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BIODEGRADACIÓN DE [BMIN] [PF₆] UTILIZANDO
Fusarium sp.

**QUE PARA OBTENER EL GRADO DE
ESPECIALIDAD EN BIOTECNOLOGIA**

Presenta:

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Dedico esta tesis con todo mi amor y cariño a:

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Miguel Esquivel Cuevas y Hortensia Viveros Paulés.

Mi hermana

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BIODEGRADACIÓN DE [bmim][PF₆] UTILIZANDO *Fusarium* sp

RESUMEN

El uso creciente de líquidos iónicos en la industria conduce a estudios de biodegradabilidad y toxicidad para prevenir la contaminación del medio ambiente por estos compuestos sintéticos. Se aisló una cepa de *Fusarium* y se probó su habilidad para tolerar y crecer en presencia de [bmim] [PF₆], un contaminante potencial de aguas residuales. La cepa de *Fusarium* fue capaz de crecer tanto en cultivo superficial y sumergido utilizando [bmim] [PF₆] como única fuente de carbono hasta 19 y 21 g [bmim] [PF₆] L⁻¹, respectivamente. Se utilizó un reactor de biopelícula de membrana aireada para los estudios de biodegradación de aguas residuales sintéticas. Se observó una biodegradación del 80% después de 28 días de incubación a 30 °C. Ésta es la primera vez que se estudia la biodegradación de [bmim][PF₆] con hongos filamentosos.

Palabras clave: Líquidos iónicos, Biodegradación de [bmim][PF₆], *Fusarium*, reactor de biopelícula de membrana aireada.



BIODEGRADATION OF [bmim][PF₆] USING *Fusarium* sp

BIODEGRADACIÓN DE [bmim][PF₆] UTILIZANDO *Fusarium* sp

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Abstract

The increased use of ionic liquids in industry has led to the study of their biodegradability and toxicity to prevent contamination of the environment by these synthetic compounds. A *Fusarium* strain was isolated and tested for its ability to tolerate and grow in the presence of [bmim] [PF₆], a potential contaminant of wastewaters. The *Fusarium* strain was able to grow in both surface and submerged liquid media using [bmim] [PF₆] as the sole carbon source up to 19 and 21 g [bmim] [PF₆] L⁻¹, respectively. A membrane-aerated biofilm reactor was used for biodegradation studies of synthetic wastewaters. Up to 80% biodegradation was observed after 28 days of incubation at 30 °C. This is the first time that [bmim][PF₆] has been subjected to fungal biodegradation.

Keywords: ionic liquids, [bmim][PF₆] biodegradation, *Fusarium*, membrane-aerated biofilm reactor.

Resumen

El uso creciente de líquidos iónicos en la industria conduce a estudios de bio-degradabilidad y toxicidad para prevenir la contaminación del medio ambiente por estos compuestos sintéticos. Se aisló una cepa de *Fusarium* y se probó su habilidad para tolerar y crecer en presencia de [bmim] [PF₆], un contaminante potencial de aguas residuales. La cepa de *Fusarium* fue capaz de crecer tanto en cultivo superficial y sumergido utilizando [bmim] [PF₆] como única fuente de carbono hasta 19 y 21 g [bmim] [PF₆] L⁻¹, respectivamente. Se utilizó un reactor de biopelícula de membrana aireada para los estudios de biodegradación de aguas residuales sintéticas. Se observó una biodegradación del 80% después de 28 días de incubación a 30 °C. Ésta es la primera vez que se estudia la biodegradación de [bmim][PF₆] con hongos filamentosos.

Palabras clave: líquidos iónicos, biodegradación de [bmim][PF₆], *Fusarium*, reactor de biopelícula de membrana aireada.

1. Introduction

Ionic liquids have been called “green solvents”, as they may be used as replacements for organic solvents depending on vapor pressure, flammability, and toxicity. Typical ionic liquids consist of halogen-containing anions (such as [AlCl₄]⁻, [PF₆]⁻, [BF₄]⁻, [CF₃SO₃]⁻ or [(CF₃SO₂)₂N]⁻). The presence of halogen atoms may cause serious concerns about the liberation of toxic and corrosive HF (e.g. [AlCl₄]⁻) or HCl (e.g. [PF₆]⁻) into the environment if the hydrolytic stability of the anion is poor or if thermal treatment of spent ionic liquids is desired (Wasserscheid *et al.*, 2002).

