Biodegradation of Medium Chain Hydrocarbons by *Acinetobacter venetianus* 2AW Immobilized to Hair-Based Adsorbent Mats

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The natural attenuation of hydrocarbons can be hindered by their rapid dispersion in the environment and limited contact with bacteria capable of oxidizing hydrocarbons. A functionalized composite material is described herein, that combines in situ *immobilized alkane-degrading bacteria with an adsorbent material that collects hydrocarbon substrates, and facilitates biodegradation by the immobilized bacterial population.* Acinetobacter venetianus *2AW was isolated for its ability to utilize hydrophobic n-alkanes (C10–C18) as the sole carbon and energy source. Growth of strain 2AW also resulted in the production of a biosurfactant that aided in the dispersion of complex mixtures of hydrophobic compounds. Effective immobilization of strain 2AW to the surface of Ottimat^{TM} adsorbent hair mats via vapor phase deposition of silica provided a stable and reproducible biocatalyst population that facilitates* in situ *biodegradation of n-alkanes. Silica-immobilized strain 2AW demonstrated* ca. *85% removal of 1% (v/v) tetradecane and hexadecane within 24 h, under continuous flow conditions. The methodology for immobilizing whole bacterial cells at the surface of an adsorbent, for* in situ *degradation of hydrocarbons, has practical application in the bioremediation of oil in water emulsions. Published 2011 American Institute of Chemical Engineers* Biotechnol Prog., *000: 000–000, 2011*
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Introduction

According to a report issued in 2002 by the National Research Council, ~380 million gallons of crude and processed petroleum finds its way into the World's oceans each year. About 60% of the total derives naturally from seepages out of the ocean floor; the rest comes from human activities.[1] Oil spills that occur as a result of accidents or environmental disturbances create significant economic issues and often long-term ecological impact.[2] The collection and recovery of oil following such events is often a laborious and time-consuming task that can be hampered as volatile components evaporate to leave compounds that may sink as dense non-aqueous phase liquids or be deposited as tar balls.[3,4] Many schemes have been proposed over the years to collect, disperse, or neutralize crude oil released into the environment. The methods may incorporate physical collection (such as adsorbents) chemical disruption (i.e., the use of surfactants), or even burning the hydrocarbons on the surface. Physical collection, merely transfers oil from one location to another (e.g., from the ocean to a landfill), where appropriate treatment and handling raise additional burdens (e.g., incineration, long-term containment). In addition, the chemical dispersants used in oil spill situations have historically proved controversial due to the potential toxicity to aquatic and marine organisms.[5–8]

A biological method of oil cleanup using natural oil-degrading microorganisms offers an environmentally sustainable alternative to chemical methods of remediation.[9–11] Although biotransformation of petroleum is readily demonstrated in laboratory experiments, the marine environment is especially difficult for bioremediation by natural attenuation due to the complex interaction of multiple factors including physical dispersion (e.g., oil movement due to wave action), weathering, temperature fluctuations, variability in water salinity, and accessibility to organic nutrients.[10,12] Bioremediation efforts, therefore, often rely on seeding an area with oil-degrading microorganisms and nutrients, but retaining the nutrients and microorganisms in close proximity with the contaminating oil can be challenging. In addition, exogenous bacteria often

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struggle to compete with the indigenous population when in direct competition.[11,13]

Various adsorbent materials have been demonstrated to adsorb petroleum hydrocarbons.[14–16] Many commercial derivatives, however, are designed only to collect oil, which subsequently requires the adsorbent to be handled as toxic waste. Certain adsorbent mats are designed to be reusable after the oil is recovered from the matrix. Many natural and synthetic polymeric matrices have been investigated for whole-cell immobilization and used in manufacturing of commodity chemicals and pharmaceuticals, but their use for the bioremediation of oil has been limited.[17–23] Herein, an adsorbent material made from human hair (Ottimat™) was selected as a model adsorbent. The material has United States Environmental Protection Agency and United States Coast Guard approval for use as an adsorbent for environmental oil contamination response (http://www.ottimat.com). The material's distributors market Ottimat as an alternative to commonly used polypropylene pads and claim comparable price and significant performance advantages, in particular the ability to "wring" and reuse hair-based mat >100 times. In an effort to demonstrate enhanced functionality of such an adsorbent material, a model oil-degrading bacterial strain was immobilized to the adsorbent via a process for vapor-phase deposition of silica.[24] The adsorbent will adsorb oil and then provide a convenient reservoir to feed substrate to the surface-immobilized bacteria. The method of cell immobilization provides a homogenous population of bacterial cells at a high and defined cell density that eliminates the significant time typically required for the establishment of the bacterial community. In this approach, the immobilized cells are also protected from weathering and the elements that may wash away the microbes in open water applications. The methodology provides means to affix bacteria with a selected metabolic or catalytic capability to compatible materials surfaces. The result mimics the biology of natural occurring oil-degrading bacteria that would exist as complex biofilms, microbial mats and coatings of oil-degrading bacteria on seaweed.[25–27]

Materials and Methods

Chemicals and materials

All reagents used were analytical grade and obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Ottimat™ bricks were supplied as a gift by World Response Group (Huntsville, AL). Uniform samples of the Ottimat™ adsorbent material were prepared for all experiments and are described as "adsorbent" throughout. A sample of used motor oil was used as a representative of a viscous mixed petroleum hydrocarbon derived from crude oil.

