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Biodegradation of n-hexadecane by *Aspergillus* sp. RFC-1 and its mechanism

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ABSTRACT

Fungi can use n-hexadecane (HXD) as a sole carbon source. But the mechanism of HXD degradation remains unclear. This work mainly aimed to study the degradation of HXD by *Aspergillus* sp. RFC-1 obtained from oil-contaminated soil. The HXD content, medium acidification and presence of hexadecanoic acid in the medium were determined by gas chromatography-mass spectrometry, and fungal growth was observed. Enzyme and gene expression assays suggested the involvement of an alkane hydroxylase, an alcohol dehydrogenase, and a P450 enzyme system in HXD degradation. A biosurfactant produced by the strain RFC-1 was also characterized. During 10 days of incubation, 86.3% of HXD was degraded by RFC-1. The highest activities of alkane hydroxylase (125.4 μmol mg^{-1} protein) and alcohol dehydrogenase (12.5 μmol mg^{-1} proteins) were recorded. The expression level of cytochrome P450 gene associated with oxidation was induced (from 0.94-fold to 5.45-fold) under the HXD condition by Real-time PCR analysis. In addition, HXD accumulated in inclusion bodies of RFC-1with the maximum of 5.1 g L^{-1}. Results of blood agar plate and thin-layer chromatography analysis showed RFC-1 released high lipid and emulsification activity in the fungal culture. Induced cell surface hydrophobicity and reduced surface tension also indicated the RFC-1-mediated biosurfactant production, which facilitated the HXD degradation and supported the degradation process.

1. Introduction

Petroleum hydrocarbons are considered the most common environmental pollutants. The increasing global demand for energy in recent years has resulted in water and soil deterioration by oil industry pollution (Hasan et al., 2010). Alkanes are highly abundant in the environment because of the broad usage of petroleum fuels and their derivatives (Meng et al., 2017). The low-molecular-weight alkanes are volatile in nature and easily degraded, whereas those with high molecular weight are highly persistent in the environment (Labinger and Bercaw, 2002).

n-Hexadecane (HXD) is a major alkane component, and is present in the aliphatic fragment of crude oil. The solubility of HXD in water is 5.21×10^{-5} mg L^{-1} at 15 °C and has high partitioning co-efficient 9.1 logKow (Stroud et al., 2007). HXD is present in highly contaminated oil sites, and it possesses well-characterized biodegradability, hence, HXD compound has been used as a model molecule for studying alkane hydrocarbon biodegradation (Schoefs et al., 2004).

There are three kinds of alkane hydroxylase monooxygenases involving in short-, medium-, and long-chain alkanes degradation. They are methane monooxygenase, membrane-bound nonheme alkane monooxygenase, and cytochrome P450 (CYP52) (Van Beilen and Funhoff, 2007). A major process of alkane degradation is the oxygenation of the terminal methyl group (Rehm and Reiff, 1981). Given that alkane-degrading microorganisms possess multiple genes for alkane hydroxylases, they can highly degrade a wide range of alkanes. Two major factors are responsible for the rapid n-alkane degradation from petroleum mixtures via microorganisms: metabolic enzyme activity for oxidizing n-alkanes and alkane transfer into cells. Little is known about the metabolic intermediates of alkane extracellularly before it is assimilated across the cell membranes, despite the understanding regarding the mechanisms by which long-chain fatty acids enter cells (Van Den Berg et al., 2004).

Many hydrocarbon-degrading microbial excrete biosurfactants, amphiphilic molecules of diverse chemical nature, which enhances the aptitude of microbial cells to use hydrophobic compounds as growth substrates (Kiran et al., 2009; Mahjoubi et al., 2013; Al-Hawash et al., 2018b). Extracellular biosurfactants and bioemulsifiers produced by

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microorganisms play a significant role in improving cell absorption by solubilization and increase the interfacial area by the lowering surface tension (Rahman et al., 2003). The microorganisms have two ways to access alkanes in the culture medium, which is adhesion to hydrocarbon droplets or biosurfactant aiding emulsification and alkanes assimilation (Al-Tahhan et al., 2000). Rojo (2009) reported that only low-molecular-weight alkanes, which are soluble and easily enter cells, can be directly absorbed from an aqueous phase, whereas medium- and long-chain alkanes transfer into cells through the produced biosurfactants or/and hydrophobic cell surfaces.

Bioremediation success depends on the inherent biodegradability of the pollutant, the pollutant accessibility to degrade microorganisms, and the biological activity optimization (Kebria et al., 2009). Some microbial species that can degrade long-chain alkanes have been proposed to play a valuable role in bioremediation of oil-polluted environments (Al-Hawash et al., 2018a). There are a few reports involved in HXD biodegradation by fungi. *Aspergillus niger* Y1 removes and biodegrades HXD from oil-contaminated environments (EL-Hanafy et al., 2015). *Aspergillus niger* ATCC 9642 can degrade and mineralize HXD (Volke-Sepulveda et al., 2003), and HXD biodegradation increases when *A. niger* ATCC 9642 is exposed to an electric current (Velasco-Alvarez et al., 2011). Dashti et al. (2008) found that the *Aspergillus* could grow on the individual oxidation products of HXD as sole carbon source.

