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Biodegradation of diethylketone by two fungi

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Abstract: Two morphologically different fungi (one green and one white) from contaminated bioreactors with an aqueous solution of diethylketone and *Streptococcus equisimilis* were isolated and characterized at molecular level by sequencing the amplified ITS region. The ITS region sequence showed 99% match with *Alternaria* genera for the green fungi and 99% match with the *Penicillium* genera for the white fungi. The performance of these two fungi to biodegrade different concentrations of diethylketone from aqueous solutions was evaluated. The biodegradation of diethylketone was studied for an initial diethylketone concentration ranging from 0.5 to 4g/L in a batch mode of operation. The biodegradation rate found for both fungi followed the pseudo-second order kinetics for initial concentrations higher than 0.5 g/L and the resulting kinetic parameters are reported. The removal percentages obtained were approximately 100%, for all the initial concentrations tested.

Keywords: fungi, diethylketone, biodegradation kinetics, genetic identification

Introduction

Contamination of water streams with hazardous compounds has attracted increasing attention in recent decades all over the world. Many of these compounds, including aromatic hydrocarbons, aldehydes and ketones, are toxic to humans because of their carcinogenic and mutagenic properties and their capacity to form toxic and phytotoxic radical intermediates (Costa et al., 2012). One common example is diethylketone (DEK), which despite being used in many anthropogenic activities as a solvent and as an intermediate in the synthesis of pharmaceuticals, flavors and pesticides among others, has had few studies regarding its biodegradability. DEK can react with OH radical being able to promote the formation of ozone and other components of the photochemical smog in urban areas (Lam et al., 2012). DEK inhalation may cause irritation in the eyes, skin, mucous membrane, cough and sneezing according to the Occupational Safety and Health hazards (OSHA) of United States Department Labor. Prolonged exposure results in tachycardia, nausea, shortness of breath, dizziness, fainting, coma and even death. DEK does not bind well to soil and thus pollutes groundwater. It is persistent in water, soil and air and has high mobility (www.cdc.gov). It has acute and chronic toxicity to the aquatic life as well.

Unlike commonly used physicochemical methods, biological techniques can be used for a broader group of compounds ensuring their decomposition to less or non-toxic compounds. Apart from bacteria, also others microorganism are capable of biodegrading organic solvents, in particularly fungi due to their diversity and remarkable ability to degrade complex and persistent natural materials such as lignin, chitin, and microcrystalline cellulose. In contrast to bacteria, fungi are able to extend the location of their biomass through hyphal growth. They are able to grow under environmentally stressed conditions such as low water activity, low nutrient availability and at low pH values where bacterial growth might be limited (Quintelas *et al.,* 2011). The biodegradation process of hazardous compounds by microorganisms is tremendously complex and may result from the combination of several factors such enzymatic degradation, binding by extracellular polymeric substances (EPS) or binding by functional groups present on the cell wall. The cell wall and extracellular exudates of fungi display a variety of Lewis-acid functional groups including carboxyl, phosphoryl, amine, hydroxyl moieties and sulphates, most of which have a strong binding affinity for cationic aqueous species (Haas et al., 1998). The major structural components of fungal cell walls are chitin and chitosan, comprised of polymerized N-acetyl-D-glucosamine and D-glucosamine, respectively along with a variety of extracellular polymers including α- and β-glucans, cellulose and a diversity of mannoproteins and glycoproteins (Haas et al., 1998), which have been implicated in the sequestering of metals (Volesky & Holan, 1995). It is known that fungi are able to produce different enzymes including pectinolyct and amylolytic systems, invertases, cellulases and hemicelullases, pectinases, proteases, laccases, phytases, α-glucuronidases, mannanases, lipases which are involved in the degradation of hazardous compounds (Ayala et al., 2008; Leitão, 2009)

This work aims the development of a technology that may decontaminate aqueous solutions containing diethylketone. The information collected from these experimental studies was used to calculate the growth kinetic constants and the degradation kinetic constants from different models, Monod (Monod, 1987), Powell (Powell, 1967), Haldane (Andrews, 1968), Luong (Luong, 1987) and Edwards (Edwards, 1970) reported in literature as models with application in biodegradation of organic pollutants. The importance of this work is notorious because not only it defends a quite straightforward technology to biodegrade high concentrations of DEK

from contaminated aqueous solutions using microorganisms, but also because up to the present knowledge these two fungi were never used to biodegrade DEK.