Isolation of hydrocarbon-degrading microbial species from environmental samples

Seaward facing, beached oil samples were collected on May 22, 2010, from the South end of the Elmer's Island Wildlife Refuge in Louisiana. Marine agar (MA) (Difco Laboratories, Detroit, MI) was used as a complete medium for bacterial cultivation. Bushnell-Haas (BH) (Becton Dickinson 3 Laboratories, Sparks, MD) agar (Difco) supplemented with 1% v/v hexadecane was used to isolate and assess microbial hydrocarbon degradation. Approximately 10 μL of the environmental sample (oil or oil–water mix) was spread on MA and BH plates using a quadrant streak method. Plates were

incubated for 18 h at 30°C. Colonies were isolated based on morphology and replated to produce pure cultures.

The individual isolates were identified using 16s rDNA sequence analysis. Following the isolation steps above, individual colonies from pure culture plates were added to 5 mL of Marine broth (MB) and incubated with shaking for 18 h at 30°C. Genomic DNA was isolated from cultures by alkaline lysis, followed by isopropyl alcohol precipitation and ethanol (70%) washes. Extracted DNA was used as a template for a polymerase chain reaction (PCR) as follows: 1 μg template, 1 U Failsafe Enzyme mix (Epicentre® Biotechnologies, Madison, WI), 2× Failsafe Premix E, 2 mM 27F, and 1492R universal bacterial primers. PCR conditions were as follows: 94°C for 5 min; 20 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 90 s, and 72°C for 7 min. The nucleotide sequences of the PCR products were determined and then checked for identity shared with 16s rDNA sequences in GenBank using the nucleotide BLAST algorithm (National Center for Biotechnology Information; NCBI).

Bacterial cultivation and characterization

Acinetobacter venetianus 2AW was cultured aerobically in BH broth (Becton Dickinson Laboratories) supplemented with tetradecane and hexadecane (1:1) at 1% v/v of each alkane unless stated otherwise. Cultures were incubated in baffled shaker flasks at 30°C with shaking (220 rpm) and growth was monitored by measuring the increase in optical density (600 nm) using a Cary 3E spectrophotometer (Varian, Palo Alto, CA). Gram staining was performed using a Protocol™ Gram stain set (Fisher Scientific, Pittsburg, PA) according to the supplier's instructions. Oxidase activity was determined using a BBL™ Dryslide™ Oxidase test (Becton Dickinson Laboratories).

Preparation of immobilized cells

A. venetianus strain 2AW was cultured as described above and harvested at late-log phase (OD$_{600}$ *ca.* 5.0) by centrifugation, washed (3×), and resuspended in phosphate buffered saline (PBS) (PBS composed of NaCl 8.0 g, KCl 0.20 g, NaH$_2$PO$_4$ 1.4 g, KH$_2$PO$_4$ 0.24 g in 1 L of deionized water [pH 7.0]) to final OD$_{600}$ of 5 (corresponding to 1.2 × 10^9 colony forming units (CFU) mL^{-1} as determined by serial dilution and plate counts). Ottimat™ adsorbent samples were cut using a core punch (22 mm diameter, 1 g dry weight) and sterilized in PBS. For silica-immobilization of bacterial cells, the sterilized adsorbent material was placed into a glass dish (50 mm × 15 mm) modified with a central glass well (28-mm diameter) designed to hold the adsorbent plugs. The harvested and washed bacterial cells were applied to the adsorbent material (5:1 v/w) to completely wet the surface. Tetramethylorthosilicate (TMOS; 1 mL) was deposited in the outer ring of the glass dish. Glass beads (3-mm diameter borosilicate, Kimble Chase, Vineland, NJ) were added to the outer well to increase the surface area for evaporation of the TMOS (Figure 1). The glass dish was covered, sealed, and incubated for 60 min at 37°C. Physisorbed controls (no immobilization with silica) were prepared in the same manner but in the absence of TMOS. Untreated sterile adsorbent material was used for abiotic controls.

