ENVIRONMENTAL BIOTECHNOLOGY

Organizers: James J. Valdes, Ananda M. Chakrabarty and Michael Bagdasarian

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Poster Sessions

Molecular Genetics and Protein Engineering of Microorganisms I

X2-001 CONSTRUCTION AND FUNCTIONAL ANALYSES OF HYBRID AROMATIC RING DIOXYGENASES, Kensuke Furukawa, Dept of Agric. Chem., Kyushu University, Hakozaki, Fukuoka 812, Japan

Aromatic ring dioxygenases involved in initial dioxygenation are ubiquitously distributed in soil bacteria. These dioxygenases usually possess relaxed substrate specificities, and often share structural and functional similarities with one another. *Pseudomonas pseudoalcaligenes* KF707 *bph* operon involved in biphenyl catabolism and *Pseudomonas putida* F1 *tod* operon involved in toluene catabolism are very similar in gene organization and structure of the corresponding enzyme, despite their discrete substrate ranges for metabolism. Construction of hybrid gene clusters between *bph* and *tod* operons revealed that a large subunit of terminal dioxygenase (encoded by *bphA1* and *todC1*, respectively), in multicomponent biphenyl dioxygenase and toluene dioxygenase, was critically responsible for the substrate specificity. Other components such as a small subunit of terminal dioxygenase (*bphA2/todC2*), ferredoxin (*bphA3/todB*) and ferredoxin reductase (*bphA4/todA*) could be exchangeable each other. *Escherichia coli* JM109 expressing a hybrid TodC1::BphA2A3A4 showed enhanced substrate ranges for benzene and biphenyl derivatives. Moreover, the same strain degraded, very efficiently, chloroethene such as trichloroethylene (TCE) and *cis*-1,2-dichloroethylene (DCE). On the other hand, *E. coli* JM109 expressing a hybrid BphA1::TodC2::BphA3A4 showed limited degradation capability for benzene and biphenyl derivatives, and no activity for chloroethene. The hybrid *todC1::bphA2A3A4* genes were integrated into chromosomal *bph* operon of *P. pseudoalcaligenes* KF707 by single crossover recombination. The resultant hybrid strain KF7109 grew well on benzene and toluene as well as biphenyl, and efficiently degraded TCE. The TCE degradation by KF7109 was inducibly enhanced when the cells were grown with biphenyl as a sole carbon source. The KF7109 was not capable of degrading tetrachlorothylene (PCE). *Pseudomonas* sp. strain Y51 isolated from soil dechlorinated PCE to DCE through TCE. The combination of anaerobic dechlorination of PCE to DCE by Y51 and aerobic degradation of DCE by KF7109 resulted in complete and efficient degradation of PCE at the concentration of as high as 0.4 mM. We constructed a hybrid dioxygenase between biphenyl dioxygenase (KF707) and naphthalene dioxygenase (encoded by NAH7 plasmid of *Pseudomonas putida* strain G7). *E. coli* JM109 expressing a hybrid dioxygenase NahAc::BphA2A3A4 showed naphthalene dioxygenase activity, but very low activity for biphenyl. JM109 expressing a hybrid dioxygenase composed of BphA1::NahAd::BphA3A4 showed no activity for biphenyl nor naphthalene. Hybrid dioxygenases were constructed between two biphenyl dioxygenases of *P. pseudoalcaligenes* KF707 and *Pseudomonas* sp. LB400. These enzymes showed different mode of oxygenation for polychlorinated biphenyls (PCBs). KF707 biphenyl dioxygenase introduced molecular oxygen at 2,3 position of non- or lesser chlorinated ring of biphenyl molecule (2,3-dioxygenation), so that 4,4'-dichlorobiphenyl was converted to the dihydrodiol, but 2,5,2',5'-tetrachlrobiphenyl was hardly degraded. On the other hand, LB400 biphenyl dioxygenase introduced molecular oxygen mainly at 3,4 position (3,4-dioxygenation) as well as at 2,3 position, thereby this strain degraded 2,5,2',5'-CB efficiently through 3,4-dioxygenation, but degraded poorly 4,4'-CB through 2,3-dioxygenation. We replaced *bphA1* (LB400) with *bphA1* (KF707) in KF707 *bph* gene cluster background. *E. coli* JM109 expressing a hybrid biphenyl dioxygenase BphA1(LB400)::BphA2A3A4 oxidized 2,5,2',5'-CB to the dihydrodiol, but oxidized hardly 4,4'-CB.

X2-002 CHECK POINTS IN PHENOL DEGRADATION AND ITS REGULATION, Victoria Shingler, Cell and Molecular Biology, Umeå University, S-901 87 Umeå, Sweden.

The applications of microbial metabolic activities to environmental detoxification and clean up purposes have stimulated interest in the genes and proteins that determine the substrate range and metabolic efficiency of individual isolates. *Pseudomonas* CF600 encodes the regulated catabolism of phenol and some of its methylated derivatives on a large megaplasmid called pVI150. The 15 structural *dmp*-genes are encoded in the *dmp*-operon, expression of which is regulated by the divergently transcribed *dmpR* regulatory gene. The first six genes of the operon are involved in the initial conversion of phenol to catechol by an unusual multi-component phenol hydroxylase, which has recently been shown to also operate in some other phenol degrading microorganisms. The remaining nine genes of the operon encode the *meta*-cleavage pathway enzymes that have counterparts in other bacteria that utilise *meta*-cleavage. One interesting aspect of the *meta*-cleavage pathway is the physical association of some of the enzymes involved. A model in which such associations may channel metabolites via the most favourable route and prevent build up of toxic intermediates will be discussed.

For a given substrate to serve as the sole source of carbon and energy two criteria must be fulfilled; firstly, the specificity of the enzymes must be such that the pathway is capable of dissimilating the substrate, and secondly, the substrate must be capable of eliciting expression of the catabolic enzymes. Hence, the compound must serve as both a substrate and a regulatory (effector) molecule. The DmpR transcriptional activator belongs to the NtrC family of regulatory proteins. The activity of DmpR is itself regulated by the presence of a distinct array of structurally related aromatic compounds. Genetically selected effector-specificity and constitutive mutants, in combination with hybrid and truncated DmpR proteins have been used to derive a model of how this regulator serves to sense its aromatic effectors in its environs. The magnitude of the transcriptional response is dependent on the nature and location of substituents on the aromatic ring. Evidence will be presented that the response of the regulator to *para*-substituted compounds is a major limiting factor in the catabolism of these compounds, and that expression of DmpR in another phenol degrading isolate expands the range of substrates that it can degrade.

X2-003 BIODEGRADATION OF HALOGENATED COMPOUNDS: PROTEIN AND METABOLIC ENGINEERING, Lawrence P. Wackett, Department of Biochemistry and Institute for Advanced Studies in Biological Process Technology, University of Minnesota, St. Paul, MN 55108.

Polyhalogenated organic compounds are a class that include many prominent pollutants. Typically, they are chemically stable and have been present in the environment briefly on an evolutionary time scale. Thus, they are environmentally persistent due to a failure of many bacteria to metabolize them. When they are metabolized, polyhalogenated compounds are generally transformed by multiple chemical mechanisms. The first dehalogenation step(s) is often a two electron into the carbon-halogen bond to yield a halide ion and a carbon-hydrogen bond. Oxygenative dehalogenation reactions occur more often with less halogenated compounds. There are a limited number of natural systems showing sequential reductive and oxygenative dehalogenation metabolism. In this context, enzyme systems with these potential reactivities have been recruited to engineer new metabolic pathways. The heme protein cytochrome $P450_{CAM}$ catalyzes fortuitous reductive dehalogenation with suitably-sized haloorganic substrates. Some substrates are tightly bound, for example hexachloroethane binds with a dissociation constant of 0.7 μM. Over one dozen halogenated compounds are reduced by cytochrome $P450_{CAM}$. Some of the products are substrates for toluene dioxygenase and these secondary reactions yield non-halogenated, non-toxic end-products. A *Pseudomonas putida* strain was engineered to maintain and express seven genes, *camABC* and *todABC$_1$C$_2$*, encoding cytochrome $P450_{CAM}$ and toluene dioxygenase. The recombinant strain metabolized pentachloroethane to glycoxylic acid and CO_2. The chlorofluorocarbons, 1,1,1,2-tetrachloro-2,2-difluoroethane and 1,1,1-trichloro-2,2,2-trifluoroethane, also can undergo sequential reductive and oxygenative dehalogenation reactions. In both cases, oxalic acid is the major end-product. 1,1,2,2-Tetrabromoethane was metabolized to a mixture of *cis* and *trans*-1,2-dibromoethene and these products were rapidly oxidized by toluene dioxygenase. Further studies are in progress to enhance the substrate specificity and catalytic efficiency of the engineered metabolic pathway.

Molecular Genetics and Protein Engineering of Microorganisms II

X2-004 CHARACTERIZATION OF XYLENE MONOOXYGENASE, BENZYL ALCOHOL DEHYDROGENASE AND BENZALDEHYDE DEHYDROGENASE ENCODED BY TOL PLASMID OF *PSEUDOMONAS PUTIDA*, Shigeaki Harayama[1,2], Jun Inoue[1], Jeffrey P. Shaw[2] and Monique Rekik[2], [1]Marine Biotechnology Institute, Kamaishi Laboratories, Kamaishi, Japan, [2]Department of Medical Biochemistry, University of Geneva, Geneva, Switzerland.

The TOL plasmid from a soil bacterium *Pseudomonas putida* encodes a metabolic pathway for the degradation of toluene, xylenes and their alcohol and carboxylate derivatives. The genes for the enzymes of this pathway are clustered in two operons on TOL plasmid pWW0. The upper operon encodes the first three enzymes of this pathway, namely xylene monooxygenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase which together transform the initial substrates (toluene or xylenes) into benzoate or toluates. Xylene monooxygenase consists of two polypeptide subunits encoded by *xylM* and *xylA*, and is responsible for the initial oxidation of the methyl side-chain of the substrates. The XylM protein was membrane-bound, and requires phospholipids and ferrous iron for its activity. XylA was an electron transport protein which transfers reducing equivalents of NADH to the XylM protein. Benzyl alcohol dehydrogenase encoded by *xylB* is a member of the zinc-containing alcohol dehydrogenase family. The mechanisms which determine the substrate specificity of this enzyme was investigated by computer modeling and site-directed mutagenesis. Benzaldehyde dehydrogenase encoded by *xylC* has a structure very similar to 2-hydroxymuconic semialdehyde dehydrogenase encoded by *xylG* on the TOL plasmid. The substrate specificities of these two aldehyde dehydrogenases overlapped.

X2-005 MONITORING ENVIRONMENTAL STRESS THROUGH INDUCTION OF *lux* GENE FUSIONS, Robert A. LaRossa[1], Tina K. Van Dyk[1], Dana R. Smulski[1], Timothy R. Reed[1], Amy C. Vollmer[2] and Shimshon S. Belkin[3], [1]DuPont Company, Wilmington, DE, [2]Swarthmore College, Swarthmore, PA and [3]Ben Gurion University, Sede-Boqer, Israel.