Material and Methods

Molecular examination and phylogenetic analysis

Two fungi with different morphology (one green and the other white) were isolated from a contaminated bioreactor with an aqueous solution with diethylketone and *S. equisimilis.* Isolation was carried out by spreading directly the fungi in a Dichloran Rose Bengal agar medium (DRBC) supplement with chloramphenicol (Ayale *et al.*, 2008), and kept at 25ºC for 5 days. The DNA from each isolated fungi, was subcultured in a Malt Extract Agar culture medium (Ayale *et al.*, 2008) (25ºC for 24h) and extracted according to the PowerSoil® Dna Isolation Kit, MO Bio Laboratories, Inc. DNA extract were used to amplified Internal Transcribe Sequences (ITS) surrounding the 5.8S-coding sequence and situated between the Small SubUnit-coding sequence (SSU) and the Large SubUnit-coding sequence (LSU) of the ribosomal operon. The ITS region was amplified by PCR using fungal primers ITS1-F5'-CTT GGT CAT TTA GAG GAA GTA A-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT GC-3'. PCR reactions were carried out in a Bio-Rad MYCYCLER thermal cycler with controlled amplification conditions as follows: initial denaturation at 95ºC for 3 minutes followed by 39 cycles of denaturation at 95ºC for 30 seconds, annealing at 50.7ºC for 30 seconds, extension at 72ºC for 36 seconds and final extension 72ºC for 5 minutes. The sequencing of the PCR purified products were conducted by Eurofins MWG Operon (Ebersberg, Germany) and subjected to a GenBank BLAST search to retrieve sequences of closely related taxa (http://www.ncbi.nlm.nih.gov/Blast/).

Biodegradation study

Each fungus was grown for 24h at 25ºC in Brain heart Infusion (Oxoid CM1135) culture medium (500mL) and then 150mL (×3) of this inoculated medium were transferred to 3 new HBI culture media (1000L). These cultures were grown for 48h

at 25ºC. After this period, the biomass was centrifuged on a Sigma 4K15 centrifuge (RCF of 7950) and the supernatant was collected on a sterile bottle for later use. The biomass pellets were resuspended on a smaller volume of the collected medium, being this volume calculated to obtain a final biomass concentration in culture up to $3g_{biomass}/L$. The 250 mL Erlenmeyer flasks were filled with a diethylketone solution (150mL) were then inoculated with this previous concentrated biomass. The diethylketone concentrations used were on the range 0.5-4g/L. At different time intervals, a sample was taken, centrifuged at 13400rpm for 10 minutes, and the supernatant was used for the estimation of diethylketone concentration. The assays were conducted in duplicate, during 5–7 days, at 25ºC and 150rpm. The samples were analysed by GC-MS Varian 4000.

Characterization procedures

The GC-MS employed herein was a Varian 4000, equipped with a flame ionization detector (FID) and mass spectrometry (MS). The separations were performed using a Meta Wax column (30 m x 0.25 mm x 0.25 µm). The operating conditions were as follows: the column was held initially at a temperature 50ºC, then heated at 10ºC/minute to 100ºC, held at 100ºC for 4 minutes, then heated again at 40ºC/minute to 200ºC and finally held at 200ºC for 2 minutes. The temperature of injector and detector were maintained at 250ºC. Nitrogen was used as a carrier gas at a flow rate of 30 mL/minute and the injections were performed in the split mode with a split ratio of 1:7. Under these conditions, the retention time for ketone was 3.2 minutes.

Results and Discussion

Identification of the isolated fungi

The sequences obtained for the white fungi showed 99% match with the *Penicillium* genera (accession number HQ850362.1) whereas the sequence blast for the green fungi showed 99% match with *Alternaria* genera (accession number KC623563.1) (Table 1). Agathos et al. (1997) related the appearance and abundance of fungi in the bioreactors to the acidic conditions inside it and their high tolerance to changes in pH, concomitant with ketones degradation.

Biodegradation assays with Penicillium sp. and Alternaria sp. fungi

For both fungi, the biodegradation of DEK is fast on the first 80 and 160 hours, respectively for the *Penicillium* sp. and *Alternaria* sp., after that it slows down until values equal or higher than 95% are reached (Figure 1).

The results obtained for the biodegradation assays with *Alternaria* sp. are best described by Haldane model (R^2=0.968). The kinetic parameters obtained are μ_m= 2.4x10^{-4} $h^{-1,}$ K_S=-0.416 and K_I=6.1.74x10^{17}. The small value obtained for the *Ks* and the high value of K_I indicate respectively the high affinity of the substrate and the lower sensitivity of the culture towards the substrate inhibition. The results obtained for the biodegradation assays with *Penicillium* sp. are best described by the Luong model (R^2=0.932). The kinetic parameters μ_m=9.6x10^{-2} h^{-}1, K_S=1.226, S_m= 5.943 and n=0.753 indicate the affinity of the substrate and that, above 5.943 g/L the biodegradation and consequently the growth cease

Regarding the biodegradation kinetics models for both assays the model that best describes the obtained results is pseudo-second order model for concentration of diethylketone higher than 0.5g/L (R^2≥0.985 and R^2≥0.995, respectively) (Table 2 and 3). The K_2 constant, for both assays decrease with the increase of the initial concentration of DEK which suggests that the biodegradation of DEK decrease over time, which is in agreement with the obtained results.