Alkane degradation studies

Adsorbent material functionalized with bacterial cells were prepared as described above and added to 50 mL growth
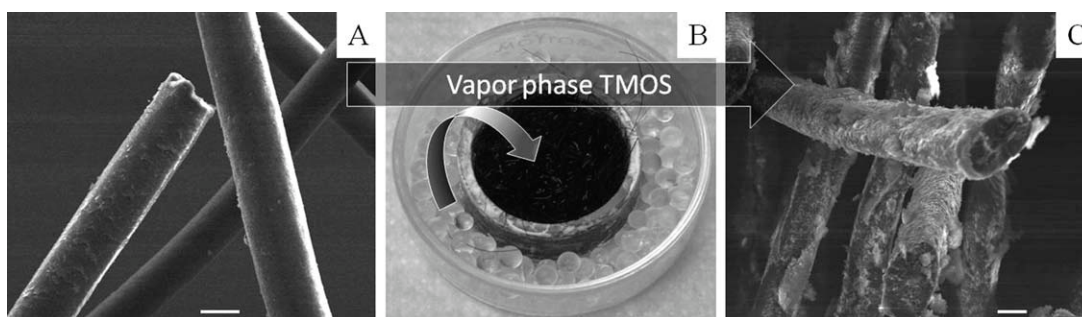
Figure 1. **Schematic for silica-immobilization of bacterial cells to adsorbent material; Untreated hair fibers within the adsorbent material (A), reaction vessel for vapor phase formation of silica on hair fibers (B), and hair fibers coated with silica (C) (Scale bars = 50 μm).**

medium (250 mL shake flask) with hexadecane and tetradecane as carbon and energy sources (1% v/v of each). The flasks were incubated at 30°C with shaking (150 rpm) for 24 h. Following incubation, the culture supernatants were clarified by centrifugation (10,000×*g* for 20 min at 4°C) and extracted into hexane (3 × 50 mL). The extracts were concentrated to near dryness on a rotary evaporator (35°C) and then dissolved in hexane (0.5 mL final volume). Hydrocarbon was similarly extracted from the adsorbent material by extraction into hexane. Hydrocarbon concentrations were determined using a 6890N Network GC system with a 5973 Mass Selective Detector (Agilent Technologies, Santa Clara, CA). Samples (1μL) were injected using split mode into a HP-5MS GC column (30 m × 0.25 mm × 0.25 μm) with a helium flow rate of 0.8 mL min^{-1}. The oven temperature was set initially at 150°C for 2 min and then increased to 280°C at 10°C min^{-1}. Tetradecane and hexadecane eluted at 3.5 and 5.7 min, respectively. Results are presented as residual hydrocarbon concentration (mM) following a fixed incubation time, unless otherwise stated. The hydrocarbon concentration was determined by comparison with standard curves generated for both alkane substrates (slope of concentration vs. peak area = $1.04 \times 10^9 \pm 2.62 \times 10^7$, $r^2 = 0.9987$).

The extraction efficiency for recovery of hexadecane and tetradecane into hexane was determined in abiotic control samples and confirmed the recovery of ˜98% of the substrate. Similarly, the ability to extract alkanes from the adsorbent mats was also tested in abiotic controls and extraction efficiency >90% (91.3 $\pm$ 12.5) was achieved.

For flow-through experiments, 1.0 g of sterile adsorbent material was functionalized with bacteria as described above and placed securely into the base of a plastic syringe (60 mL, Becton Dickinson Laboratories). Sterile PBS (1 L) was pumped through the adsorbent samples at a flow rate of 5 mL min^{-1} using an up-flow continuous flow method. The elution of unattached cells from the hair mats was measured by optical density and serial dilution of the mobile phase. The PBS wash was then switched to a feed containing 0.5% (v/v) tetradecane and hexadecane (1:1 ratio in 1 L of 10% BH broth) and recycled continuously for 24 h (5 mL min^{-1}). Unreacted alkanes in the adsorbent mats and in the liquid fraction were extracted into hexane as previously described for batch experiments and analyzed using GC-MS.

Purification and characterization of the biosurfactant activity

To purify the biosurfactant activity, strain 2AW was cultured as described above and the cells harvested at late log phase by centrifugation (10,000×*g* for 20 min at 4°C). The

cell-free supernatant was mixed with ammonium sulfate (60% saturation) and incubated for 1 h at room temperature as described previously for the purification of biosurfactants.[28] The precipitate was collected by centrifugation (5,000*g* for 30 min at 4°C) and dialyzed extensively against deionized water (5 L total) at 4°C using a Slide-A-Lyzer dialysis cassette with a 10K molecular weight cutoff membrane (Thermo Fisher Scientific, Rockford, IL). The dialyzed biosurfactant was stored at −20°C. The emulsifying activity of the biosurfactant was measured using a previously described method for emulsification of hexadecane and 2-methylnapthalene.[28]

