have been suggested to occur sequentially involving at least three separate enzymes; an aryl-aldehyde oxidoreductase carboxylic acid reductase that converts (L) into (H), an alcohol dehydrogenase aldehyde reductase ADH that converts (H) into the alcohol (A), and an acyl transferase that catalyzes the formation of acetyl ester (K) from the alcohol product (Chen and Rosazza, 1994). However, (K) has not been obtained using free cells of the *C. acutatum* as biocatalyst.

3.3 Biotransformation of (A) with immobilized spores within calcium alginate and chitosan beads

The immobilization with alginate and chitosan beads has been preferred over other materials due to several advantages, like biodegradability, hydrophilicity, natural origin, low density, mechanical resistance and stability over an experimental pH range (Arica *et al*., 2004). The present study showed that formed beads with calcium alginate and chitosan were in spherical shape, being those from alginate the more regular (Figure 4). The beads diameter obtained was almost 5 mm. Visual observations scarcely showed free cells; it means that under these conditions almost no cells were detached due to the complete entrapment of fungal spores inside the beads. In contrast to PUF experiments, in this case the amount of cells apparently was kept constant along the assays, since no evidence of growth was detected inside of the beads. Thereby, the lifetime and quality of the cells to transform the substrate could play an important role in this bioprocess (the stationary and death phases of the fungus probably altered the enzymatic pool available). In this sense, results showed that the immobilization of spores within calcium alginate and chitosan beads were not satisfactory to obtain (G).