Conclusions

The obtained results showed the potential of two fungi from contaminated reactor with DEK and S. *equisimilis*, identified as fungi belonging to the *Penicillium* and *Alternaria* genera, to efficiently biodegrade DEK in aqueous solutions (98 and 99%, respectively). The growth kinetic models that best describe the growth of *Penicillium* sp. and *Alternaria* sp. are respectively the Luong and the Haldane model. The degradation kinetic model that best fit the results obtained for *Penicillium* sp is the pseudo-second order whereas for the *Alternaria* sp the pseudo-second order model only describes adequately the assays with initial concentrations of DEK higher than 0.5g/L.

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http://www.ncbi.nlm.nih.gov/Blast/

www.cdc.gov

Figures and Tables

Table 1 ITS1-F-5.8S RNA-ITS4 sequence obtained for the two isolated fungi.

Fungi	Genetic identification (%)	Accession number
White	*Penicillium sp.* (99%)	HQ850362.1
Green	*Alternaria sp.* (99%)	KC178625.1

Table 2 Constant parameters of zero-order, pseudo-first, pseudo-second and three-half-order kinetic models for different initial diethylketone concentrations and for the biodegradation study with the *Penicillium sp.* fungi.

S (g/L)	Zero order kinetic			Pseudo-first order kinetic		
	K_0	S_0	R^2	K_1		R^2
0.5	-0.0719	0.7678	0.9601	-0.0066		0.832
1	-0.0151	0.5252	0.722	-0.0141		0.965
2	-0.0202	3.9174	0.9834	-0.0097		0.957
4	-0.0214	4.2364	0.9896	-0.0141		0.962
S (g/L)	**Pseudo-second order kinetic**			**Three-half order kinetic**		
	K_2		R^2	K_{31}	K_{32}	R^2
0.5	-0.0794		0.984	-	-	-
1	0.0138		0.996	0.009	-6E-5	0.875
2	0.0073		0.985	-	-	-
4	0.01368		0.998	0.011	-8E-5	0.682

Table 3 Constant parameters of zero-order, pseudo-first, pseudo-second and three-half-order kinetic models for different initial diethylketone concentrations and for the biodegradation study with the 90 *f*ungi.

S (g/L)	**Zero order kinetic**			**Pseudo-first order kinetic**	
	K_0	S_0	R^2	K_1	R^2
0.5	-0.018	0.7816	0.964	-	-
1	-0.012	1.562	0.941	-0.006	0.971
2	-0.012	1.562	0.941	-	-
4	-0.0168	5.279	0.840	-	-

S (g/L)	**Pseudo-second order kinetic**		**Three-half order kinetic**		
	K_2	R^2	K_{31}	K_{32}	R^2
0.5	-0.070	0.754	-	-	-
1	0.015	0.996	0.008	-4E-5	0.824
2	0.003	0.998	-	-	-
4	0.015	0.996	0.005	-2E-5	0.928

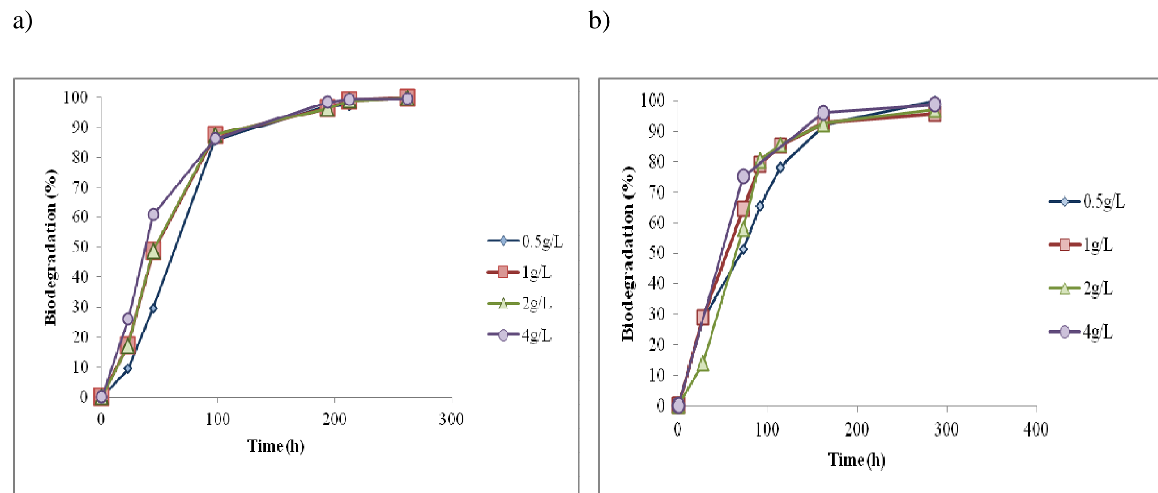

Figure 1 Biodegradation percentage *versus* time, for all the diethylketone concentrations tested. a) Biodegradation of diethylketone by the *Penicillium sp.* fungi; b) Biodegradation of diethylketone by *Alternaria sp.* fungi.