Microscopy and cell viability

Samples for scanning electron microscopy (SEM) were washed overnight in PBS at room temperature to remove any cells that were not attached. Samples were fixed in glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.4) and dehydrated with a step-wise increase in ethanol concentration (50, 70, 80, 90, and 100%) before drying in an Autosamdri®-815 critical point dryer (Tousimis Research Corporation, Rockville, MD). SEM samples were coated with gold for 30 s using a Desk V sputter coater (Denton Vacuum, Moorestown, NJ) and imaged using a Hitachi S2600N SEM (Hitachi High Technologies America, Pleasanton, CA). Imaging of Gram stained cells was performed on a Motic BA-300 light microscope equipped with a 5 MP camera (Motic North America, Richmond, BC, Canada). Bacterial viability was assessed using the LIVE/DEAD® *Bac*Light™ bacterial viability kit (Invitrogen, Carlsbad, CA). Samples were washed in saline (0.85%) to remove phosphate buffer before fluorescent staining and also to remove excess dye after staining. Samples were visualized using a Zeiss Axioskop 40 FL (Carl Zeiss Microimaging, Thornwood, NY) fluorescence microscope for green (live) and red (dead) fluorescence. Neutral lipids within the bacterial cells and the extracellular biosurfactant were stained with Nile Red (7-diethylamino-3,4-benzophenoxazine-2-one) by incubating Nile Red (10 μL of 1 mg mL^{-1} stock in acetone) with 0.5 mL samples. Samples were stained for 5 min, washed, and then examined using fluorescence microscopy.

Bacterial viability was assayed using the BacTiter-Glo™ (Promega, Madison, WI) assay based on the quantification of ATP luminescence. Washed cells at a range of dilutions (OD$_{600}$ from 0.0005–0.5) were prepared by serial dilution and mixed with an equal volume of BacTiter-Glo reagent in black polystyrene 96 well assay plates (Costar®, Corning, NY). Luminescence was measured at 25°C at 5 min intervals
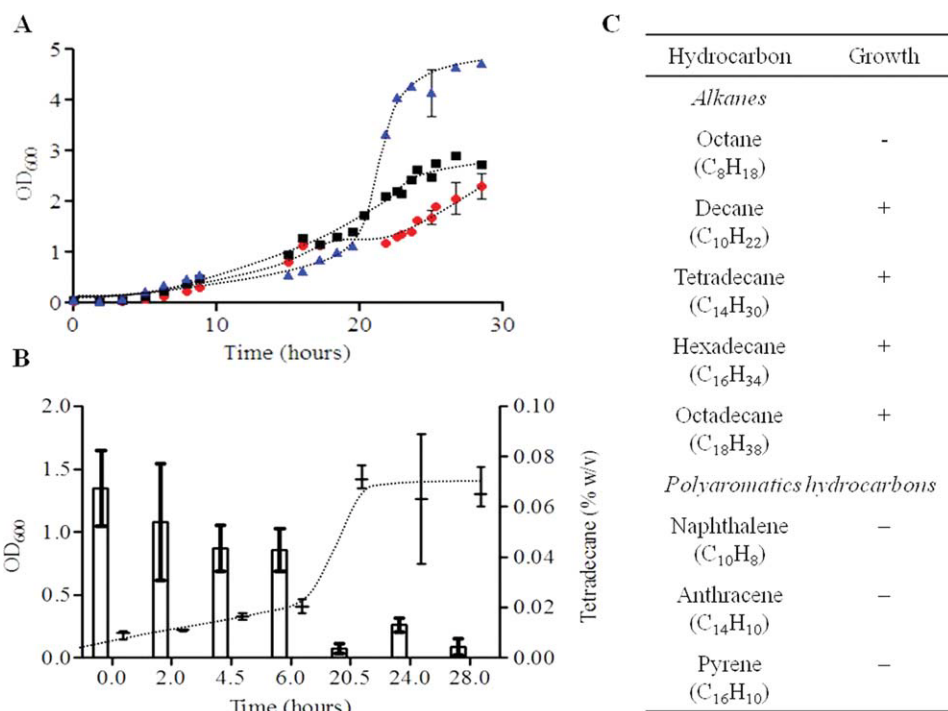
Hydrocarbon	Growth
Alkanes	
Octane (C_8H_{18})	−
Decane ($C_{10}H_{22}$)	+
Tetradecane ($C_{14}H_{30}$)	+
Hexadecane ($C_{16}H_{34}$)	+
Octadecane ($C_{18}H_{38}$)	+
Polyaromatics hydrocarbons	
Naphthalene ($C_{10}H_8$)	−
Anthracene ($C_{14}H_{10}$)	−
Pyrene ($C_{16}H_{10}$)	−

Figure 2. **Growth of *Acinetobacter venetianus* 2AW with *n*-alkanes of various carbon chain lengths; dodecane (●), tetradecane (■), and hexadecane (▲).**