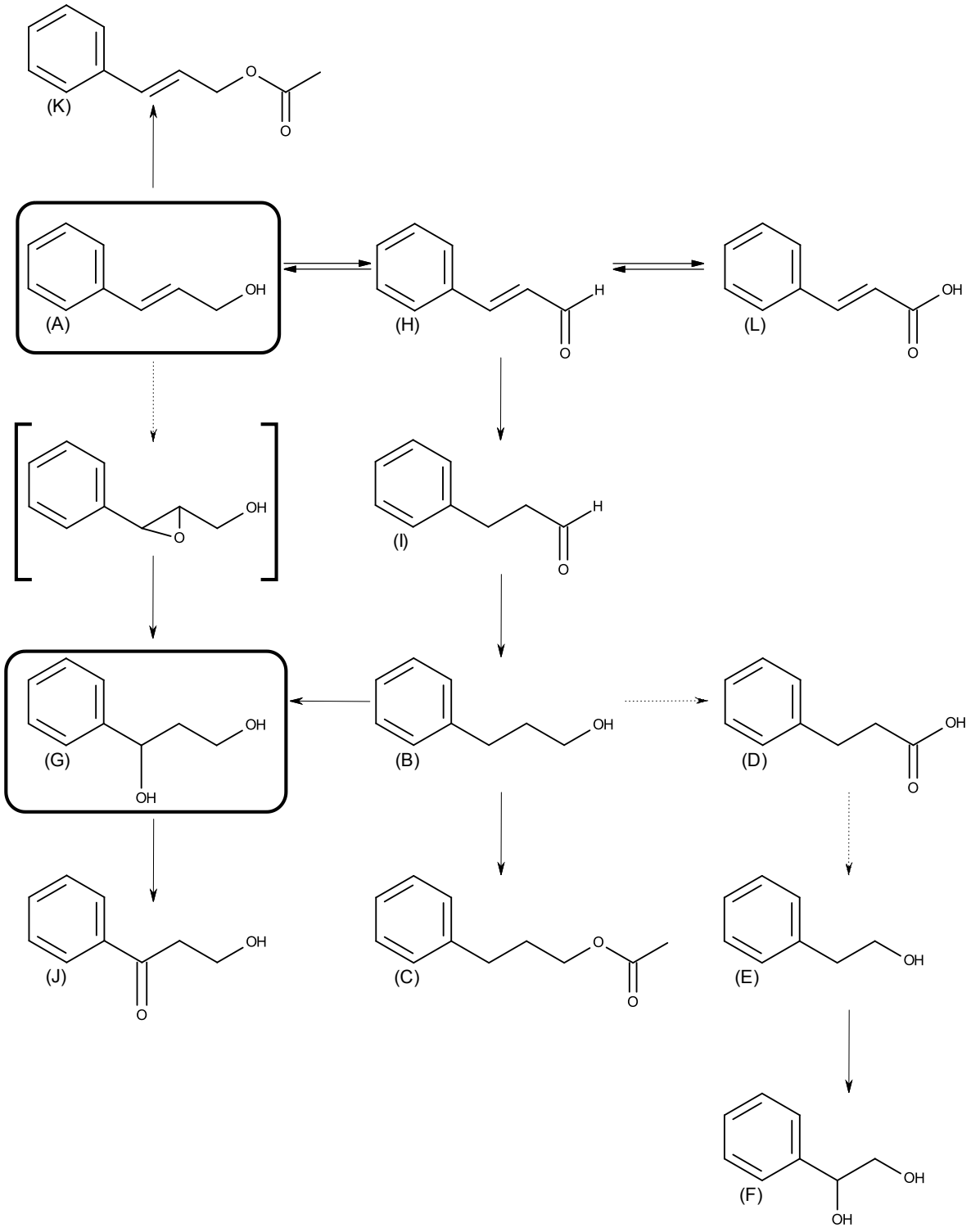

Fig. 3. Possible metabolic pathway of (A) by *C. acutatum*.