Recently, Ranke *et al.* (2007) pointed out that such “green” arguments are not sufficient for the design of sustainable chemical products and referred to the concept of ecotoxicological risk profiles, which comparatively assesses substances according to the following five risk indicators: release, spatiotemporal range, bioaccumulation, biological activity, and uncertainty. Using ecotoxicological tests, Wells and Coombe (2006) revealed that several ionic liquids have very high toxicity towards freshwater algae and the freshwater invertebrate *Daphnia magna*. They concluded that considerable care should be exercised in the choice of ionic liquids to be used in chemical processes at the

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design stage to avoid contamination of aqueous effluent streams.

Imidazolium ionic liquids have been widely studied and the most commonly used are dialkylimidazolium ionic liquids, [bmim] [BF₄] and [bmim] [PF₆]; however, only a small amount of toxicological and/or ecotoxicological data is available for ionic liquids (Jastorff *et al.*, 2003). Recent toxicity studies of several imidazolium (Romero *et al.*, 2008) and a new group of redesigned (Stasiewicz *et al.*, 2008) ionic liquids pointed out the toxic effect of the chain length, alkyl and alkoxyethyl, respectively.

In order to synthesize biodegradable ionic liquids, some studies have focused on the biodegradability of the commonly used dialkylimidazolium ionic liquids and the effect of the imidazolium cation (Gathergood *et al.*, 2004) and anion (Garcia *et al.*, 2005) on biodegradability. Solutions containing 2 mg L⁻¹ [bmim] [PF₆] and inoculated with microorganisms isolated from wastewaters showed no biodegradation (0%) after 28 days incubation at 20 °C, using a method (Sturm test, OECD 301B) approved by The Organization for Economic Cooperation and Development. In this test, the chemical being evaluated is added to aerobic aqueous medium inoculated with wastewater microorganisms and the evolution of CO₂ is measured for a defined period and reported as a percentage of the theoretical maximum. Stepnowski and Zaleska (2005) reported the use of three common advanced oxidation processes (UV, UV/H₂O₂ and UV/TiO₂) in the degradation of imidazolium ionic liquids in aqueous solution. The authors claimed that the greatest degradation efficiency for all the compounds studied was achieved with the H₂O₂/UV system.

The use of biofilm reactors has been described by Qureshi *et al.* (2005) not only for the production of various chemicals by fermentation, but also for wastewater treatment. Cell densities in the reactor can be increased as a result of biofilm formation. In nature, this is a natural process where microbial cells attach to the support. The authors compared various reactor types for production of ethanol, butanol, lactic acid, acetic acid/vinegar, succinic acid, and fumaric acid in addition to wastewater treatment in the biofilm reactors. More recently, Judd (2008) reviewed the current status of membrane bioreactor technology for wastewater treatment. It was found (González-Brambila and López-Isunza, 2007) that supplying oxygen from inside the membrane and simultaneous sparing of air to the residual water reduced oxygen transfer limitations across the biofilm. Membrane-aerated biofilm reactors (MABR) have also been used in the treatment of wastewaters containing perchloroethylene (Ohandja and Stuckey, 2006) and controllable nitrification (Terada *et al.*, 2006).

The aim of this work was to test the ability of an isolated *Fusarium* strain to grow in the presence



Fig. 1. Photograph of the filamentous *Fusarium* sp. incubated in agar media supplied with [bmim] [PF₆].

of [bmim] [PF₆] as the sole carbon source in surface and submerged culture. The biodegradation of [bmim] [PF₆] in synthetic wastewater using a reactor MABR was also studied.

2. Materials and methods

2.1. Chemicals

The ionic liquid used for biodegradation studies was 1-butyl-3-methyl-imidazolium hexafluorophosphate [bmim] [PF₆] at 98% purity, purchased from Solvent Innovation GmbH (Köln, Germany).

2.2. Culture media

SNA (Spezielle Nahstoffarmer Agar) medium for classification and identification of the Nectria group (*Fusarium*, *Cylindrocladium*, *Cylindrocarpon*, *Acremonium*) was used to isolate and propagate the *Fusarium* sp. strain. To test *Fusarium* strain tolerance to the ionic liquid and for biodegradation experiments, the carbon source in the SNA medium was changed to [bmim] [PF₆] (see Table 1). In all culture media used pH was adjusted to 7 using HCl or NaOH 0.1 M solutions.