(A); Correlation of bacterial growth and tetradecane degradation (B); growth substrates for strain 2AW (C).
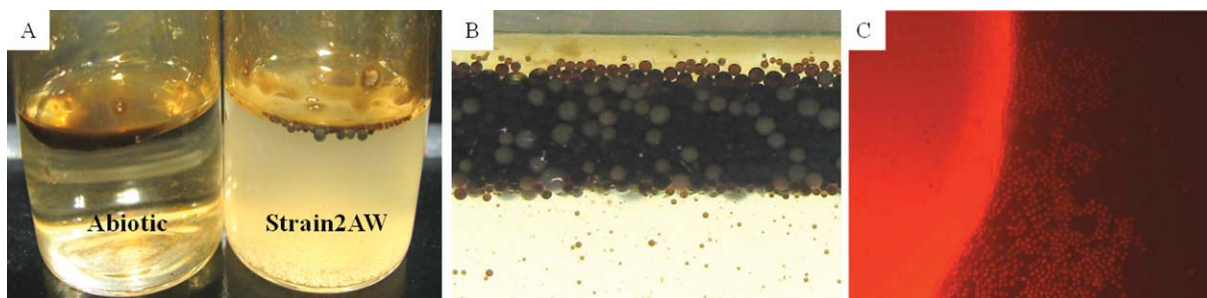
Figure 3. ***Acinetobacter venetianus* 2AW growing in liquid culture with used motor oil as the sole carbon and energy source vs. an abiotic control (A); the appearance of micelles at the oil/water interface (B); the adhesion of *Acinetobacter venetianus* 2AW to oil droplets as observed by Nile red staining (C).**

over a period of 40 min using a Synergy 4 hybrid plate reader (Biotek, Winooski, VT). The same cell dilutions were cultivated overnight at room temperature on agar plates and cell counts determined by colony counting. Relative luminescence units (RLU) were plotted against colony counts (CFU mL^{-1}) to create a standard curve for viable cell counts (slope = 0.0168 ± 0.0003, R^2 = 0.9969).

Results and Discussion

Isolation and characterization of alkane-degrading bacteria

The enrichment cultures seeded with beached oil samples provided a single strain that grew aerobically with medium chain alkanes (C_{10}–C_{18}) but showed no growth with polycyclic aromatic hydrocarbons (Figure 2). The cell morphology showed coccobacilli (short rods) that stained Gram negative and tested oxidase negative. The 16S *r*DNA sequence shared 99% identity with *A. venetianus* (GenBank Accession AM909651) and the isolate was subsequently designated *A. venetianus* 2AW (GenBank Accession JF412205).The ability of strain 2AW to use long-chain alkanes as a carbon source

demonstrates its utility in the degradation of complex petroleum products such as crude oil. Similarly, significant bacterial growth was observed within 24 h with motor oil as the sole carbon and energy source (Figure 3). The abiotic culture showed no increase in optical density over time and the oil remained as a "slick" on the liquid surface. The culture inoculated with strain 2AW, however, showed visible microbial growth over a period of 24 h (OD$_{600}$ = 0.64 ± 0.12) and the oil at the liquid–air interface dispersed into small micelles. The ability of the culture to disperse the oil layer is evident long before visible growth of the culture is observed (Figure 3B). The micelles were stable and could be collected, stained, and visualized without losing their mechanical integrity. Microscopic visualization of the micelles revealed an oil-in-water emulsion consisting of oil droplets with bacteria cells adhered directly to the hydrocarbon (Figure 3C).

Characterization of the biosurfactant activity

Nile red staining and microscopy revealed orange-red fluorescence with bacterial cells grown on alkanes. The
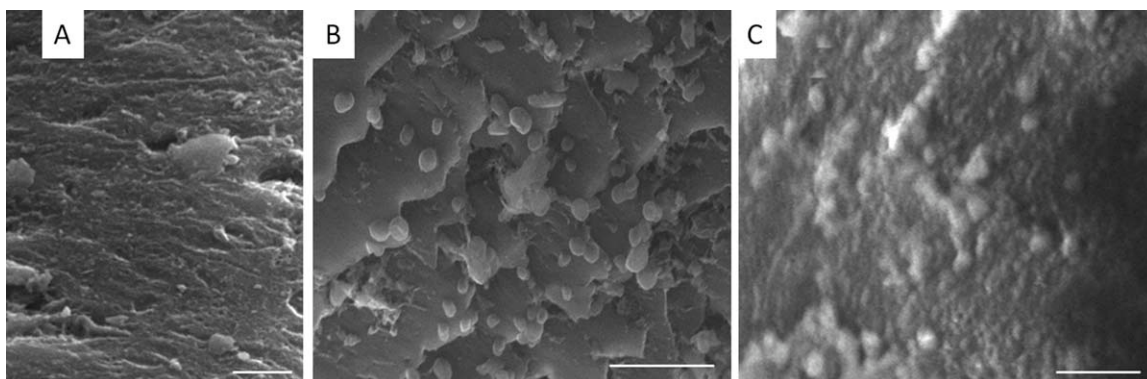
Figure 4. SEM images of hair surface (A), with physisorbed (B), and silica-immobilized (C) *Acinetobacter venetianus* 2AW (Scale bars = 5 μm).