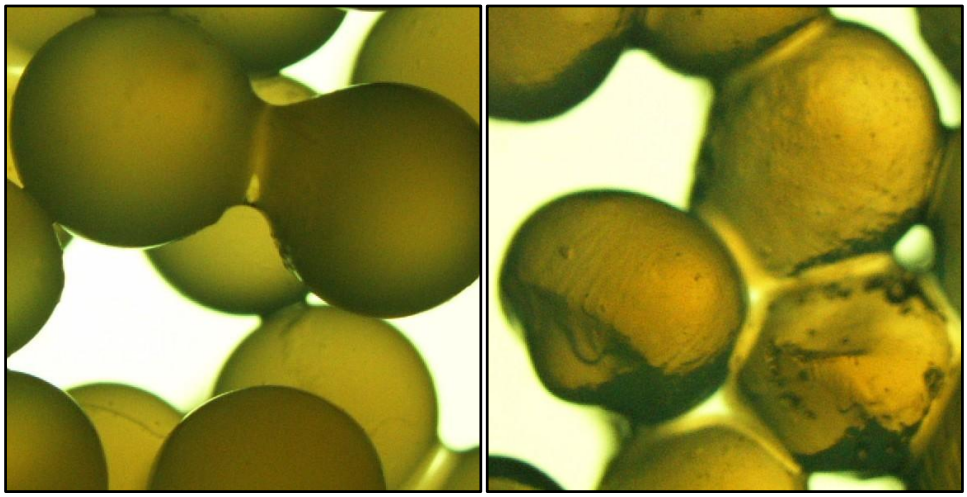

Fig. 4. Calcium alginate (left) and chitosan (right) beads

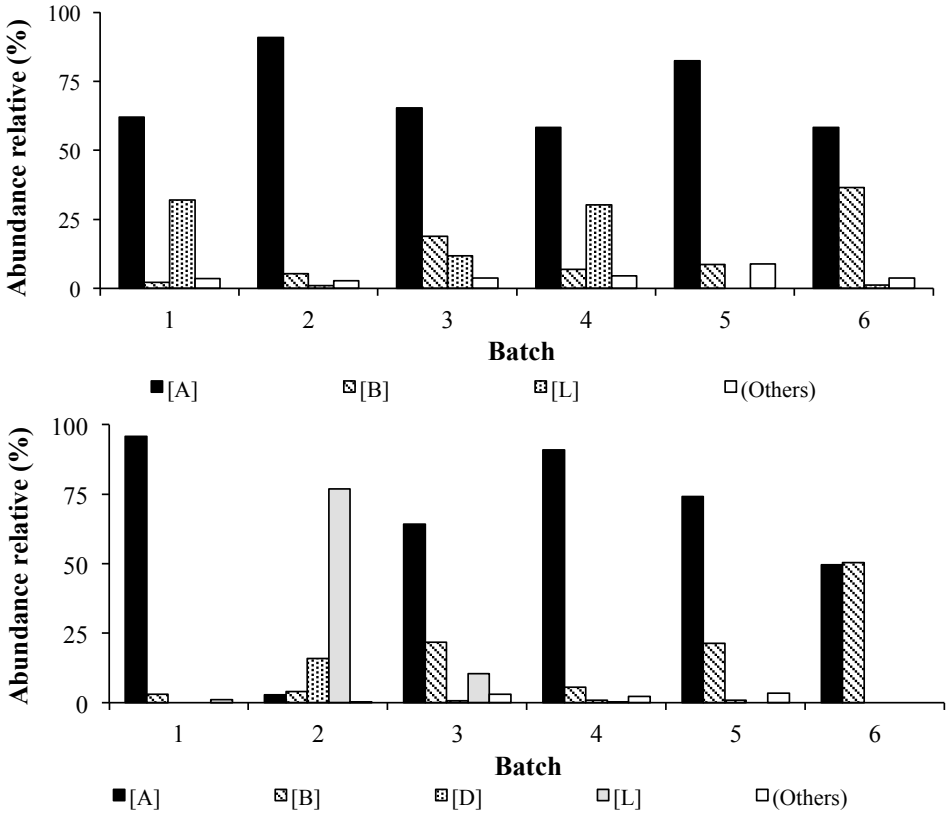

Fig. 5. Time course for the biotransformation of (A) by *C. acutatum* supported within calcium alginate (top) and chitosan beads (bottom). Cinnamyl alcohol (A); 3-phenyl-1-propanol (B); hydrocinnamic acid (D); cinnamic acid (L); others (1-phenylpropane-1,3-diol (G), cinnamaldehyde (H), 3-phenylpropanal (I), 3-hydroxy-1-phenyl-1-propanone (J), 2-phenylethanol (E), benzoic acid).

As shown in Figure 5 (top), (A) was weakly metabolized by *C. acutatum* using alginate as matrix during 15 days and in the course of six batches. However, under these conditions the main products were (B) and (L), with maximum relative abundance of 36.6% and 32.1%, respectively. These results were unexpected in comparison with those obtained with free cells (Velasco *et al*., 2010), where the conversion of (A) into (L) was not detected. The transformation of (A) to (L) and (L) to (A) (Velasco *et al*., 2012) with the same fungus supports the reversibility of the interconversion (A) $\rightleftharpoons$ (H) $\rightleftharpoons$ (L).

According to Figure 5 (bottom), (A) was poorly metabolized using *C. acutatum* entrapped within chitosan beads, especially in the first and fourth cycle. Under these conditions, the conversion of (A) was lesser than using the free cells system (Velasco *et al*., 2010) but it was slightly higher than using *C. acutatum* immobilized in alginate beads. It is strange that during the second cycle, (A) was almost completely transformed, being (L) the major metabolic product. This behavior was possibly due to the conditioning of the fungus into the support, the aging of the cells and the new environment. However, there further studies are necessary with the aim to clarify this unexpected performance in the second batch. Then, it was seen a major transformation of (A) into (B), and its concentration increases with the progress of the process until reaching a relative abundance of 50.4%. Visual observation of *C. acutatum* entrapped in chitosan and alginate did not show the presence of free cell mass, indicating less leakage of cells. Thus, the viscosity of the culture broth is reduced considerably, helping the oxygen transference and causing less operational problems.