Table 1. Culture media used to isolate, maintain and propagate *Fusarium* sp.

Compound	SNA (g L ⁻¹)	Modified SNA (g L ⁻¹)
Sucrose	0.2	-
Glucose	0.2	-
[bmim][PF ₆]	-	1.0
KH ₂ PO ₄	1	1
KNO ₃	1	1
MgSO ₄ 7H ₂ O	0.5	0.5
KCl	0.5	0.5
Agar	20	20

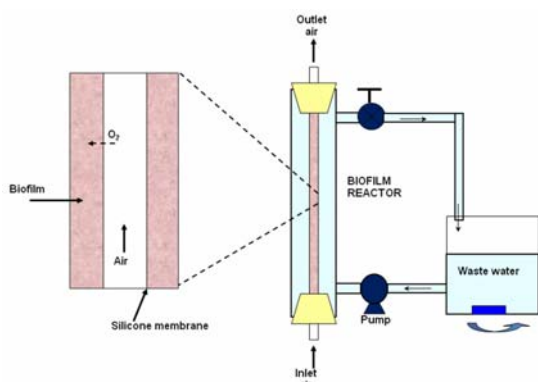


Fig. 2. Membrane-aerated biofilm reactor for biodegradation studies of synthetic ionic liquid-contaminated wastewater.

2.3. Microorganism

The microorganism used for ionic liquid biodegradation studies was the strain *Fusarium* sp. The strain was isolated from contaminated ionic liquid samples at Pilot Plant 4 of the Biotechnology Department of Universidad Autónoma Metropolitana, Iztapalapa Campus. The strain was propagated and conserved in SNA agar medium in Petri dishes at 30 °C. A photograph of the strain is shown in Fig. 1, showing its filamentous morphology and pink pigmentation.

2.4. Biofilm reactor

The MABR used was previously described by Brambila-González and López-Isunza (2007). The Pyrex tube-bioreactor had the following dimensions: 200 ml volume, 300 mm height, 30 mm outside diameter, 2 mm thickness. A silicon rubber membrane (290 mm long, 1.5 mm inner radius, 0.3 mm thick) was used. The silicone rubber membrane tube was immersed in the glass bioreactor. One membrane side was connected to the air and oxygen supply valves and the other side was partially blocked to force the gases to flow through the wall membrane (Fig. 2).

2.5 Analysis

2.5.1. Determination of [bmim] [PF₆] by spectroscopy

Ionic liquid concentration, [bmim] [PF₆], was determined by spectroscopy in the UV region at 210 nm using a Spectrophotometer (Perkin-Elmer). Aniruddha et al. (2005) reported on the optical behaviour of a typical imidazolium ionic liquid and showed that [bmim] [PF₆] has significant absorption in the UV region, and also shows very interesting fluorescence behavior. The peak fluorescence of [bmim] [PF₆] is strongly dependent on the excitation wavelength. The unusual fluorescence response of

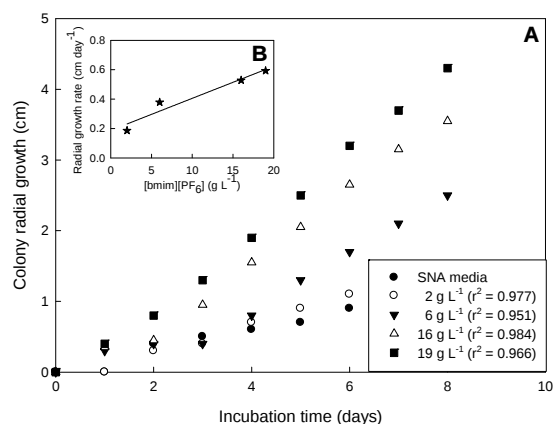


Fig. 3. (A) Colony radial growth of *Fusarium* sp. growing in SNA and SNA modified medium using [bmim] [PF₆] as the sole carbon source at different concentrations. (B) Effect of [bmim] [PF₆] concentration on the radial growth rate ($r^2 = 0.941$).

[bmim] [PF₆] is attributed to the presence of several energetically different associated forms of the imidazolium ions in the ionic liquid. Distilled water, which absorbed at 200 nm, was used as a blank.