Global regulatory mechanisms have evolved to monitor transient excursions from optimal conditions and to allow bacteria to survive in hostile environments. Bacteria thus sense extreme conditions and respond accordingly. One such response is the induction of stimulons, discrete sets of genes whose expression is elevated by a particular stress. Stimulon triggers may be relatively general (e.g. DNA damage) or specific (e.g. DNA alkylation). Fifteen *Escherichia coli* promoter elements representing twelve distinct regulatory circuits were fused to the *Vibrio fischeri lux* structural genes on a broad host range plasmid. Promoter fusions representing the heat shock, SOS, adaptive response, peroxide-stimulated, superoxide-stimulated and fatty acid biosynthetic regulons were constructed to monitor cellular damage. Promoters induced by growth limitation, entry into stationary phase or starvation for carbon, nitrogen, amino acids or phosphate provided tools to monitor the physiological state of the cell. *E. coli* cells carrying these fusions responded appropriately to stimuli with known modes of action as determined by measuring increases in light emission as a function of time after toxicant application. These responses were under the control of the expected global regulatory proteins. This panel of fifteen fusion-containing strains may be a useful tool for monitoring environmental cleanup efforts, determining chemical modes of action and controlling fermentations. This panel has been applied in monitoring the efficiency of several treatment plants for the purification of industrial waste waters.

X2-006 RATIONAL ENZYME REDESIGN FOR IMPROVING BIODEHALOGENATION, Rick L. Ornstein, Environmental Molecular Sciences Laboratory, Pacific Northwest Laboratory*, Richland, Washington 99352

Adequate structural, mechanistic, and genetic information is currently available for only a small number of important biodehalogenating enzymes to warrant rational redesign for alternative specificity and improved efficiency. Two classes of enzymes meet the prerequisite requirements necessary for rational enzyme redesign: cytochromes P450 and haloalkane dehalogenase. Computational molecular dynamics simulations, starting with a high-resolution experimental three-dimensional enzyme structure, can play a valuable if not unique role in uncovering inherent and essential structure-function-dynamic relations. The best approach for understanding such relations is an iterative, self-consistent, coupled experiment-theory protocol whereby unexpected simulated conformational states lead to experimentally testable predictions. Recent results will be described for cytochromes P450 and haloalkane dehalogenase.

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Marine Pollution and Biotechnology

X2-007 THE USE OF MICROBIAL METHODS TO TREAT MINING WASTES CONTAINING CYANIDE, Peggy J. Arps[1,2] and Michael G. Nelson[1], [1]School of Mineral Engineering, University of Alaska Fairbanks, Fairbanks, AK 99775-5800, [2]Applied Microbiology and Biotechnology, Inc., Glenwood Springs, CO 81602-1604.

Manufacturing and mining wastes containing cyanide are a major problem for economically developed and developing countries. Although the volume of cyanide waste is comparatively small, its toxicity to living organisms is greater than that of most other industrial wastes. The biological treatment of industrial cyanide wastes was first considered about 40 years ago and implemented several years later. Until then, all cyanide waste treatment facilities utilized chemical methods, the most prevalent being alkaline chlorination. The use of cyanide by the mining industry to recover gold from its ores has been steadily increasing as the number of low-grade gold deposits under production has grown worldwide. As with other industries employing cyanide, chemical methods were chosen to treat the cyanide-containing wastes produced at the mines. Typically sodium hypochlorite, hydrogen peroxide, or sulfur dioxide/air/copper were used to oxidize the free and complexed cyanides present on site. Biological treatment systems for cyanide mining wastes have been a more recent development. To date, several systems have been tested and built at a handful of mining sites throughout the western United States. The microbial strains and bioremediation strategies selected for each of these operations will be described in detail. Additional cyanide degrading bacteria have been isolated and studied by various laboratories, and these will also be reviewed. Our laboratory, in collaboration with the U.S. Bureau of Mines Salt Lake City Research Center, has been investigating a bacterial isolate, designated UA7 (identified as *Pseudomonas pseudoalcaligenes*), that is capable of degrading cyanide. UA7 demonstrated clear cyanide-lowering capabilities, even at cyanide concentrations much higher than those normally used in mining. Samples containing cyanide up to 900 ppm incubated overnight with UA7 showed no cyanide remaining. Attempts to isolate indigenous cyanide degrading bacteria from the nearby Ryan Lode Mine were unsuccessful, so UA7 was tested and found capable of degrading cyanide present in effluent water from the leach heaps at the mine. *Pseudomonas pseudoalcaligenes* is currently being used in field tests involving direct inoculation of a small cyanide leach heap at the Ryan Lode Mine to determine whether the bacteria can overwinter and retain their ability to degrade cyanide the following spring. Preliminary results from that study will be presented.

X2-008 INTEGRATED WASTE MANAGEMENT: BIOTECHNOLOGY APPLICATIONS. Murray Moo-Young, Yusuf Chisti and Bill Anderson, Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada, N2L 3G1

Biotechnology has been proposed as a "natural" way of treating waste residues because of its benign process requirements and low-impact ecological effects. However, this technology often relies on its integration to other forms of technology to be cost effective in large-scale waste management systems. We consider two illustrative case studies: bioconversion of agricultural crop residues, which are potential environmental pollutants, into proteinaceous animal feed SCP products; and bioremediation of petrochemical-contaminated clayey soils. In the first case, physicochemical pretreatment of the recalcitrant lignocellulosic materials is crucial to the techno-economics. In the second case, photocatalytic or chemical pretreatment of the xenobiotics seem to be essential to the overall biodegradation process. In both cases, bioreactor design is over-ridingly important (1,2,3). As with other bulk waste-treatment scenarios, the relevant processing facilities could be challenged by the "not-in-my-backyard" syndrome of many communities.

1. Moo-Young, Chisti, Vlach. "Fermentation of Cellulosic Materials to Mycoprotein Foods", Biotech. Adv. **11**, 469-479 (1993).
2. Moo-Young, Chisti. "Bioreactor Applications in Waste Treatment", Res. Con. Recycle, **11**, 13-24 (1994).
3. Allsop, Chisti, Sullivan, Moo-Young. "Dynamics of Phenol Degradation by *P. putida*", Biotech. Bioeng. **41**, 572-580 (1993).

Biochemistry and Physiology of Biodegradation

X2-009 MULTIPLE REACTIONS CATALYZED BY MULTICOMPONENT AROMATIC HYDROCARBON DIOXYGENASES, David T. Gibson, Daniel S. Torok, Kyoung Lee, Sol M. Resnick, and John D. Haddock, The Department of Microbiology and Center for Biocatalysis and Bioprocessing, The University of Iowa, Iowa City, IA 52242.

Pseudomonas putida F1, *Pseudomonas putida* NCIB 9816, and *Pseudomonas* species strain LB400 can grow with toluene, naphthalene, and biphenyl, respectively, as the sole source of carbon and energy. Each strain catalyzes the enantiospecific incorporation (>98% e.e.) of both atoms of molecular oxygen into the aromatic nucleus of their respective substrates to form *cis*-dihydrodiols in which the adjacent hydroxyl groups have a *cis*-relative stereochemistry and an *R* absolute stereochemistry for the hydroxyl group adjacent to the methyl and phenyl substituents of toluene and biphenyl dihydrodiols and for the hydroxyl group adjacent to the bridgehead carbon in naphthalene dihydrodiol. Thus the three *cis*-dihydrodiol enantiomers are structurally similar even though application of the Cahn-Ingold-Prelog priority rules gives the impression that this is not the case. The enzymes catalyzing the *cis*-hydroxylation of toluene, naphthalene, and biphenyl each contain three proteins. These multicomponent enzyme systems have been designated toluene 2,3-, naphthalene 1,2-, and biphenyl 2,3-dioxygenases, respectively. Studies with mutant strains, recombinant strains that express the individual dioxygenases, and purified enzymes have shown that in contrast to their enantioselectivity in forming *cis*-dihydrodiols from aromatic substrates, these enzymes are capable of oxidizing a wide range of chemicals, many of which bear little structural relationship to the aromatic hydrocarbon growth substrates. For example, toluene dioxygenase oxidizes trichlorethylene, indole, indan, indene, and dihydronaphthalene. In addition, each enzyme can catalyze different reactions depending on the substrate oxidized. Types of reactions observed to date include dioxygenation, monooxygenation, desaturation/dehydrogenation, dechlorination, *N*- and *O*-dealkylation, and sulfoxidation. Cytochrome P-450, methane monooxygenase and 4-methoxybenzoate demethylase also catalyze diverse reactions with different substrates. These enzymes have been studied in detail and reactive iron-oxygen species have been identified that can account for all of the reactions observed. Recent studies suggest that the *Nitrosomonas europaea* ammonia monooxygenase may also be similar to methane monooxygenase in terms of the range of reactions observed. Although toluene, naphthalene, and biphenyl dioxygenases catalyze similar reactions to those catalyzed by cytochrome P-450, and the monooxygenases described above, they appear to be unique in their ability to catalyze the enantiospecific incorporation of dioxygen into the aromatic nucleus. This difference in catalytic specificity probably reflects the nature of the binding sites for aromatic hydrocarbon substrates and the type of reactive oxygen species involved.

X2-010 SULFATE STARVATION-INDUCED REGULATION OF GENE EXPRESSION IN GRAM-NEGATIVE BACTERIA, Thomas Leisinger, Michael Kertesz, Alasdair Cook, Stefan Beil, Jürgen Ragaller, Martina Weiss, and Jan van der Ploeg, Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland.

Bacteria growing in soil environments obtain their sulfur for growth almost entirely in the form of organically bound sulfur, and not as inorganic sulfate. We have recently observed that under sulfate starvation conditions bacteria produce a set of proteins whose synthesis is repressed by sulfate or cysteine (1). The identity of these sulfate-repressed proteins is under investigation in *Pseudomonas aeruginosa* PAO and in *E. coli*. The methods used include determination of the N-termini of proteins purified by 2D-polyacrylamide electrophoresis, enzyme activity measurements, and - in *E. coli* - screening of translational *lacZ*-fusions in the chromosome for sulfate starvation-regulated expression of β-galactosidase fusion proteins.

In *P. aeruginosa* 12 proteins are upregulated under conditions of sulfate-starvation, but none of these could be correlated to a number of enzyme acitivities involved in the assimilation of non-sulfate sulfur sources. Arylsulfatase, however, proved to be coregulated with the sulfate starvation-induced proteins and thus was used as a marker enzyme. We have purified and characterized this enzyme from strain PAO, and have cloned and sequenced its structural gene *atsA*. The AtsA protein was 38% identical to the arylsulfatase from *Klebsiella aerogenes*. In contrast to other authors, we have found no evidence for multiple forms of the enzyme or the *atsA* gene in *P. aeruginosa*. Transposon insertion mutants of *P. aeruginosa* were generated which synthesize arylsulfatase at high levels in the presence of sulfate, and these are being used to explore sulfate regulation of *atsA* expression.