fluorescence apparent in the micrographs emanates from stained lipid moieties and indicates the production of a biosurfactant in 2AW (Figure 3C). Similar fluorescence staining has been observed for the extracellular bioemulsifier (emulsan) produced by the prototypical oil-degrading bacteria, *A. venetianus* RAG-1.[29–33] Many *Acinetobacter* spp. produce biosurfactants (also termed bioemulsifiers) that aid in the adhesion to, and degradation of, hydrophobic substrates by reducing surface tension and increasing micelle formation.[12,30,34,35] Thus, *Acinetobacter* spp. and their associated biosurfactant activity are commonly employed for *in situ* remediation of oil spills.[5]

The biosurfactant activity associated with *A. venetianus* 2AW was isolated from culture supernatant and examination results showed it shared similarity to biosurfactants produced from other *Acinetobacter* spp. Many biosurfactants are complex mixtures of polysaccharides, proteins, and glycolipids.[34] The emulsan from the *A. venetianus* RAG-1, e.g., is an extracellular glycoprotein that displays esterase activity.[29] The emulsan-like activity from strain 2AW was protein-based and provided esterase activity, consistent with previously described emulsifying action. The preparation demonstrated a relatively high bioemulsifying activity of 330 ± 77 U mg^{-1} protein ($n = 3$), based on a standard method for emulsification of hexadecane and 2-methylnapthalene and compared with the reported activity of the bioemulsifier (alasan) from *A. radioresistens* KA53 (712 U mg^{-1}).[28] The behavior and appearance of strain 2AW cultures were consistent with its production of biosurfactant compounds that will enhance the organism's use of complex hydrocarbons for growth.

Immobilization of A. venetianus 2AW to absorbent mats

We examined the utility of combining bacterial metabolism and physicochemical sorption in biodegradation applications by using a method to fix strain 2AW on a bio-derived material that adsorbs oil. Commercially available adsorbent mats fabricated from pressed human hair fibers (Ottimat^{TM}) were selected as the model adsorbent and scaffold for bacterial immobilization. The adsorbent matrix sequesters hydrocarbons within the material, allowing degradation to take place *in situ*. *A. venetianus* 2AW was prepared at a defined cell density (1.2×10^9 CFU mL^{-1}) and the cells incubated with the material either in the absence (physisorbed) or presence of TMOS vapors (silica-immobilized). The vapor-phase TMOS forms a matrix of silica particles that aids attachment and retention of the bacterial cells directly to the hair fibers

of the adsorbent mats (Figure 4).[24] The chemical vapor deposition of silica was recently demonstrated as a versatile and simple method for the immobilization of a wide range of biological molecules, including whole bacterial cells.[24,36]

High-resolution imaging of the materials using SEM revealed an even coating of bacterial cells at the surface of the hair fibers that constitute the adsorbent material. The physisorbed bacterial cells are clearly visible on the hair fibers and become immobilized by an even coating of silica (Figure 4). The retention of cellular activity following immobilization was determined by monitoring the removal of *n*-alkane substrates. The residual concentrations of hexadecane and tetradecane were determined after 24 h incubation with bacteria-coated adsorbent (Figure 5). The rates of alkane removal were significantly greater for silica-immobilized cells compared with the physisorbed and abiotic controls. Approximately 60% of tetradecane and hexadecane remained after 24 h incubation with physisorbed cells from an initial concentration of 0.5% v/v (19 and 17 mM for tetradecane and hexadecane, respectively). For silica-immobilized cells, however, <16% of the initial tetradecane and 14% of the initial hexadecane remained after the same time period, relative to the abiotic control. The calculated rates correspond to conversion of 0.8 and 0.7 mM h^{-1} for tetradecane and hexadecane, respectively, at a flow rate of 5 mL min^{-1} and with a corresponding residence time of 1 min.

It was clear from SEM imaging that a high loading of bacterial cells was achieved on the adsorbent by physisorption alone, but the cells were visibly removed from the adsorbent by gentle washing. Silica-immobilized cells, by comparison, were not readily removed from the adsorbent material. As such, immobilization of bacterial cells was investigated as a mechanism to enhance biodegradation during continuous flow conditions.