It was reported that among the filamentous fungi, *Aspergillus* spp. dominate in most of the petroleum contaminated sites (Bairagi et al., 2011; Ye et al., 2011; Zhang et al., 2016; EL-Hanafy et al., 2017). We obtained a filamentous fungus *Aspergillus* sp. RFC-1 degrading crude oil and PAHs (Al-Hawash et al., 2018d). In the present study, in order to determine the mechanism involved in HXD degradation by *Aspergillus* sp. RFC-1, its growth and respiration were examined using a microtiter plate methodology, the roles of two potential key enzymes in degradation were investigated, and the expression of the CYP52 gene in HXD oxidation was determined. The other factors in relation to HXD degradation, including cell surface hydrophobicity, emulsification index, surface tension, biosurfactant production and intracellular hydrocarbon accumulation were also probed.

2. Materials and methods

2.1. Microorganism and culture media

Aspergillus sp. RFC-1 (NCBI GenBank accession numbers: KY328441) was isolated from crude petroleum-contaminated soil samples of Rumaila oilfield Basra, Iraq (Al-Hawash et al., 2018d).

Mineral salt medium (MSM) was composed of the following (per g L^{-1} distilled water): K_2HPO_4 $3H_2O$ 1.0, KH_2PO_4 0.4, NaCl 0.5, $(NH_4)_2SO_4$ 0.2, $NaNO_3$ 0.1, and $MgSO_4{\cdot}7H_2O$ 0.025. The pH was adjusted to 7.0 with 1N HCl. Potato dextrose broth was composed of 20 g L^{-1} dextrose and potato extract prepared from 200 g L^{-1} potatoes. The strain was maintained on agar slants with 15 g L^{-1} agar. Culture media were sterilized in an autoclave at 121 °C for 20 min.

A full loop of culture maintained on slants was inoculated in 100 mL of potato dextrose medium in a 250 mL Erlenmeyer flask, incubated at 30 °C with shaking at 130 rpm for 3 days, and subcultured under the same condition for 2 days. The mycelial suspension was centrifuged at 7000 rpm for 5 min at 4 °C. Subsequently, the mycelial pellets were collected and washed two times with sterile MSM to remove nutrient materials and some undesirable impurities. Afterward, fungal mycelia (considered as pristine RFC-1) were re-suspended in MSM, transferred into a sterile Waring Blender Cup, and homogenized for inoculum.

2.2. Scanning electron microscopy (SEM) and fourier transform infrared spectroscopy (FTIR)

The SEM was used to characterize mycelial surface morphology. After completing degradation experiment, mycelia from 1% HXD and no HXD (pristine RFC-1) were collected and dried to SEM analysis using the method described by Bairagi et al. (2011). The samples were observed under a JEOL, JSM-52500 LV SEM (Japan), and different sections of the samples were evaluated.

To elucidate the changes of chemical bonds (functional groups) and chemical characteristics relevant to HXD uptake by the fungal biomass, FTIR was used to analyze the mycelia from 1% HXD and no HXD (pristine RFC-1) using an FTIR spectrophotometer. Pressed mycelia were prepared by grinding them with IR-grade KBr in agate mortar (1:100) and recorded in the region of 4000–400 cm^{-1}.

2.3. Fungal growth in MSM with HXD

The growth curve of strain RFC-1 was assessed by microtiter plate using Langvad' method which showed a linear correlation between the absorbance reading at 630 nm and dry biomass weight of filamentous fungi (Langvad, 1999). A homogenized mycelial suspension (50 µL) was used to inoculate individual wells of a 96-well microtiter plate, which contained 200 mL of sterile MSM supplemented with different concentrations of HXD (0.5–3% v/v). The microtiter plates were incubated at 30 °C for 10 days. At two-day intervals, the microtiter plates were removed, and growth was measured at 630 nm wavelengths by a microplate reader (Thermo Fisher, Waltham, Massachusetts, USA) (Giti et al., 2005).

To determine the time course of the fungal growth, 50 µL of the homogenized mycelial suspension was inoculated into a 96-well microtiter plate, which contained 200 µL of sterile MSM supplemented with 1% HXD (v/v) under the same conditions as above for 10 days, without HXD serving as a control. All tests were carried out with three independent replicates.

2.4. Assay of fungal respiration in the presence of HXD

Respiration assay was performed according to the method of Alef and Nannipieri (1995). To determine dehydrogenase activity, the strain RFC-1 was grown at 30 °C in MSM with different concentrations (0.5–3% v/v) of HXD in microtiter plates and the samples were obtained at two-day intervals until 10 days. A 50 mL aliquot of an electron acceptor, which is a triphenyl tetrazolium chloride (TTC) solution containing 0.25 g of TTC in 100 mL Tris buffer (0.1 mol L^{-1}, pH 7), was added to each well. The plates were incubated at room temperature for 1 day for color development. The optical density (O.D.) was measured against a blank at 490 nm on the microplate reader.

To determine the time course for the respiration activity, the strain RFC-1 was grown at 30 °C in MSM with 1% HXD (v/v) in microtiter plates under the same procedures mentioned above. All tests were carried out in three independent replicates.

2.5. Protein estimation

To determine the fungal cell protein, the mycelia of RFC-1 grown in MSM with 1% HXD were harvested at two-day intervals. After washing with sterile MSM, the mycelia were re-suspended in cell lysis solution (Suzhou Keming Biotechnology, Jiangsu, China), ground for 2 min in an ice bath by a Dounce glass grinder homogenizer, and centrifuged at 12,000 rpm for 30 min at 4 °C to get intracellular crude extract, and subsequently preserved at 0 °C for protein content determination and enzyme assay. The protein content in the strain RFC-1 was measured by a microplate reader at 595 nm, followed by the method of Bradford (1976), using bovine serum albumin as a standard. All tests were carried out with three independent replicates.

2.6. Biodegradation of HXD

To determine the potential of the strain RFC-1 for substrate