Overall, the amount of (B) using free whole cells of *C. acutatum* (Velasco *et al*., 2010) was higher than that of the immobilized cells. In addition, it was noticed that maximum conversion of (A) to (B) took place with the attachment of the cells in the PUF during the fifth cycle, 69%. Previous studies have estimated that the effective diffusion coefficient in foams is at least 10^4-fold those expected in gel beads, and in turn are similar among alginate and chitosan (Prociak *et al*., 1999; Falk *et al*., 2004; Holte *et al*., 2005;). The low activity of entrapment cells compared to the attachment on PUF might be due to diffusional restriction imposed by the gels: the lower the porosity, the more hindered the mass transfers, and the amount and quality of cells available to carry out the biotransformation of the substrate. While the spores entrapped in gels probably do not increase the amount of cells in the assay and they aged after each batch, the fungus on PUF added new cells to the experiment. Additionally, in entrapment methods the substrate has to diffuse through not one, but two semi-permeable barriers (gel matrix and cell membrane) which can affect the performance of biotransformation (Peart *et al*., 2012).

Finally, although during PUF assays were employed spores and whole cells of *C. acutatum*, the assays performed in bead supports did not employed whole microorganism as starting biomass due to the difficult to guaranty the homogeneous distribution of the cells in all the beads. Also, while cell growth was detected during PUF evaluations, no evidence of spore germination was observed within the beads. This fact should be considered in further studies, since a previous report suggests the biotechnological potential use of spores as promising biocatalyst (Murata, 1993). Thereby, it is important to consider the cell features in order to express specific genes that codify for those enzymes involved in each bioconversion. Likewise, further studies are necessary in order to understand the stereochemistry of the process to obtain this compound.

Conclusions

The biotransformation of cinnamyl alcohol through the immobilization of *Colletotrichum acutatum* on three different solid supports (attachment on polyurethane foam and entrapment in calcium alginate and chitosan beads) showed marked variances in the chemical profile. Polyurethane foam proved to be a good support in terms of the amount of cinnamyl alcohol transformed and the relative abundance of 1-phenylpropane-1,3-diol produced. This diol was the major metabolic product during the first 3 (spores) or 4 (whole cells) batches. However, visual observation showed the presence of free cells, indicating cell leakage. The decreased yield in the production of the diol after the third or fourth cycle can be due to the excessive aggregation of mycelia in multiple layers around the cubes, which affects the mass transfer. On the other hand, although the method of entrapment in calcium alginate and chitosan showed less amount of cell free leakage, both supports were not adequate to obtain 1-phenylpropane-1,3-diol in the course of this biotransformation. The above can be attributed to the diffusivity of substances is dramatically reduced compared to what can be observed in free cells or in polyurethane foam. Likewise, the amount and quality

of the cells available to carry out the biotransformation after each batch certainly hinder the comparison between these supports. Interestingly, some minor metabolites, which have not been found using free cells, were detected using *C. acutatum* attached to polyurethane cubes.

Acknowledgement

Special thanks to Dirección de Investigación Sede Medellín (DIME) and Universidad Nacional de Colombia for their financial support.

References

Afanador-Kafuri, L., Minz, D., Maymon, M., Freeman, S. (2003). Characterization of *Colletotrichum* isolates from tamarillo, passiflora, and mango in Colombia and identification of a unique species from the genus. *Phytopathology 93*, 579-587.

Arica, M.Y., Kacar, Y., Genc, O. (2001). Entrapment of white-rot fungus *Trametes versicolor* in Ca-alginate beads: preparation and biosorption kinetic analysis for cadmium removal from an aqueous solution. *Bioresource Technology 80*, 121-129.

Arroyo, M., de la Mata, I., Garcia, J.L., Barredo, J.L. (2017). Biocatalysis for industrial production of active pharmaceutical ingredients. In: *Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications*, (Brahmachari G., ed.), Pp. 451-474. Academic Press, New York.