2.5.2. Other determinations

pH was measured using a pH meter (Cole-Parmer). Conductivity of wastewater during biodegradation was measured on-line using a T-cell and a Conduct meter (Meter OVFL CL3).

3. Results and discussion

3.1 Petri dish experiments

Fusarium was isolated on Petri dishes with SNA agar medium at 30 °C. Experiments to test growth and tolerance were carried out at 30 °C. The strain was inoculated and incubated on Petri dishes with modified SNA medium (Table 1), in which the carbon source (sucrose and glucose) was substituted by [bmim] [PF₆]. Different concentrations from 2, 6, 16 and 19 g [bmim] [PF₆] L⁻¹ were tested and in all cases colony radius was measured. Radial growth versus incubation time is plotted in Fig. 3A. Radial growth increased when [bmim] [PF₆] concentration increased, probably due to an increment in the carbon source. *Fusarium* grew on media containing high concentrations of [bmim] [PF₆] up to 19 g L⁻¹ without apparent inhibition. Colony radial growth from 2 to 8 days of incubation was adjusted by linear regression and the slopes obtained (radial growth rate) were plotted against [bmim][PF₆] concentration (Fig. 3B). A linear increment of the radial growth rate was observed with respect to [bmim] [PF₆] concentration. Fungal growth in surface and solid state fermentation has been reported to occur at high substrate and contaminant concentrations, probably

Table 2. Biomass production and estimated yields of *Fusarium* sp treated with different carbon sources in submerged cultures at 30 °C.

Carbon source	g L ⁻¹	Biomass mg L ⁻¹	Yield (biomass/[bmim] [PF ₆]) mg biomass (mg C-mol) ⁻¹	Yield (biomass/butyl) mg biomass (mg C-mol) ⁻¹
Glucose+sucrose	0.4	102.9	7.520	7.520
[bmim] [PF ₆]	9.8	121.9	0.442	3.533
[bmim] [PF ₆]	19.6	150.35	0.272	2.179

due to slow mass transfer rates (Volke *et al.*, 2006).

3.2. Erlenmeyer experiments

The solubility of [bmim] [PF₆] is relatively high, up to 21 g L⁻¹, and when it is used in biphasic systems, [bmim] [PF₆] must be recovered from wastewaters from bioconversion processes. This can be done by cooling and decanting, or by using membrane separation (Fernández *et al.*, 2008). However, wastewaters may contain considerable amounts of this ionic liquid. Submerged culture experiments carried out using modified SNA medium in Erlenmeyer flasks at 0, 9.8, and 19.6 g [bmim] [PF₆] L⁻¹ produced 102.9, 121.9, and 150.35 g biomass L⁻¹, respectively. It was confirmed that *Fusarium* is able to grow at the highest concentration of [bmim] [PF₆]. However, biomass concentration did not increase as expected. Biomass yield, based on C-mol of [bmim] [PF₆], decreased up to 17 times from SNA medium to modified SNA medium containing 9.8 g [bmim] [PF₆] L⁻¹ (Table 2). When ionic liquid concentration was increased to 19.6 g L⁻¹, biomass yield decreased by a factor of 27.64. These results suggest either strong inhibition of biomass formation by [bmim] [PF₆] or that *Fusarium* is only able to metabolise a fraction of [bmim] [PF₆]. To test this, yields were recalculated on the basis that only butyl was consumed (Table 2). These new yields indicated that biomass yield is only reduced by 2.12 and 3.45 times at 9.8 and 19.6 g [bmim] [PF₆] L⁻¹, respectively. It is important to point out that the pH of the culture medium was around 7±0.2, indicating that non-acidification occurred by HF liberation from [bmim] [PF₆]. Docherty *et al.* (2007) reported that pyridinium ionic liquids can be fully mineralized, but imidazolium-based ionic liquids were only partially mineralized, and [bmim][Br] were not biodegraded by an activated sludge microbial community after 43 days of incubation at room temperature.