At the onset of continuous flow experiments, the adsorbent mats were washed extensively with buffer. The presence of cells in the mobile phase was highest for physisorbed samples (OD$_{600}$ = 0.145 ± 0.011) compared with the silica-immobilized samples (OD$_{600}$ = 0.072 ± 0.013) and the abiotic control (OD$_{600}$ = 0.009 ± 0.002). Adsorbent with physisorbed cells and silica-immobilized cells continuously exposed to recycled flow of tetradecane/hexadecane provided activity consistent with the batch incubation experiments. Significantly, more alkane was removed by the silica-immobilized samples after continuous flow conditions than for physisorbed samples (Figure 6). Concentrations of hexadecane and tetradecane adsorbed to the mat were negligible
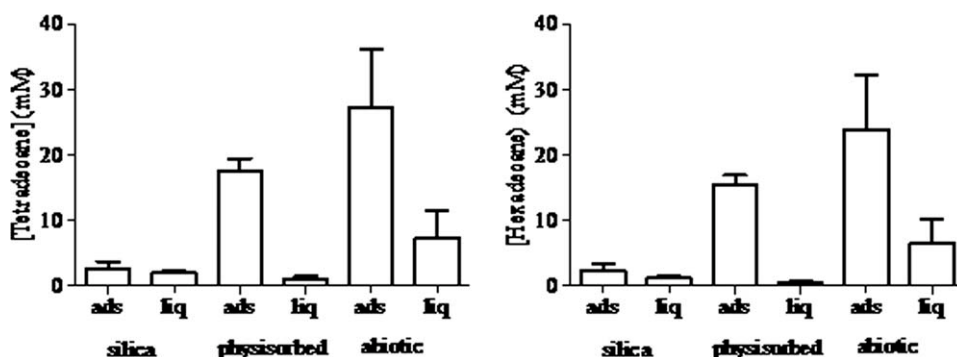
Figure 5. Degradation of tetradecane (left panel) and hexadecane (right panel) by *Acinetobacter venetianus* 2AW in batch mode when physisorbed or silica-immobilized to adsorbent mats vs. an abiotic control.

Residual alkane was extracted from the adsorbent mats (ads) and the liquid phase (liq) after 24 h incubation with alkane in batch mode.
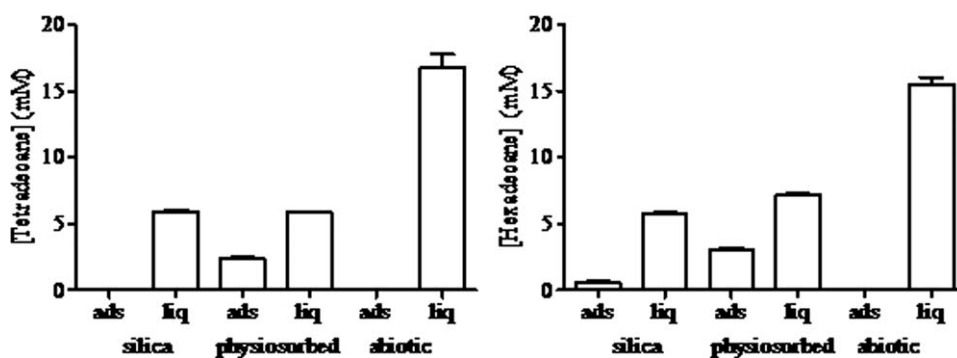

Figure 6. Degradation of tetradecane (left panel) and hexadecane (right panel) by *Acinetobacter venetianus* 2AW physisorbed or silica-immobilized to adsorbent mats vs. an abiotic control.

Residual alkane was extracted from the adsorbent mats (ads) and the liquid phase (liq) after incubation with alkane under continuous flow for 24 h.
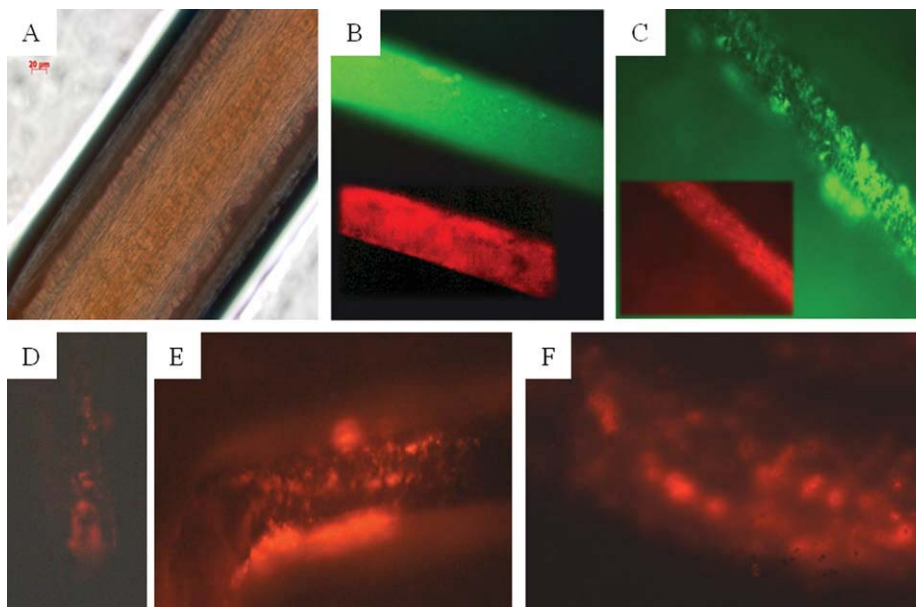