Seven sulfate starvation-induced proteins from *E. coli* were purified by preparative 2D-polyacrylamide gel electrophoresis, and their N-terminal sequences were determined. Five of the sequences obtained were novel, and two were identified by comparison with computer databases as sulfate-binding protein and cysteine synthase. The latter was also present after growth with sulfate, but was slightly upregulated under sulfate-limitation. Three sulfate-regulated translational *lacZ*-fusions in the *E. coli* chromosome were also analyzed. Sequencing of the DNA-regions upstream of the insertion sites revealed two sequences whose function is unknown as well as *dmsA*, the gene encoding the catalytic subunit of dimethyl sulphoxide reductase. Together with the largely novel N-terminal sequences found for the sulfate starvation-regulated proteins excised from 2D-gels, it appears that we have discovered a group of genes which is not expressed under routine laboratory conditions, and whose members are therefore as yet quite uncharacterized.

(1) Kertesz, M., T.Leisinger, and A.M.Cook (1993). J.Bacteriol. 175: 1187 - 1190.

X2-011 GENETICS OF SULFATE-REDUCING BACTERIA FOR IMPROVEMENT IN BIOREMEDIATION, Judy D. Wall[1], Barbara J. Rapp-Giles[1], R. Samuel English[1], John Argyle[2], Tiffany Murnan[1], and Tara Wickman[1], [1]University of Missouri-Columbia, Columbia, and [2]University of Washington, Seattle.

Biotechnological intervention in the environment aims at allowing a biodegradative or bioremedial process to occur, to improve its rate, or to change the nature of the end products. Intervention is being approached at the level of engineering bacteria for special metabolic capabilities or altering the environment so that naturally occurring microbes with special capabilities have an opportunity to flourish. Information from basic genetic studies increases the likelihood that the intervention will be successful. Degradation in anaerobic environments is often rate limiting in the mineralization or removal of noxious materials from the environment. Coincidently, our understanding of anaerobic metabolism and genetics is still far behind that of the aerobic environment. Therefore we are pursuing a genetic approach to metabolic regulation in the strictly anaerobic sulfate-reducing bacteria.

The importance of the sulfate-reducing bacteria in corrosion, biodegradation, bioremediation and biogeochemical conversions is only presently being established. We have begun a basic genetic analysis of a *Desulfovibrio desulfuricans* strain G100A to look at the metabolism of hydrogen, a substrate important not only for SRB growth but also for bacterial community development. Among the tools that have been successful with *D. desulfuricans* G100A are genetic exchange by conjugation with IncQ plasmids, the construction of useful shuttle vectors with an endogenous replicon, insertion of a Tn7-based single copy delivery system for studying regulation of genes, a random mutagenesis tool, and pulsed field electrophoresis of chromosomal DNA for the establishment of a preliminary physical map. With these tools, we have initiated studies on the *cycA* gene encoding the cytochrome c_3. Results of the genetic and physiological experiments will be discussed.

Bioprocessing of Industrial and Agricultural Waste I

X2-012 ADVANCES IN THE BIODEGRADATION OF CHEMICAL WARFARE AGENTS AND RELATED MATERIALS, Joseph J. DeFrank, Tu-chen Cheng, Jan E. Kolakowski, and Steven P. Harvey, U.S. Army Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD 21010-5423.

The use of biochemical/biological processes offers considerable potential for the destruction of chemical warfare (CW) agents. For the two major areas of interest, battlefield decontamination and destruction of stockpiles, different processes are required. Because of the extremely toxic nature of the CW agents, rapid detoxification is required if personnel or equipment become contaminated. The use of catalytic enzymes could provide the military with decontamination systems that are not only effective, but also non-toxic, non-corrosive and environmentally safe, unlike some of the materials currently used for this purpose. A number of enzymes that detoxify organophosphorus nerve agents (Soman, Sarin, Tabun) were identified and purified, and are the subject of considerable study. More recently, an enzyme was identified that will catalytically degrade the organophosphothioate agent VX. Protein engineering studies are under way to improve the activity of this enzyme against VX. For dissemination of the decontaminant, a number of systems are being examined (liquid spray, foam) and preliminary results have shown some promise. In accordance with an agreement between the United States and the former Soviet Union, both nations have agreed to destroy their stockpiles of CW agents. While incineration was the original method of choice by the U.S. to destroy its 25,000 tons of agent, public and political opposition have resulted in the evaluation of alternative technologies. One of the technologies selected for evaluation is chemical neutralization (for VX and mustard) followed by biodegradation of the products. Both VX and mustard were shown to be amenable to rapid hydrolysis at temperatures below 100°C. Microbial systems are currently under development that will mineralize the agent hydrolysis products in a process similar to that used in treatment of municipal or industrial waste streams. The end products of this process will be biomass, carbon dioxide, salts and water. This technology should provide a less expensive, safer, and more publicly acceptable means of destroying the CW stocks of both the U.S. and Russia.

X2-013 MOLECULAR MONITORING OF XENOBIOTIC-DEGRADING MICROORGANISMS, AND THEIR ACTIVITY DURING BIOREMEDIATION OF CONTAMINATED SOIL, Charles W. Greer[1], Judith Godbout[1], Yves Comeau[2], Luke Masson[1], and Roland Brousseau[1], [1]Biotechnology Research Institute, National Research Council of Canada, Montreal, Canada, H4P 2R2, [2]École Polytechnique, Montreal, Canada, H3C 3A7.

The feasibility of using bioremediation as a clean-up technology, requires a thorough characterization of the chemical, physical and biological properties of the contaminated environment. Molecular tools and techniques such as gene probes, reporter genes and PCR techniques, are being applied to characterize the indigenous microorganisms in contaminated materials, and to assess the fate of bacteria introduced into these environments.

Catabolic gene probes, derived from the coding regions of genes from known pollutant degradation pathways, are being used to identify and quantify pollutant-degrading microorganisms, and to isolate and characterize them. The probes have been used to assess indigenous bacterial populations in soil, bedrock and groundwater contaminated with gasoline and diesel fuels to determine the potential for biotreatment. Organisms that are positive for the *xylE* gene appear to be very widespread, and are routinely detected in contaminated and non-contaminated soils. Monitoring of petroleum hydrocarbon-degrading bacteria, using *xylE* and *alkB* gene probes, during the course of on-site biotreatment operations, revealed that their levels were temperature dependent, and probably reflected substrate bioavailability. Microbial activity was assessed during the monitoring program using electrolytic respirometry and mineralization of a ^{14}C-labeled representative contaminant. The mineralization test provides an estimate of the instantaneous biodegradation activity in the soil, since bioavailability of the substrate is not a restriction.

A reporter gene marking system, that integrates a *lacZY-luxAB* construct into the chromosome of a target organism, was constructed to facilitate the monitoring of bacteria introduced into different soil environments. A 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading *Pseudomonas* sp. and a biosurfactant-producing *Pseudomonas* isolated from petroleum contaminated soil, have been marked with the reporter system. Marked strains have been detected at levels lower than 10 colony forming units per gram of soil, and because of the nature of the construct, DNA can be extracted directly from the soil and analyzed by PCR. Results show that the marked 2,4-D-degrading bacterium behaved identically to the parent strain in liquid culture and in soil. Physical and chemical properties of the soil (pH, temperature, moisture content, soil composition, etc...) influenced the survival and activity of the introduced strains, whereas freezing and thawing of the soil had little impact on strain survival. The ability to isolate and mark indigenous bacteria from contaminated sites can provide valuable data on their in situ behavior following re-introduction.

Bioprocessing of Industrial and Agricultural Waste II

X2-014 ADVANCES IN BIOREMEDIATION OF SOIL AND GROUNDWATER AT U. S. DEPARTMENT OF ENERGY SITES, Terry C. Hazen[1], [1]Savannah River Technology Center, Westinghouse Savannah River Company, Aiken, SC.

Bioremediation has proven to be one of the most cost effective and environmentally sound remediation technologies available at sites where it will work. The petroleum industry has been using bioremediation to handle oil sludges for more than 40 years (petroleum land-farming), and a patent was issued for in situ bioremediation of gasoline spills in 1974. Even so, this technology is perceived as being "new". Recent media reports of the use of bioremediation at the Valdez spill and Gulf of Mexico blowout have further sensationalized it's use. Since relatively few controlled, full scale demonstrations of bioremediation in situ have ever been done and in theory, in situ bioremediation looks extremely attractive, a lot of remediation companies are currently selling magic bugs and magic nutrient mixes to unwary clients and government agencies. In an effort to critically evaluate the effectiveness of remediation technologies and foster their development the US Department of Energy through the Office of Technology Development started the Integrated Demonstration Program. The Savannah River Site has just completed an Integrated Demonstration on "Clean-up of Soils and Groundwater Contaminated with Chlorinated VOCs." This full-scale in situ bioremediation demonstration was one of the largest and most comprehensive bioremediation demonstrations ever done. More than 20 laboratories, several companies and several government agencies were involved in the planning, execution and evaluation of this demonstration. The demonstration showed how gaseous nutrients (methane, nitrous oxide and triethyl-phosphate) could be injected into a aquifer via a horizontal well to stimulate indigenous bacteria (methanotrophs) to degrade trichloroethylene and other microbes to reduce tetrachloroethylene to trichloroethylene. The 14 month demonstration showed how nucleic acid probes, fluorescent antibodies, and phospholipid fatty acid analyses could be used to directly characterize and monitor bioremediation in the sediment and groundwater. A number other assays were cross compared with varying degrees of success. The direct functional group assays were extremely effective at showing quickly, who was present, how important they were to the remediation and how happy they were. Evaluations and modeling by several laboratories showed that this aerobic methane stimulation in situ bioremediation process was at least 40% more effective than any physical stripping process (also tested at this site), and 5 times more effective than any pump and treat process. The process removed 78% of all of the TCE and PCE present during the demonstration, with initial concentrations higher than 1000 ppb and final concentrations in the most effected areas reaching less than 2 ppb. The demonstration modeling showed that the cleanup at this site would be complete in less than 4 years, while in situ air stripping (physical process) would require more than 10 years and conventional pump-and-treat technologies would require more than 20 years. The demonstration showed that in situ bioremediation could reach a cleaner endpoint faster than any technology currently available. Since methane monooxygenase the enzyme responsible for degrading the contaminants can degrade more than 250 different compounds it is expected that this technology is very broadly applicable to waste sites throughout the US. Cost analyses showed that only 1500 lbs of contaminant needs to be biodegraded to recover all costs of natural gas, nutrients and additional equipment used for this demonstration. The data generated by this demonstration has allowed more than 10 companies to immediately license and apply it at sites in several states.

X2-015 BIOLOGICAL TREATMENT SYSTEMS FOR REMEDIATION AND POLLUTION CONTROL, Ronald Unterman, Envirogen, Inc., 4100 Quakerbridge Road, Lawrenceville, New Jersey.

The release of organic compounds into our environment poses one of the most significant problems confronting our society today. These problems include both historical contamination that requires remediation, as well as the current production and release of industrial chemicals. These compounds include solvents, pesticides, wood preservatives, fire retardent fluids, pulp and paper wastes, and industrial byproducts. This seminar will present the status of current studies into the development of new and more effective biocatalysts with emphasis on their application in pilot and commercial biotreatment systems for soil, water, and air emissions. Specific examples will include the bioremediation of PCB and TNT contaminated soils and sediments, and TCE, nitrobenzene and aniline contaminated ground water, as well as biotreatment systems for chlorinated solvents in industrial waste water and VOCs in industrial air effluents. To date, field system development has focused on the use of superior, naturally occurring strains under optimized process conditions, however, future applications will include the use of genetically engineered microorganisms in bioreactors and eventually in ground water and field applications.