Tolerance of *Fusarium* sp strain to ionic liquid could be explained since several genera of *Fusarium* have been reported to biodegrade several complex compounds. Regalado *et al.* (1997) reported a soil-inhabiting *Fusarium proliferatum* strain, which was capable of transforming or degrading nonlabeled and ¹⁴C-labeled industrial, natural, and synthetic lignin. Dumestre *et al.* (1997) reported that *Fusarium solani* IHEM 8026 showed good potential for cyanide biodegradation under alkaline conditions. Mendonça *et al.* (2004) assayed the mycelium of *Fusarium flocciferum* for its ability to degrade

aromatic compounds, namely, gallic, protocatechuic, vanillic, syringic, caffeic, and ferulic acids and syringic aldehyde, commonly found in agro-industrial wastes. Magaña-Reyes *et al.* (2005) used *Fusarium solani* to degrade methyl tert-butyl ether (MTBE) and other oxygenated compounds from gasoline, including tert-butyl alcohol (TBA). Recently, Chulalaksananukul *et al.* (2006) claimed that *Fusarium* sp. E033 was able to survive in the presence of benzo(a)pyrene concentrations up to 1.2 mM (300 mg L⁻¹) and to biodegrade it (65–70%) using 0.4 mM (100 mg L⁻¹) benzo(a)pyrene. Also, Arriaga *et al.* (2006) reported the biodegradation of hexane solubilized in silicone oil by the fungus *Fusarium solani* in various bioreactor configurations.

3.3. Membrane-aerated biofilm reactor experiments

To study [bmim] [PF₆] biodegradation, synthetic wastewater treatment experiments were carried out using a MABR. In order to form the biofilm, the MABR was inoculated with 6×10⁸ spores suspended in 250 mL of SNA medium. The biofilm was allowed to develop for 15 days at 30 °C. After this time, SNA medium was replaced with a synthetic medium, in which the carbon source was substituted by [bmim] [PF₆]. The reactor was operated with a recycle rate of 12.6 mL min⁻¹ and an overall hydraulic retention time of 0.185 h, and the ascending liquid velocity parallel to the membrane was 0.5 m h⁻¹. The biodegradation of [bmim][PF₆] was measured by UV. Fig. 4 shows that the initial [bmim][PF₆] concentration (21 g L⁻¹) decreased in about 80% after 28 days of incubation at 30 °C. After this period, no further changes in [bmim] [PF₆] concentration were observed. Ohandja and Stuckey (2006) claimed that with proper control of parameters such as the oxygen level, the nature and concentration of the electron donor, and modification of the reactor design, the MABR has considerable potential to be able to completely mineralize and biodegrade perchloroethylene in wastewaters. Studying controllable nitrification, Terada *et al.* (2006) showed that the oxygen supply rate (OSR) with three polyacrylonitrile membrane modules was equivalent in these membrane modules and that OSR was affected only by air pressure, thus enabling control of aeration simply by adjustment of air pressure. More than 80% oxygen utilization efficiency was achieved under all operational conditions. However, in order to enhance yields and reduce biodegradation times, further studies of bio-

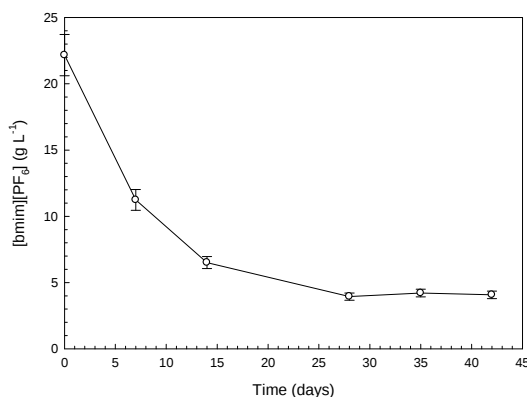


Fig. 4. Biodegradation of [bmim] [PF₆] by *Fusarium* sp. using a membrane-aerated biofilm reactor. Error bars represent the standard deviation of [bmim] [PF₆] concentration analyzed by triplicate.

degradation products and optimization of biofilm reactor operations are needed.

Conclusions

An isolate of *Fusarium* was able to grow in solid surface and submerged liquid modified SNA media containing [bmim] [PF₆] as the sole carbon source. Results obtained on Petri dishes and in Erlenmeyer flasks indicate that [bmim] [PF₆] is biodegraded by *Fusarium*. Using a membrane-aerated biofilm reactor, up to 80% biodegradation occurred after 28 days, with [bmim] [PF₆] concentrations in a modified SNA media up to 21 g L⁻¹. Further studies of biodegradation products and optimisation of biofilm reactor operations are needed to enhance biodegradation.

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