Asther, M., Bellon-Fontaine, M.N., Capdevila, C., Corrieu, G. (1990). A thermodynamic model to predict *Phanerochaete chrysosporium* INA-12 adhesion to various solid carriers in relation to lignin peroxidase production. *Biotechnology and Bioengineering 35*, 477-482.

Barreto, R.V.G., Hissa, D.C., Paes, F.A., Grangeiro, T.B., Nascimento, R.F., Rebelo, L.M., Craveiro, A.A., Melo, V.M.M. (2010). New approach for petroleum hydrocarbon degradation using bacterial spores entrapped in chitosan beads. *Bioresource Technology 101*, 2121-2125.

Behan, J.M., Clements, C.F., Hooper, D.C., Martin, J.R., Melville, J.B., Perring, K.D. (1996). Perfume compositions. U.S. Patent 5,554,588, Sept. 10.

Carballeira, J.D., Quezada, M.A., Hoyos, P., Simeó, Y., Hernaiz, M.J., Alcantara, A.R. and Sinisterra, J.V. (2009). Microbial cells as catalysts for stereoselective red-ox reactions. *Biotechnology Advances 27*, 686-714.

Chen, Y. and Rosazza, J. (1994). Microbial transformation of ibuprofen by a *Nocardia* species. *Applied and Environmental Microbiology 60*, 1292-1296.

De Castro and Rhee. (2008). Selective nosylation of 1-phenyl-1,3-diol and perchloric acid mediated Friedel-Crafts alkylation: key steps for the new and straightforward synthesis of tolterodine. *Synthesis 12*, 1841-1844.

Falk, B., Garramone, S. and Shivkumar, S. (2004). Diffusion coefficient of paracetamol in a chitosan hydrogel. *Materials Letters 58*, 3261-3265.

Fronza, G., Fuganti, C., Grasselli, P., Mele, A. (1991). On the mode of bakers' yeast transformation of 3-chloropropiophenone and related ketones. Synthesis of (2S)-[2-^{2}H]propiophenone, (*R*)-fluoxetine, and (*R*)- and (*S*)-fenfluramine. *The Journal of Organic Chemistry 56*, 6019-6023.

Holte, Ø, Tønnesen, H.H. and Karlsen, J. (2006). Measurement of diffusion through calcium alginate gel matrices. *Pharmazie 61*, 30-34.

Klein, J. and Medlik A. (1971). Metalation reactions. XI. Preparation of 1,3-diols by hydroboration of allyllithium derivatives. *Journal of the American Chemical Society 93*, 6313-6314.

Kumar, P., Upadhyay, R.K. and Pandey, R.K. (2004). Asymmetric dihydroxylation route to (*R*)-isoprenaline, (*R*)-norfluoxetine and (*R*)-fluoxetine. *Tetrahedron Asymmetry 15*, 3955-3959.

Mahmoud, W. and Rehm, H.J. (1987). Chlortetracycline production with immobilized *Streptomyces aureofaciens. Applied Microbiology and Biotechnology 26*, 333-337.

Mulla, S.I., Talwar, M.P., Hoskeri, R.S. and Ninnekar, H.Z. (2012). Enhanced degradation of 3-nitrobenzoate by immobilized cells of

Bacillus flexus strain XJU-4. *Biotechnology and Bioprocess Engineering 17*, 1294-1299.

Murata, K. (1993). Use of microbial spores as a biocatalyst. *Critical Reviews in Biotechnology 13*, 173-193.

Orozco-Santos, M., Manzo-Sánchez, G., Guzmán-González, S., Farías-Larios, J. and Timmer, L.W. (2004). Crecimiento y cambios morfológicos de *Colletotrichum acutatum* Simmonds, agente causal de la antracnosis del limón mexicano (*Citrus aurantifolia* Christm, Swingle) incubado en diferentes medios de cultivo sólidos y líquidos. *Revista Mexicana de Fitopatología 22*, 423-428.