X2-016 ANAEROBIC BIOPROCESSING OF ORGANIC WASTES, W. Verstraete and D. de Beer, Center Environmental Sanitation, University of Gent, Coupure L 653, B-9000 Gent, Belgium.

Summary: For treating wastewaters, a novel and highly performing new system has been introduced in the last decade i.e. the upflow anaerobic sludge blanket system (UASB). This reactor concept requires the anaerobic consortium to grow in a dense and eco-physiologically well organized way. The microbial principles of such granular sludge growth are presented. Using a thermodynamic approach, the formation of different types of aggregates is explained. The application of this bioprocess in worldwide wastewater treatment is indicated. Due to the long retention times of the active biomass the UASB is also suitable for the development of the complex bacterial consortia capable to degrade xenobiotics in wastewater. To treat organic suspensions, there is currently a tendency to evolve from the conventional mesophilic continuously stirred tank system to the thermophilic configuration. This permits higher conversion rates and better hygienization levels. New approaches in order to combine methane production with nutrient removal are discussed. Integration of ultrafiltration in the anaerobic digestion process results in high quality liquid effluent. Finally, with respect to organic solids, the recent trend in society towards source separated collection of biowaste has opened a broad range of new application areas for solid state anaerobic fermentation. Several full-scale systems are currently in operation in Europe. This technology opens new perspectives for recycling various fractions of domestic wastes and furthermore has important implications for the producers of consumer goods.

Engineered Bioremediation Systems

X2-017 PENETRATION OF POROUS MEDIA BY BACTERIA IN VARIOUS NUTRITIONAL STATES, J. W. Costerton[1], A. B. Cunningham[1], Bryan Warwood[1], Paul Sturman[1], and Randy Hiebert[2], [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, [2]MSE Incorporated, Butte, MT 59702.

The injection of vegetative bacteria into porous media results in a very shallow plug because of the pronounced tendency of these organisms to adhere to available surfaces. Even coarse sand (2.5 darcies) is rapidly plugged by vegetative bacteria. However, nutrient deprived bacteria penetrate porous media much more readily, and starved ultramicrobacteria penetrate hundreds of meters through sand and soil greater than 110 millidarcies in penetrability. It is clear that starved bacteria constitute the vehicle of choice in the delivery of allochthonous bacteria for *in situ* bioremediation.

X2-018 IN-SITU BIOREMEDIATION OF CHLORINATED-SOLVENTS, Perry L. McCarty, Western Region Hazardous Substance Research Center, Department of Civil Engineering, Stanford University, Stanford, California 94305-4020

Studies over the past few years have demonstrated that both aerobic and anaerobic microorganisms can degrade chlorinated aliphatic hydrocarbons (CAHs), largely through cometabolism. Evidence is presented for natural anaerobic transformation of trichloroethene (TCE) contamination with concentrations > 50 mg/l at the St. Joseph, Michigan, Superfund site to cis-1,2-dichloroethene (cDCE), vinyl chloride (VC), and ethene. Flux estimates indicated 60 kg TCE/year was moving off-site from the spill location, and that 20 percent of the CAHs were being converted to ethene in the anaerobic zone. The driving force for the dehalogenation occurring appears to be unidentified organic material discharged previously to a lagoon, and seepage into the groundwater. Efforts are underway to enhance the anaerobic process in order to achieve more complete transformation to ethene.

Since anaerobic transformation of tetrachloroethylene (PCE) and TCE often lead to incomplete removal of hazardous organics, field and laboratory evaluations of aerobic transformations of TCE and its anaerobic metabolites have been conducted. Our earlier field studies at the Moffett Federal Airfield, Mountain View, California, with injection of 4 mg/l methane indicated that methane-oxidizing microorganisms could effectively degraded VC (95%) and tDCE (90%), but degradation of cDCE (50%) and TCE (20%) were not so complete. However, our more recent field studies have demonstrated that aerobic microorganisms grown on phenol (12.5 mg/l) can efficiently cometabolize TCE (90%), cDCE (95%), and VC (>95%). Transformation of tDCE is less efficient (50%). Percentage removal of TCE remained constant (88%) with TCE concentrations ranging between 62 and 500 μg/l, but decreased to 70% at 1000 μg/l. However, when 65 μg/l 1,1-DCE was added together with TCE, transformation product toxicity proved significant, reducing TCE degradation to less than 50% and only 50% destruction of 1,1-DCE was obtained. When 9 mg/l toluene was suddenly added as the primary substrate to replace phenol, excellent toluene removal was immediate and there was no adverse impact on the excellent TCE removal being obtained. Production of o-cresol following toluene introduction indicated that toluene ortho monooxygenase was the enzyme effective in TCE transformation. Injected phenol and toluene were destroyed >99.995% biologically to below the detection limit (1 μg/l) within 2 m of injection, representing a travel time of 18 hr. Use of 97 mg/L hydrogen peroxide in place of 33 mg/L pure oxygen produced equally effective treatment results and reduced clogging potential near the injection well.

These field results suggest that both aerobic and anaerobic biological processes can be used to effectively degrade CAHs. Initial use of anaerobic degradation to convert CAHs to less halogenated forms for final polishing by aerobic processes may be optimal for many situations.

X2-019 **DESIGNING BACTERIA TO DEGRADE TRICHLOROETHYLENE USING SOLUBLE METHANE MONOOXYGENASE FROM *Methylosinus trichosporium* OB3b,** Deokjin Jahng, Adam K. Sun, Craig S. Kim and Thomas K. Wood, Department of Chemical and Biochemical Engineering, University of California, Irvine, Irvine, CA 92717-2575

Soluble methane monooxygenase (sMMO) from *Methylosinus trichosporium* OB3b can degrade many halogenated-aliphatic compounds that are found in contaminated soil and groundwater. This enzyme oxidizes the most-frequently-detected pollutant, trichloroethylene (TCE), at least 50 times faster than other enzymes. However, slow growth of the strain, strong competition between TCE and methane for sMMO, and repression of the *smmo* locus by low concentrations of copper ion limit use of this natural bacterium. To overcome these obstacles, the 5.5 kb *smmo* locus of *M. trichosporium* OB3b was cloned into a wide-host-range vector (to form pSMMO20), and this plasmid was electroporated into *Pseudomonas* strains. The best TCE degradation results were obtained with *P. putida* F1/pSMMO20. The plasmid was maintained stably, and the sMMO proteins were observed clearly using SDS-PAGE and Western immunoblotting. The recombinant cell mineralizes both TCE and chloroform (as indicated by measuring chloride ion concentrations with a chloride-ion-specific electrode), degrades TCE with a V_{max} of 5 nmol/(min mg protein), grows much faster than *M. trichosporium* OB3b, and degrades TCE without competitive inhibition. However, significant problems were encountered with sMMO activity in the initial constructs in that sMMO expression in *P. putida* F1/pSMMO20 was inconsistent: 20% of the cultures degraded 35% of 20 μM TCE in 5 hrs compared to 80% of the cultures which degraded only 15% of the TCE. Furthermore, although pSMMO20 was completely stable in 50-mL shake-flask studies, segregational instability was encountered in 20-L fermentations. To address these problems, the recombinant sMMO component responsible for the inconsistent degradation has been identified, and pSMMO20 has been stabilized by inserting the *hok/sok* killer locus which kills plasmid-free cells as they are formed. In addition, new hosts which are phylogenetically more closely-related to *M. trichosporium* OB3b (e.g. *Rhizobium, Agrobacterium,* and methanotrophs) have been evaluated for enhanced sMMO expression. The effect of copper on sMMO activity has also been investigated.

Policy Issues

X2-020 GENETIC CONSTRUCTS FOR HAZARDOUS MATERIALS. C. M. Mello[1], P. Szafranski[2], T. Sano[2], C. L. Smith[2], C. R. Cantor[2], and D. L. Kaplan[1], [1] US Army Natick RD & E Center, Biotechnology Division, Natick, MA 01760, [2] Boston University, Center for Advanced Biotechnology, Boston MA 02215.

The release of genetically engineered microorganisms (GEMs) into the environment has sparked debate due to the potential destruction of the current ecosystem and uncontrolled transfer of foreign plasmids to other organisms. To address these concerns and potentially permit the use of GEMs for *in situ* bioremediation, the development of a genetically engineered self destructive microorganism has been investigated (1,2). We will present the design, construction and evaluation of a new suicidal system for the containment of GEMs. This system is based on (i) the streptavidin gene, encoding cell-killing function due to sequestration of biotin, (ii) regulatory elements which assure tight control of the lethal expression and (iii) the catabolic activity of the TOL plasmid from *Pseudomonas putida*. This suicidal element has been evaluated in both *Escherichia coli* and *P. putida*. The genetic stability of these constructs and the regulatory coupling of this suicide component to the metabolic activity of the TOL plasmid are currently under investigation.

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Environmental Biotechnology

X2-100 HALOPEROXIDASES FROM LAMINARIA OF NORTH ATLANTIC M.Almeida[1], M.Humanes[1], R.Melo[1], J.A.Silva[2], J.J.R. Fraústo da Silva[2]. 1- Departamento de Química, Faculdade de Ciências de Lisboa, Rua Ernesto Vasconcelos, Campo Grande, Edifício C1, 1700 Lisboa, Portugal. 2- Centro de Química Estrutural, Instituto Superior Técnico, Av. Rovisco Pais 1,1096 Lisboa Codex, Portugal.

Marine organisms, specially seaweed, release to the sea water large quantities of halometabolites which are probably related to defense mechanisms(1). In some cases volatile halogenated compounds (for instance bromoform) can be released to the atmosphere(2).

These compounds are thought to result from the metabolism of a enzyme named haloperoxidase. This enzyme catalyses the oxidation of halogens ions with simultaneous halogenation of organic substrates.

The presence of vanadium in the prosthetic group of some of these enzymes, for instance in the brown seaweed *Ascophyllum nodosum* was ascertain by Vilter(3) and, since then, vanadium haloperoxidases enzymes were reported for several alga, for a terrestrial fungi(4) and for one lichen(5).

These enzymes are of potential technological interest.

The portuguese coast presents a wide variety of brown alga and we began a comparative study on the vanadium haloperoxidase activity from the brown alga belonging to the family of Laminaria.

Different types of activity were detected for these seaweed - iodo- and bromoperoxidase, sometimes in the same algae; also it was detected a dependency of the activity values on the local of recollection.

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X2-101 BIODEGRADATIVE TREATMENT OF NIXTAMALIZATION WASTEWATERS (NEJAYOTE), USING INMOBILIZED CELLS ANOXIC REACTORS. Mario M. Alvarez and J. Fernando Ramírez. Centro de Biotecnología. Instituto Tecnológico y de Estudios Superiores de Monterrey. Sucursal de Correos "J'. Monterrey, N.L. 64849, México.