Figure 7. Imaging of hair fiber under phase contrast (A), LIVE/DEAD® staining of physisorbed (B) and silica-immobilized *Acinetobacter venetianus* 2AW (C), Nile red staining of physisorbed (D), and silica-immobilized *Acinetobacter venetianus* 2AW (E, F).*

*Abiotic controls show no fluorescence when incubated with Nile Red.

compared with physisorbed samples and is attributed to the ability of silica-immobilized cells to effectively reduce the concentration of alkane directly at the adsorbent surface. The alkanes principally remained in the liquid phase with the abi-

otic controls; little was retained on the adsorbant surface compared with samples with strain 2AW. The behavior may be attributed to the biosurfactant activity supplied by the *Acinetobacter* cells. In batch culture, physical agitation is

Table 1. Viable Cell Counts of *Acinetobacter venetianus* 2AW on Adsorbent Mats

	RLU	CFU mL^{-1}*
Physisorbed cells	15649 ± 5350	$7.35 \times 10^5 \pm 1.22 \times 10^5$
Silica-immobilized cells	36375 ± 4500	$1.42 \times 10^6 \pm 7.11 \times 10^4$
Abiotic control	361 ± 270	0

* Corresponding CFU mL^{-1} calculated from a standard curve of Relative luminescence units (RLU) vs. cell culture serial dilution.

sufficient to break up the hydrocarbon and eliminate this effect. In the control, the alkanes remain as a biphasic solvent rather than an emulsion that may be readily retained by the adsorbent surface.

Under continuous flow conditions, silica-immobilized cells demonstrated a significantly higher biocatalytic turnover rate than physisorbed cells, which indicates that there was higher retention of cells in the silica matrix. Nonetheless, the result suggests that silica treatment did not limit metabolic activity of the organisms. Initial quantification of adsorbed bacteria using protein measurements was confounded by the high concentration of protein in the hair fibers. Initial SEM visualization (Figure 4) confirmed that bacterial cells were associated with the hair fibers, but further attempts to elucidate cell viability by fluorescent staining of the bacterial cells *in situ* proved inconclusive due to the high background absorbance of fluorescent dye to the silica matrix and the hair fiber itself. Nile red staining of the silica-immobilized hair for biosurfactant activity helped identify areas of high lipid concentration. The dye was associated with cellular material attached at the hair surface and indicated the retention of biosurfactant activity, even with silica-immobilized cells (Figure 7). High resolution imaging of the adsorbent surface and selective staining of the bacteria using fluorescent *in situ* hybridization (FISH) may be considered in future work to better resolve specific bacterial colonization.[37] The cell viability for adsorbent samples was ultimately determined by quantification of ATP luminescence and revealed significantly higher retention of viable cells following silica-immobilization when compared with physisorbed cells (50% by comparison) or an abiotic control (0%) (Table 1).

Conclusions

Silica-immobilization of *A. venetianus* 2AW creates a stable, defined bacterial population that degrades long-chain alkanes without the significant colonization times typically associated with natural attenuation or open seeding processes. Immobilization of cells to an adsorbent matrix did not reduce cell viability and appeared to enhance the efficiency of hydrocarbon degradation. The improved breakdown may be attributed to the materials sequestering the hydrocarbon substrates near the bacteria, thus enhancing degradation; and second, the immobilization process prevents the cells from being washed away in flow-through experiments. Continuous degradation at the surface of the adsorbent enhances the utility of the material and increases the total capacity for adsorption, as some portion of the adsorbed material is continuously removed. The methodology described is rapid, reproducible, and encapsulates whole bacterial cells without hindering their physiology. The bacterial activity was demonstrated by biosurfactant production and active alkane degradation. Adsorbent mats including the immobilized bacterial cells provide a system for coupled adsorption/biodegradation in open-water oil spills or treatment of large contaminated volumes such as ballast water or holding ponds. The practical application of the mat is supported further by the adsorption of a broad range of the hydrocarbons found in crude or processed petroleum. Moreover, the natural materials making up the mat are freely available and pose no long-term environmental concerns.

Notation

BH = Bushnell-Haas
FISH = fluorescent *in situ* hybridization
MB = Marine broth
PBS = phosphate buffered saline
PCR = polymerase chain reaction
RLU = Relative luminescence unit
SEM = scanning electron microscopy
TMOS = Tetramethylorthosilicate

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