Peart, P.C., Chen, A.R.M., Reynolds, W.F. and Reese, P.B. (2012). Entrapment of mycelial fragments in calcium alginate: A general technique for the use of immobilized filamentous fungi in biocatalysis. *Steroids 77*, 85-90.

Prasad, K.K., Mohan, S.V., Pati B.R. and Sarma, P.N. (2006). Immobilization of *Pleurotus ostreatus* 1804 on PUF cubes: Influence of mycelial growth pattern on laccase yield. *Indian Journal of Biotechnology 5*, 84-88.

Prociak, A., Sterzyñski, T. and Pielichowski, J. (1999). Thermal diffusivity of polyurethane foams measured by the modified Ångström method. *Polymer Engineering and Science 39*, 1689-1695.

Romano, A., Romano, D., Ragg, E., Constantino, F., Lenna, R., Gandolfi, R. and Molinari, F. (2006). Steroid hydroxylations with *Botryodiplodia malorum* and *Colletotrichum lini. Steroids 71*, 429-434.

Sanromán, A., Feijoo, G. and Lema, J.M. (1995). Immobilization of *Aspergillus niger* and *Phanerochaete chrysosporium* on polyurethane foam. In: *Immobilized cells: Basics and Applications Progress in Biotechnology*. Vol. 11, (Wijffels, R. H.; Buitelaar, R. M.; Bucke, C.; Tramper, J., Eds.); Pp. 132-135. Elsevier: The Netherlands

Savina, J.P., Kohler, D. and Brunerie, P. (1999). Method for extracting 2-phenylethanol. U.S. Patent 5,965,780, Oct. 12.

Sendovski, M., Nir, N. and Fishman, A. (2010). Bioproduction of 2-phenylethanol in a biphasic ionic liquid aqueous system. *Journal of Agricultural and Food Chemistry 58*, 2260-2265.

Siddiqui, M.F., Andleeb, S., Ali, N., Ghumro, P.B. and Ahmed, S. (2009). Up-flow immobilized fungal column reactor for the treatment of anthraquinone dye Drimarene blue K2RL. *African Journal of Biotechnology 8*, 5570-5577.

Sun, J., Lin, Y., Shen, X., Jain, R., Sun, X., Yuan, Q. and Yan, Y. (2016). Aerobic biosynthesis of hydrocinnamic acids in *Escherichia coli* with a strictly oxygen-sensitive enoate reductase. *Metabolic Engineering 35*, 75-82.

Tian, X., Ye, R., Wang, J., Chen, Y., Cai, B., Guan, S., Rong, S. and Li, Q. (2015). Effects of aroma quality on the biotransformation of natural 2-phenylethanol produced using ascorbic acid. *Electronic Journal of Biotechnology 18*, 286-290.

Velasco, R., Gil, J.H., García, C.M. and Durango, D.L. (2010). Production of 2-phenylethanol in the biotransformation of cinnamyl alcohol by the plant pathogenic fungus *Colletotrichum acutatum. Vitae 17*, 272-280.

Velasco, R., Gil, J.H., García, C.M. and Durango, D.L. (2012). Structural modification of *trans*-cinnamic acid using *Colletotrichum acutatum. Revista de la Facultad de Ingeniería Universidad de Antioquia 63*, 20-29.

Velasco, R., Mesa, R., Gil, J., Garcia, C. and Durango, D. (2015). Transformation of *trans*-anethole using the plant pathogenic fungus *Colletotrichum acutatum* as biocatalyst. *Revista Mexicana de Ingeniería Química 14*, 653-666.

Wang, J., Lu, H., Zhou, Y., Song, Y., Liu, G., Feng, Y. (2013). Enhanced biotransformation of nitrobenzene by the synergies of *Shewanella* species and mediator-functionalized polyurethane foam. *Journal of Hazardous Materials 252-253*, 227-232.