The performance of continuous reactors on the biodegradative treatment of Nejayote (wastewater resulting from corn processing: nixtamalization) using mixed cultures in anoxic environments, has been tested. Preliminary tests have been performed on a batch system in order to evaluate the mixed culture response at two organic load levels: 10,021 mg/lt and 16,690 mg/lt, and two temperatures (27 and 35°C). In these experiments, a 2 by 2 experimental design with 4 central points was applied. A significant effect of organic load was observed. A significant interaction between the two tested factors was also found (90% confidence level). The effects of temperature, organic COD feed level, and volumetric flux were evaluated using a triple column reactor (540 cm length and 2 cm inner diameter), packed with activated carbon. Results show that, above a temperature of 35°C, and within the feed flow range of 1.67-3.42 ml/min, a cellular washout ocurred. There is a significant effect of feed flow rate on the degradative reactor efficiency (95% confidence level). COD feed does not have a significant effect. At steady state, a single packed column reactor (4.4 cm inner diameter and 113 cm of packed bed height) with a feed flow rate of 1.67 ml/min, was found to remove 20-30% COD with six hours of hydraulic residence time (HRT). Perturbation responses to changes in COD feed conditions were also evaluated in the experimental rig. It was observed that the direction of stimulus has an effect on the response delay time of the system. When an increment in feed COD level was applied, the new steady state was reached in 7.3 HRT's. When the change was in the opposite direction, the new steady state was observed after 2.5 HRT's. An empiric relationship between the COD longitudinal profile and entrapped biomass concentration along the column was found.

X2-102 LINEAR PLASMIDS IN Rhodococcus corallina V49 John A. C. Archer and Hywel Griffiths, Department of Genetics, University of Cambridge, Cambridge CB23EH England

The Nocardioform Actinomycete Rhodococcus exhibits several structural and physiological adaptations to oligotrophic soil conditions which facilitate successful competition against other soil organisms. Rhodococcus possesses an extremely versatile hydrocarbon catabolic metabolism which has been characterised only at the biochemical level. To investigate the underlying genetic basis for Rhodococcus catabolic abilities, the structure of the Rhodococcus genome has been investigated using pulse field gel electrophoresis. This technique can resolve very large DNA molecules on the basis of their size and topological form. An immobilized lysis technique for Rhodococcus has been successfully developed and the total DNA content can be visualised by contour clamped homogenous field electrophoresis. R. corallina V49 contains six linear plasmids with apparent molecular sizes of 800 Kb, 550 Kb, 440 Kb, 380 Kb, 350 Kb, 280 Kb with an additional 90 Kb supercoiled plasmid. From these gels, a total genomic content of 5.88 Mb has been calculated. These experiments have revealed the surprising result that up to 40% of the total DNA content of R. corallina is comprised of linear plasmid DNA. Current experiments seek to determine the relationship between these plasmids and the chromosome as well as their phenotypic effects on the host bacterium.

X2-103 MOLECULAR DYNAMICS STUDY OF HALOALKANE DEHALOGENASE AND IMPLICATIONS FOR ITS RATIONAL REDESIGN, Gregory E. Arnold and Rick L. Ornstein, EMSL, PNL*, P.O. Box 999, K1-96, Richland, Washington 99352.

Xanthobacter grows on 1,2-dichloroethane as a sole carbon and energy source (DB Janssen and coworkers). The haloalkane dehalogenase enzyme catalyzes the first metabolic step in the 1,2-dichloroethane *Xanthobacter* degradation pathway. The enzyme mechanism is very similar to that of a serine protease except that an aspartic acid has replaced the nucleophilic serine in the catalytic triad. Several crystal structures of this 310 amino acid enzyme have been solved (BW Dijkstra and coworkers). The x-ray structures, where substrate is bound or a covalent intermediate has been trapped, indicate that the active site pocket is occluded from the surface of the enzyme. The underlying dynamics operative in controlling the access and exit channel(s), for substrate and product, are not well understood. To better understand this process, we are currently analyzing the results from three 300 ps trajectories of fully solvated enzyme that used the following three crystal structures as starting points: 1) substrate free, 2) substrate bound, and 3) the trapped covalent intermediate. Results from the simulations will be presented and inferences for improving the rate and altering dehalogenation specificity, by subsequent rational redesign of the enzyme, will be discussed.

* The Pacific Northwest Laboratory is a multiprogram national laboratory operated for the U.S. Department of Energy by Battelle Memorial Institute under contract number DE-AC06-76RLO1830

X2-104 USE OF PHAGE SURFACE DISPLAY FOR PROTEIN ENGINEERING OF FUNCTIONAL PROTEINS. Michael Benedik[1], Baby Djojonegoro[1] and Richard Willson[1,2]. Department of Biochemical and Biophysical Sciences[1] and Department of Chemical Engineering[2]. University of Houston, Houston, TX 77204-5934.

Filamentous phage display is finding widespread use to identify affinity ligands especially from libraries of short synthetic peptide. The technology is based on the observation that filamentous bacteriophage can incorporate foreign peptides into their structures as fusions to the gpIII adsorption protein and still assemble normally with the foreign peptide "displayed" on the phage surface. However, in addition to small peptides, entire proteins can in fact be displayed by phage surface display. This allows for chromatographic selections based on separation properties, which in the case of enzymes can be ligands such as substrates or inhibitors.

As a model system for the optimization of separation ligands by bacteriophage surface display, we have constructed a phage surface expression system for a single immunoglobulin-binding domain (B) of Protein A of *Staphylococcus aureus*. Phage displaying the Protein A domain retain normal IgG binding. They are selectively retained on human IgG sepharose by a specific Protein A-IgG as demonstrated by the ability to competitively inhibit this interaction either with soluble Protein A or polyclonal human IgG.

Phage expressing Protein A can be purified in a few rounds of selective adsorption form a vast excess of wild type phage, demonstrating the possibility of using this approach for the identification and optimization of affinity ligands after mutagenesis of a phage display lysate. This should allow the massive screening for mutant forms of Protein A with alterations in binding and elution properties.

We are also extending this approach to complete enzymes using competive inhibitors as affinity matrices to mimic substrate binding. This approach will be useful for the isolation of novel mutants of such enzymes mutants.

X2-105 TRICHLOROETHYLENE DEGRADATION BY TOLUENE OXIDIZING BACTERIA COLONIZING A VAPOR PHASE BIOFILTER. A. Breen[1], T. Ward[1], J. C. Loper[1], R. Govind[2], J. Haines[3] and D. Bishop[3]. [1]University of Cincinnati, College of Medicine, [2] University of Cincinnati, College of Engineering and [3] Risk Reduction Engineering Laboratory, U.S. EPA Cincinnati, OH.

Trichloroethylene (TCE) degrading heterotrophic bacteria, colonizing a vapor phase bench-scale biofilter, inoculated with municipal sewage sludge, were characterized. Biofiltration is a promising technology for the removal of hazardous compounds from a gas stream. A number of volatile organic compounds, including toluene and TCE, were removed from an artificial waste gas stream. When other VOCs were removed from the gas stream TCE degradation continued for 18 months. In order to characterize the degradative population(s) samples were removed from the biofilter matrix and tested for degradative capability in batch vial assays using ^{14}C-TCE. Nitrapyrin inhibitor studies did not implicate ammonia oxidizing bacteria hence, alternative bacteria were examined. The presence of aromatic compound degrading heterotrophs was tested by inoculation of biofilter biomass into toluene and benzene vapor desiccators. Three monocultures representing predominant organisms revealed two Gram positive bacteria and a Gram negative bacterium at three sampling times tested. Lipid analysis was used to further characterize these isolates. Conversion of TCE to CO_2 during an 18 hour incubation was as follows; *Pseudomonas* sp. TA2 converted 41% of added TCE to CO_2, *Rhodococcus* sp. TA1 converted 24% and *Norcardia* sp. AR1 converted 13%. The biofilter harbors a stable community which appears to be resilient to the deleterious effects of TCE oxidation.

X2-106 BIODEGRADATION OF BENZENE UNDER ANAEROBIC CONDITIONS. Elizabeth A. Edwards, Beak Consultants Limited, 42 Arrow Rd., Guelph, Ontario, Canada, N1K 1S6.

All BTEX compounds (benzene, toluene, ethylbenzene and xylenes) are not equally biodegradable under anaerobic conditions. While the anaerobic degradation of toluene and xylene is well documented, few studies have presented evidence for anaerobic benzene degradation This is unfortunate since benzene is the most toxic BTEX compound and a proven human carcinogen. The objective of this study was to screen sediment from contaminated sites for the presence of microorganisms capable of biodegrading benzene under anaerobic conditions, specifically under methanogenic, sulfate-reducing, iron reducing, and denitrifying conditions, in laboratory microcosms. Each microcosms consisted of sediment (50 g), pre-reduced, defined mineral medium (50 ml), and benzene (50 µM initial concentration). To confirm benzene biodegradation, ^{14}C-labeled benzene was added to some microcosms. The results of this investigation show that both sediments tested contain microorganisms capable of degrading ^{14}C-labeled benzene to $^{14}CO_2$ under anaerobic conditions. The fastest rate of degradation was about 1 µM per day. Active microcosms turned black from precipitation of ferrous sulfide, suggesting sulfate-reducing bacteria may be involved. Considerable variation in the rates of degradation was observed, even between replicates, suggesting that the microorganisms involved have very particular growth requirements and are not uniformly distributed in the sediment.

X2-107 THE EFFECT OF IRON CONCENTRATION ON THE OUTER MEMBRANE PROTEIN PROFILE OF SULPHATE REDUCING BACTERIA, M.J. Feio[1], V. Zinkevich[2], I.B. Beech[2], A.R. Lino[1,3] and J.J. G. Moura[4], 1- ITQB- Instituto de Tecnologia Química e Biológica, APT. 127, 2780 Oeiras, Portugal, 2- Chemistry Department, University of Portsmouth, UK, 3- Depart. de Química da Faculdade de Ciências de Lisboa, 1700 Lisboa, Portugal, 4- Depart. de Química da Faculdade de Ciências e Tecnologia, UNL, 2825 Monte da Caparica, Portugal

Biocorrosion is a process with enormous environmental and economical impact, namely in sector such as oil and paper mill industries.
Although many micro-organisms can play a role in the biocorrosion process, the major one is reserved to Sulphate Reducing Bacteria (SRB) often involved in the anaerobic microbial influenced corrosion of iron and iron containing alloys. The mechanism associated with the process is not yet fully understood, but is generally believed that metal solubilisation is based in a cathodic depolarisation process, leading to localised corrosion due to the heterogeneity of bacterial colonisation [Hamilton, 1985].
The understanding of the mechanism involved in bacterial cell attachment to metal surfaces along with the Fe(II) adsorption to the cell surface demands some knowledge about the functional relationships of the cell envelope.
In this work we describe the effect of the iron concentration in the outer membrane protein profile of different SRB strains. A wide range of iron concentrations were tested as soluble salts and the obtained results were compared with those obtained in the presence of mils steel coupons, both at the outer membrane protein and excreted exopolymer levels. These comparative studies carried out by SDS-PAGE confirmed previous results and suggest a induction/repression of some representative membrane proteins as function of iron concentration and metal surface presence. More elaborate studies concerning purification and characterisation of these proteins are now under development.

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X2-108 DETECTION AND QUANTIFICATION OF 2,3-CATECHOL DIOXYGENASE GENES IN DIFFERENT SOIL HORIZONS BY PCR, Mats Forsman, Anna Wiklund, Anna Nyrén, and Per Wikström, Department of Microbiology, National Research Establishment, S-901 82 Umeå, Sweden.

With the aim to monitor xenobiotic degrading microbial subpopulations in soil, a method for rapid extraction of DNA, amenable for amplification by the PCR, from soil was developed. The efficiency and reproducibility of the DNA extraction method was evaluated, by competitive PCR, on five different defined soil horizons (OH, OF, A1, A2, and B1) with characteristics applying for a wide range of soils. The organic content in the soils was the major factor affecting release of DNA from the soils. Native bacterial DNA from soil samples were detected by using universal primers specific to conserved regions of procaryotic 16S rRNA genes. A degenerate primer pair, for use in the PCR, with specificity for catechol-2,3-dioxygenase genes was designed. The specificity of the primer pair was initially demonstrated on different characterized catechol-2,3-dioxygenase genes and subsequently on natural bacterial isolates from ongoing field-scale bioremediation processes. The kinetics of catechol-2,3-dioxygenase genes in PAH contaminated soil undergoing an *ex situ* compost process was determined over a period of 15 weeks by competitive PCR.

X2-109 MICROBIAL DESULFURIZATION OF STERICALLY HINDERED COMPOUNDS AND ANALYSIS OF THE EFFECT ON FUELS BY X-RAY ABSORPTION SPECTROSCOPY, M. J. Grossman†, R. C. Prince†, M. K. Lee†, J. D. Seniust, R. E. Bare†, V. Minak- Bernero†, I. J. Pickering*, G. N. George*, C.W. Greer#, A. M. Jones#, J. Hawari#, and P. C. K. Lau#, †Exxon Research and Engineering Co., Annandale, NJ 08801, *SSRL Stanford CA 94309, #Biotechnology Research Institute, Montréal, Québec H42R2

Sterically hindered organic sulfur compounds such as 4,6 diethyl - dibenzothiophene (4,6-DEDBT) show the greatest resistance to hydrodesulfurization, the method of sulfur removal currently employed by the petroleum industry.

Microorganisms capable of selective sulfur removal from sterically hindered organic sulfur compounds were isolated from intertidal marine sediments by enrichment culture on 4,6-DEDBT as sole sulfur source. Isolate ECRD-1, a non-spore forming irregular rod shaped Gram-positive bacterium obtained from this enrichment, selectively desulfurized 4,6 DEDBT with the production of 2-hydroxy-3,3-diethyl biphenyl. 4,6 DEDBT sulfone was detected as an intermediate in the desulfurization pathway. No hydrocarbon degradation was observed. The substrate range of isolate ECRD-1 was shown to include dibenzothiophene (DBT), substituted benzothiophenes and benzyl-phenyl sulfide. Analysis of 2-hydroxy biphenyl produced by ECRD-1 grown on DBT in the presence of $H_2{}^{18}O$ and $^{18}O_2$ demonstrated that $^{18}O_2$ was the source of incorporated oxygen.

ECRD-1 was able to use a number of petroleum products as a sulfur source including crude oil, a middle distillate cut of crude oil (232 - 343 °C), bitumen and bitumen vacuum gas oil (343-496 °C). A 232 - 343 °C cut of Oregon Basin crude oil was provided as a sole sulfur source to ECRD-1 in liquid culture. Analysis of the treated oil with GC FID and GC SCD demonstrated a 34% reduction in total sulfur. Compounds across the entire boiling range of the oil were affected by the treatment. Sulfur K-edge X-ray absorption-edge spectroscopy analysis showed that 50% of the remaining sulfur was in an oxidized form demonstrating that more than two-thirds of the initial sulfur had been affected by the microbial treatment.

X2-110 THE USE OF ANTIBODY FRAGMENTS FOR SENSITIVE DETECTION OF ORGANIC POLLUTANTS IN THE ENVIRONMENT, William J.Harris, Fergus R. Byrne, Barbara M.Graham, Andrew A. Shelton, Dianne Learmonth and Andrew Porter, Department of Molecular and Cell Biology, University of Aberdeen, Aberdeen AB9 1AS, Scotland, UK.

There is an urgent need for new user-friendly and cost effective technologies to detect low levels of organic pollutants in drinking water and other environmental sources. Methods to routinely measure levels of pollutants present at less than 10ng per litre are required to meet legislative demands and consumer safety concerns. Existing analytical methods which are suitable include GLC- mass spectrophotometric analysis but this requires access to expensive and sophisticated instrumentation available at central facilities.

Immunoassays are routinely used in medical diagnostics and have good potential for environmental detection methodologies. In general however immunoassays do not quantitatively detect organic pollutants present in the 1-10ng per litre range, particularly for poorly water-soluble pollutants. Antibody fragments(Fab,Fv,scFv) representing as little as 20% of intact immunoglobuin can be made in *E.coli* and designed to retain high binding affinity in adverse environments. The use of antibody fragments could increase sensitivity of assays by 10-100 fold.

We have designed gene constructs which allow the assembly of bivalent single chain antibody dimer fragments in *E.coli* and have expressed antibody fragments against paraquat, atrazine, diuron, and polychlorinated biphenyls. Their use in ELISA based immunoassays will be described.

X2-111 **DNA PROBE ANALYSES FOR PREDICTING BIOREMEDIATION POTENTIAL**, William E. Holben[1] and Robert J. Steffan[2], The Agouron Institute, LaJolla, CA[1], Envirogen, Inc. Lawrenceville, NJ[2]

Methods capable of specifically detecting organisms encoding biodegradative pathways of interest allow an estimation of the quantity, distribution and diversity of the biodegradative potential of the microbial community at the site being studied. An evaluation of the genetic capacity of the community is key for decision-making in determining feasible bioremediation strategies. For example, limited diversity of indigenous biodegrading organisms would suggest a limited potential for the satisfactory removal of pollutant by indigenous microorganisms in most habitats since organisms specialized for many niches would be needed. To make this evaluation we employ methodologies for the isolation and purification of microbial community DNA from soil and sediment samples and for the detection of specific DNA sequences of interest in this complex mixture of community DNA. In addition, a new methodology that allows us to fractionate the DNA of the entire bacterial community in an environmental sample is utilized. Probe analysis of individual fractions allows for both an assessment of diversity and an increase in sensitivity compared to analysis of unfractionated total community DNA. In addition, probes specific to indigenous organisms at various sites have been generated by isolating unique amplified fragments generated by arbitrarily primed polymerase chain reaction (AP-PCR). DNA probes developed by this method are very specific and allow for the differentiation of closely related strains of bacteria. A further advantage is that this method generates probes for indigenous isolates without requiring detailed strain characterization. These techniques can be used to assess the quantity, distribution and diversity of bacterial populations with degradative functions of interest and develop an index of in situ bioremediation potential for environmental samples. The model compounds are BTEX (benzene, toluene, ethylbenzene and xylene) which are hazardous wastes representing fuels. These compounds were chosen because they represent a major environmental toxicant for which appropriate functional gene probes already exist. The results of hybridization studies with functional gene probes and AP-PCR generated probes are compared to biotreatability assay data to assess the ability of DNA probes to determine bioremediation potential.

X2-112 METABOLIC ENGINEERING OF THE TOLUENE DEGRADATION PATHWAY, Graham A. Jackson[1,2], Sunil Shrestha[1,2], John M. Ward[1],[1]Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, [2]The BBSRC Advanced Centre for Biochemical Engineering, Departmental of Chemical and Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE.

The ability to alter the substrate range of a bacterial aromatic degradative pathway by the formation of hybrid gene clusters comprising subunits from different aromatic dioxygenases has recently been described (Hirose *et al,* 1994). Here we describe a different methodology for broadening the substrate range of such a pathway based upon the co-expression of two wild-type dioxygenases within the same cell. The *nah*AaAbAcAd encoded naphthalene dioxygenase from *Pseudomonas putida* PpG1064 plasmid NAH7 has been cloned into the broad host-range vector pMMB66EH, forming the plasmid pSS2. This dioxygenase has been shown to possess a broad substrate range, and is capable of catalysing the dioxygenation of benzene, toluene, biphenyl and naphthalene to the respective *cis*-dihydrodiols. The plasmid pSS2 has been introduced into an *E.coli* JM107 strain containing a recombinant pBGS18 plasmid, pQR150, that expresses the entire *meta*-cleavage operon from the *Pseudomonas putida* -mt2 TOL plasmid pWW0. *E.coli* cells containing both plasmids pSS2 and pQR150 were grown to late log phase, and then harvested by centrifugation. Resuspended cells were then incubated at 30°C in the presence of different aromatic solvents, and samples taken at appropriate intervals. Samples were subsequently assayed spectrophometrically for the presence of *meta*-ring-cleavage compounds, as an indication of metabolism of aromatic substrates. The observation of characteristic yellow bacterial pellets upon centrifugation, together with spectrophometric data, has shown that such a dual-dioxygenase system is capable of degrading benzoate, biphenyl, naphthalene and toluene at least as far as ring-cleavage. In comparison, control experiments with *E.coli* expressing pQR150 alone have shown that the substrate specificity is restricted to benzoate alone. Such indications illustrate that the substrate range of the TOL plasmid *meta*-cleavage pathway can be extended by the co-expression of a second aromatic dioxygenase. It would also appear that several enzymes of the TOL *meta*-cleavage pathway display a broad substrate specificity, and that the specificity of the pathway is initially restricted by the limited substrate range of the benzoate dioxygenase.

X2-113 HIGHLY ENERGETIC ORGANONITRO POLLUTANTS: ASSESSMENT OF THE AEROBIC BIODEGRADATION POTENTIAL OF RDX, TNT, GAP, AND NC. Alison M. Jones, Charles W. Greer, and Jalal Hawari, Biotechnology Research Institute, National Research Council Canada, Montreal, PQ, Canada, and Sonia Thiboutot and Guy Ampleman, Department of National Defence, Defence Research Establishment Valcartier, Courcelette, PQ, Canada.

Contamination of soils and waters with energetic organonitro compounds derived from explosive munitions manufacture and handling is of significant environmental concern. Conventional technologies for decontamination of organonitro compound-contaminated environments are both expensive and sub optimal. *In situ* biodegradation may be a viable solution to large-scale land reclamation. Laboratory studies were conducted to evaluate the biodegradation potential of four energetic compounds - RDX, TNT, GAP, and NC - under aerobic conditions. A bacterium, designated strain A, was enriched and purified from RDX-contaminated soil by its ability to use RDX as sole nitrogen source. Using [^{14}C]-labelled RDX, the ability of strain A to mineralize RDX was verified. Bioaugmentation of RDX-contaminated soil with strain A enhanced the rate and extent of biodegradation. Moreover, biostimulation with various nutrients further enhanced the biodegradative ability of strain A in soil microcosms. In contrast, although TNT is more recalcitrant and toxic, [^{14}C]TNT mineralization activity was detected in TNT-contaminated soil. This activity was concentration dependent: in soil, as the concentration of TNT increased, mineralization decreased and was abrogated at 100 ppm TNT. An enrichment culture developed from this soil yielded a bacterium able to transform TNT when provided as sole nitrogen source, but which possessed limited mineralization activity. Studies are underway to characterize the TNT biotransformation pathway in this isolate, and to promote TNT mineralization. Although it remains unknown whether any of the soils we are working with are contaminated with GAP or NC, some soils harboured [^{14}C]GAP or [^{14}C]NC mineralization activity. Enrichment cultures developed from these soils used GAP or NC as sole carbon sources for growth. These data firmly establish that the bioremediation of RDX-contaminated soil is possible under aerobic conditions, and that the potential also exists for the bioremediation of TNT, GAP, and NC.

X2-114 IMPROVEMENT OF PREFERENTIAL LIGNIN DEGRADATION BY *PLEUROTUS OSTREATUS*, Zohar Kerem and Yitzhak Hadar, Department of Microbiology and Plant Pathology, The Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel.

The global commercial production of *Pleurotus* amounts to about 900,000 tones annually, with production second only to *Agaricus* . Large amounts of spent raw materials are a by-product of this industry. The practical utilization of the polysaccharides of the lignocellulosic complex is limited due to the high content of lignin. The focus of this study is on the effect of manganese on preferential biodegradation of lignin during solid state fermentation by *Pleurotus ostreatus* . The effects of manganese (at concentrations of 0 to 2.7 mM) on the production of extracellular enzymes involved in ligninolysis by *P. ostreatus* were studied during chemically defined, solid-state fermentation. Mn was found to precipitate in all treatments. Physiological activities of the fungus expressed as glucose consumption and CO_2 evolution peaked during the first week of fermentation, and were not affected by Mn level. Laccase, Mn peroxidase and catalase activities, and H_2O_2 production were all affected by Mn level, albeit differently. In general, the specific enzymes involved in ligninolysis were enhanced by increasing concentrations of Mn during the secondary growth phase. Degradation of [^{14}C]lignin prepared from cotton branches to soluble products, as well as its mineralization to $^{14}CO_2$, was enhanced by the addition of Mn. Degradation of [^{14}C]cellulose was not affected by the addition of Mn. Cotton stalks were amended with Mn(II) at concentration range of 30 to 620 µg Mn/ g.d.w., and fermented with *P. ostreatus*. Enhancement of selectivity was demonstrated by analyzing the residual matter at the end of the fermentation period using crude fiber analysis. The cellulose fraction in the original matter was 1.8 times higher than lignin. This ratio (cellulose:lignin) doubled during the 32 days of SSF, from 3.0 in the control to 5.6 and 6.6, following the addition of Mn to the medium. Enhancement of selective lignin degradation could be the result of either increasing the activities of ligninolytic enzymes or producing Mn(III), which might preferentially degrade aromatic structures in the lignocellulosic complex.

X2-115 **EVALUATION OF SSF, SLF AND SmF FOR ALPHA-AMYLASE PRODUCTION BY *Aeromonas caviae* (CBTK 185) UTILISING BANANA WASTES,** Chundakkadu Krishna and M. Chandrasekharan, Centre for Biotechnology, Cochin University of Science and Technology, Cochin - 682 022, Kerala, INDIA

Worldwide, attempts are being made for the maximum economic utilization of agricultural wastes including banana wastes through microbial conversion into valuable proteins and enzymes. Although ripe banana wastes are used as animal feed, green wastes are rarely used because of its poor digestibility and related toxicological problems. In the present investigation, a new approach to producing alpha-amylases from these starchy wastes was developed. A newly isolated hyper-enzyme producing strain of *Aeromonas caviae* (CBTK 185) from vegetable wastes was found to grow well on banana wastes and produce amylase in significant quantity under solid state fermentation (SSF). SSF with banana stalk and slurry fermentation (SLF) consisting of 10% banana stalk was compared with submerged fermentation (SmF) using banana stalk extract fortified with mineral medium. SSF had the maximum activity of 70,712 units/gm in 24h against 1580 units in slurry fermentation and 682.3 units in submerged fermentation. Substantial increase in activity, 44.8-fold, was noticed on comparing with activity in slurry fermentation when the pretreated banana stalk medium contained particles of 1mm size, had 70% moisture content, pH 7.0, and incubation temperature of 35 ± 2°C for 24h. The present results indicate the excellent scope for utilizing this hyper-enzyme producing strain of *Aeromonas caviae* (CBTK 185) and banana wastes for commercial production of amylases through solid state fermentation. This is the first report on utilizing these wastes and this bacterial strain for the production of alpha- amylases.

X2-116 THE METABOLIC PATHWAYS UTILIZED BY PHANEROCHAETE CHRYSOSPORIUM FOR THE DEGRADATION OF THE CYCLODIENE PESTICIDE, ENDOSULFAN. Seth W. Kullman and F. Matsumura, Department of Environmental Toxicology, University of California, Davis, CA 95616.

The white rot fungus Phanerochaete *chrysosporium*, has been shown to degrade and mineralize a wide variety of industrial and agricultural pollutants. The ability to degrade such structurally diverse compounds has generally been attributed to the production of extracellular enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP). However, recent studies have shown that some xenobiotics are still metabolized under conditions that do not favor the production of LiP and MnP. To test for the involvement of additional enzyme systems in pollutant degradation, we have used the cyclodiene pesticide, endosulfan as a chemical probe. Cultures of the white rot fungus *Phanerochaete chrysosporium BU-1* were grown in nitrogen deficient, carbon deficient and nitrogen rich media. Rapid metabolism of this chlorinated pesticide occurred under each nutrient condition tested. However, the extent of degradation and the metabolic products formed differed for deficient and rich media. The pathway for endosulfan degradation was characterized by analysis of the fungal metabolites produced. The major endosulfan metabolites were identified by GC-ECD and GC-MS as endosulfan sulfate, endosulfan diol, endosulfan hydroxyether, and a novel metabolite identified as endosulfan dehydroether. The nature of the metabolites formed under these conditions indicates that this organism utilizes both oxidative and hydrolytic pathways for degradation of this pesticide. Endosulfan metabolism under nitrogen deficient conditions favored the production of both oxidation and hydrolysis products. In contrast, endosulfan metabolism under nitrogen rich conditions produced only hydrolysis products, endosulfan diol, endosulfan hydroxy ether, and endosulfan dehydroether. Incubations of individual metabolites with the fungus confirmed that endosulfan diol is formed by an initial hydrolysis of the parent compound endosulfan and not from hydrolysis of endosulfan sulfate. Piperonyl butoxide, a known cytochrome p-450 inhibitor, significantly inhibited the oxidation of endosulfan to endosulfan sulfate and enhanced hydrolysis of endosulfan to endosulfan diol. We suggest that the metabolism of endosulfan is mediated by two divergent pathways one hydrolytic and the other oxidative. Judging by the inaction of extracellular fluid and partially purified lignin peroxidase to metabolize endosulfan, we conclude that degradation of this compound may not involve the action of extracellular peroxidases.

X2-117 ENGINEERING MICROORGANISMS TO GROW ON HALOGENATED ALIPHATICS: A MODEL SYSTEM.

Cleston C. Lange, Lawrence P. Wackett, and Rick L. Ornstein*. University of Minnesota, St. Paul, MN 55108 and *Battelle/Pacific Northwest Laboratories, Richland, WA 99352.

Halogenated organics constitute the single largest group on the USEPA's list of priority pollutants, and of these, halogenated aliphatics are the largest subgroup. Many of the halogenated aliphatics listed exhibit carcinogenic and/or teratogenic properties,and their persistence in the environment poses a significant health hazard. A few of the halogenated aliphatics support growth of microorganisms, but most do not, and enrichments for such microorganisms have proven futile. Because of the recent release of these pollutants into the environment through human activities, it can be assumed that the evolution of the required degradative pathways has not yet occurred. However, it would be of great benefit to isolate, or genetically engineer, such microorganisms. We have taken a novel approach to resolve this problem by using microorganisms expressing cytochrome P450cam and/or toluene dioxygenase, both known to fortuitously dehalogenate halogenated aliphatics. Using a well-founded knowledge base concerning the dehalogenation reactions, we have set out to develop a system that would allow genetically engineered microorganisms to obtain metabolic energy from a number of "ideal" halogenated aliphatic substrates.

X2-118 REGULATORY GENES AS BIOMONITORING TOOLS AND USE OF INSERTION SEQUENCES FOR STRAIN IDENTIFICATION, Peter C.K. Lau, Diane Labbé, James Garnon, Hélène Bergeron and Ying Wang, Environmental Genetics Group, Biotechnology Research Institute, Montreal, Quebec, Canada H4P 2R2

PCR primers derived from the NahR regulatory gene of the naphthalene degradation pathway in *Pseudomonas putida* G7 successfully amplified a homologous gene from *Pseudomonas pseudoalcaligenes* KF707 but not from an isofunctional *Pseudomonas* sp. LB400. Supported by DNA hybridization, it is evident that different transcriptional regulatory systems have been evolved to control the expression of similar or virtually identical phenotypes in xenobiotic-degrading pathways. This has important ramification in applied remediation. The *bphR* gene of KF707 was sequenced and found to be associated with two insertion sequences, one of which hybridized to *P. putida* strain mt2 DNA but not to LB400 or other strains. We have also isolated an insertion sequence from a Gram-positive (*Rhodococcus* sp. M5) biphenyl/polychlorinated biphenyl-degrader. This element (IS*1284*) with sequence homology to IS*117* of *Streptomyces coelicolor* A3(2) is associated with biphenyl-PCB degradation, and it can distinguish M5 from other rhodococci.

X2-119 ISOLATION AND CHARACTERISATION OF PAH-DEGRADING SOIL BACTERIA. **Gareth Lloyd-Jones and David W. Hunter. Landcare Research Ltd, Private Bag 3127, Hamilton, New Zealand.**

Bacteria capable of degrading naphthalene and phenanthrene are readily isolated from soils, whereas high molecular weight PAHs are more recalcitrant. The genetic and regulatory mechanisms involved in bacterial catabolism specific to the high molecular weight PAHs is poorly defined. We are studying PAH catabolism by bacterial strains capable of growing at the expense of the PAHs; phenanthrene, fluoranthene and pyrene. Strains were isolated from PAH-contaminated soil taken from a bioremediation exercise at a decommissioned coal carbonization plant, and from a timber treatment yard, by direct plating or via enrichment culture.

A Gram-negative phenanthrene degrading isolate capable of growing at the expense of biphenyl, naphthalene, m-xylene, benzoate, salicylate and m-toluate was selected for further study. This strain was able to oxidise indole to indigo, and possessed *meta*-cleavage activity towards 3-methylcatechol, but not catechol. Using these colorimetric screening reactions facilitated the cloning of the genes encoding the dioxygenase enzymes involved in the catabolism of these compounds by this strain.

Strains isolated for ability to grow at the expense of fluoranthene and pyrene were predominantly Gram-positive (tentatively identified as belonging to the CMN group [Coryneforms, *Mycobacterium*, *Nocardia* and *Rhodococcus*]). These Gram-positive isolates had narrow growth substrate spectra, growth tending to be restricted to the PAH on which they were isolated as the only aromatic hydrocarbon supporting growth, the n-alkanes hexadecane and dodecane also served as growth substrates.

The survival of these Gram-negative and Gram-positive isolates in soils amended with PAHs will be compared under a range of conditions. Using gene probes specific to each of these PAH-degrading bacteria we will be able to detect the presence of PAH-genes in polluted soils, this data will be useful in predicting the amenability of these soils to bioremediation.

X2-120 DEGRADATION OF AN ORGANOPHOSPHATE INSECTICIDE IN HIGHLY CONTAMINATED SOILS AND IN LIQUID WASTES, Walter W. Mulbry and Jeffrey S. Karns, Soil Microbial Systems Laboratory, USDA/ARS, Beltsville, MD 20705

Insecticide wastes generated from livestock dipping operations are well suited for biodegradation processes since these wastes are concentrated, contained, and have no other significant toxic components. About 400,000 L of cattle dip wastes containing approximately 1500 ppm of the organophosphate coumaphos [O,O-diethyl O-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) phosphorothioate] are generated yearly along the Mexican border from one USDA program designed to control disease carrying cattle ticks. Use of unlined evaporation pits for the disposal of these wastes has resulted in highly contaminated soils underlying these sites. Previous work has shown that microbial consortia present in selected dip wastes can be induced to mineralize coumaphos. Our results demonstrate that similar consortia are present in coumaphos contaminated soils from eight waste sites and that these organisms are capable of rapidly mineralizing coumaphos in these soils using soil slurries. In addition, our results show that these consortia are able to colonize pea gravel in trickling gravel filters and can be used in these filters to quickly metabolize coumaphos from dip wastes. These simple systems offer low cost means to detoxify coumaphos containing wastes and to bioremediate soils contaminated with this organophosphate compound.

X2-121 STABLE EXPRESSION OF DICAMBA CATABOLISM IN *PSEUDOMONAS SP.* (STRAIN PXM) Parnell O'Brien and Douglas J. Cork, Department of Chemical and Biological Sciences, Biology Division, Illinois Institute of Technology, Chicago, Illinois.

Pseudomomas sp. (Strain PXM) has the ability to degrade Dicamba, a well known herbicide used to control broadleaf grasses. *Pseudomonas sp.* (Strain PXM) has been found to contain a large plasmid of approximately 200 Kb, and this plasmid has been correlated with the stable expression of Dicamba and dichlorosalicylic acid degrading activity. We have examined parameters which insure stable expression of this plasmid in batch and chemostat cultures. When used as alternative limiting carbon sources, succinate and benzoic acid induce time dependent loss of Dicamba degrading activity; whereas salicylic acid stabilizes the expression of Dicamba catabolism. Based on our preliminary data we present an optimal policy for the stable expression of the Dicamba degrading phenotype.

X2-122 RADIOIMMUNOASSAY AS A SENSITIVE TOOL FOR MEASURING SPECIFIC GENOTOXIC DAMAGE IN ENVIRONMENTAL SAMPLES, Ralph J. Pledger, Wade H. Jeffrey* and David L. Mitchell, Department of Carcinogenesis, The University of Texas M.D. Anderson Cancer Center, Science Park/Research Division, Smithville, TX 78957 and *University of West Florida, Center for Environmental Diagnostics and Bioremediation, Gulf Breeze, FL 32561.

A radioimmunoassay (RIA) has been developed which permits the accurate quantification of DNA damage from environmental samples. Polyclonal sera are currently available with specificity to pyrimidine cyclobutane dimers, 6–4 photoproducts and benzo[a]pyrene diolepoxide (BPDE–I) adducts. The benzo[a]pyrene assay is highly specific for BPDE–I with negligible binding to BPDE–II, BPDE–III and the BPDE–I tetrol. Other sera are under development. Using the first two sera UV induced DNA damage in marine microplankton was quantified during three cruises on the Gulf of Mexico. A distinct diurnal pattern of DNA damage and repair which correlated with solar fluence was observed from shipboard experiments and natural samples from surface waters. Depth profiles of damage in the DNA of natural populations generally showed the expected decrease in damage with extinction of UV radiation, although some deviation in this pattern was observed probably reflecting mixing or heterogeneity in the populations sampled from the water column. Laboratory studies with the fish species *Medaka oryzias* revealed that BPDE–I adducts could be detected and quantified from gills, intestines and liver of benzo[a]pyrene exposed organisms. In contrast, when exposed to the same regime the fish species *Xiphophorus maculatus* had barely detectable levels of BPDE–I adducts in the gill and intestine but approximately the same levels in the liver. We propose that this RIA is a powerful tool which will have many important applications in environmental biotechnology. [This work was supported by EPA grant CR822020–01].

X2-123 THE USE OF IMMOBILISED ANTIBODY FRAGMENTS TO REMOVE POLLUTANTS FROM THE ENVIRONMENT, Andrew J.Porter, Fergus R.Byrne, Barbara M. Graham, Andrew A. Shelton, and William J. Harris, Department of Molecular and Cell Biology, University of Aberdeen, Aberdeen AB9 1AS, Scotland,UK.

There is an urgent need for new methods to remove low levels of organic pollutants from the environment. Current remediation methodologies can provide a first level of clean up and reduce organic pollutant levels to 5-10ug per litre ranges, at least 100 fold above the targeted level of 0.1ug per litre. Activated carbon can remove such residual levels but recycling of activated carbon requires high temperature exposure and could not be contemplated on a large scale.

Antibodies have binding affinities of the order of 10-9M and can chelate organics present at ng per litre concentrations, and could be used for second level clean- up. Further, bound pollutants can be easily stripped from immobilised antibodies allowing recycling and repeated re-use of the adsorbent. Intact antibodies are however too large in molecular weight and to expensive for such a remediation role.

Antibody fragments(Fab,Fv,ScFv) representing as little as 20% of intact immunoglobulin molecules can be made in *E.coli* in quantities and at a cost acceptable for remedial uses. Such fragments can be designed retain high binding affinity.

We have designed gene consructs which allow the assembly in *E.coli* of bivalent antibody fragments against paraquat, atrazine and diuron. Immobilisation of these fragments has been carried out and shown to effectively remove trace pesticides from aqueous environments.

X2-124 GENETIC ANALYSIS OF HYDROCARBON DEGRADATIVE SYSTEMS IN *RHODOCOCCUS CORALLINA* V49, Justin Powell and John Archer, Department of Genetics, University of Cambridge, UK.

The Nocardioform actinomycetes form a supra-generic group of mycolic acid-containing Gram-positive bacteria which consists of the genera *Corynebacterium, Nocardia, Rhodococcus, Tsukamurella, Gordona* and *Mycobacterium*. *Rhodococcus* is highly competetive under oligotrophic conditions and can degrade a wide range of hydrocarbons including many xenobiotic molecules. These hydrocarbon degradative systems and their regulation are genetically little studied in comparison to those in *Pseudomonas spp.*

We aim to isolate genes from *Rhodococcus corallina* V49 involved in the degradation of toluene, elucidate the mechanisms responsible for their genetic regulation and characterise the regulation in terms of substrate specificity and catabolite repression.

We have shown that growth of V49 on toluene as a sole carbon source induces an enzyme with catechol 2,3-dioxygenase activity and another (probably an aromatic ring dioxygenase) with an indole to indigo transformation activity. We are screening for these transformation actvities in plasmid libraries expressing V49 DNA constructed both in *E. coli* and *Corynebacterium glutamicum*. In addition we have shown that *R. corallina* V49 contains numerous DNA fragments which cross hybridise to *Pseudomonas* genes involved in aromatic catabolism. We are currently working towards isolating these sequences from a V49 lambda library.

X2-125 CLONING AND CHARACTERIZATION OF GENES FOR THE PARTICULATE METHANE MONOOXYGENASE, Jeremy Semrau, Andrei Chistoserdov, Jose Lebron, Andria Costello, Tonya Peeples and Mary Lidstrom, Keck Laboratories 138-78, California Institute of Technology, Pasadena, CA 91125 and J. Colin Murrell, Department of Biological Science, University of Warwick, Coventry CV4 7AL UK

Methane-oxidizing bacteria (methanotrophs) are capable of cooxidizing halogenated methanes and ethylenes via the methane-oxidation system, the methane monooxygenase (MMO). Two forms of the MMO are known, a membrane-bound form, found in all methanotrophs (particulate, or pMMO) and a cytoplasmic form found as an additional MMO in a few strains (soluble or sMMO). Both enzymes oxidize halogenated methanes and ethylenes, but the sMMO is a high rate, low affinity enzyme, while the pMMO is a low rate, high affinity enzyme. The sMMO has been well-studied, but the pMMO is much less well-known. We have initiated molecular studies of the pMMO by cloning and characterizing the genes encoding the pMMO subunits. N-terminal amino acid sequences were generated for the 45 kDa pMMO subunit from three different methanotrophs, and a conserved region was used to generate an oligonucleotide probe. This probe was used to clone *Methylococcus capsulatus* Bath DNA fragments that contained the gene encoding this subunit, *pmoB*. Sequencing of these fragments revealed the complete *pmoB* as well as the gene encoding the 27 kDa pMMO subunit, *pmoA*, which is present immediately upstream of *pmoB*. Our results show that these genes have high similarity with the analogous genes for the ammonia monooxygenase. In addition, we have also shown that they are present in duplicate copies in the chromosome, and these gene copies seem to be highly similar.

X2-126 COMPLEXOLIZE AS A MECHANISM OF BIODEGRADATION OF INDUSTRIAL WASTE, CONTAINING MANGANESE DIOXIDE. Serebryanaya M.Z., Petrova L.N. Ukraine, Dniepropetrovsk, State University, Institute of Geology.

There are many guantities of industrial waste, containing manganese dioxide. Degradation of this component is correlation with reduction Mn IV - Mn II. Many heterothrophic microorganisms can transformate manganese dioxide in solvable form. The majority of manganese leaching microorganisms are producers of organic acids. We investigated interaction between manganese dioxide and some organic acids, such as citric, α-ketoglutaric, malic, formic, acetic, lactic, piruvatic and succinic. It was shown that mono-, di- and threecarbon acids dissolved manganese dioxide in case if there are oxy- or keto-groups in their structure. Dissolving manganese dioxide by this types of organic acids is correlated with decrease of Eh and increase of pH of solution. Metal-organic acids complex was purified y precepitation in vine spirit. Using elementary analysis it was shown that process of complexolise is accompanied by decarboxylation of organic acid and joining one atom of Mn. It was shown by electron-paramagnetic resonance that valence of manganese in complex is II. Manganese-organic acids complexes are not stable in alkaline media, under this condition (pH>7,5-8,0) manganese precepitated as a manganese hidrooxide. Organic acids residue after precepitations of manganese was leachable of manganese dioxide in acid medium (pH<4,0-4,5). This solution can be utilized once more for leaching manganese dioxide containing wastes.

X2-127 CONTROLLED ENGINEERING OF THE TOLUENE METABOLIC PATHWAY, Rose Sheridan[1], Lucy Regan[1], Graham A. Jackson[1,2], John M. Ward[2] and Peter Dunnill[1], [1]The BBSRC Advanced Centre for Biochemical Engineering, Department of Chemical and Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE. U.K, [2]Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT. U.K.

Metabolic pathways are essentially self-regulating systems, responding to dynamic microenvironments. Control is usually exerted at a number of points along the pathway. The bioengineer often is required to increase the flux through a pathway, maximise a product and/or minimise a waste or toxic by-product. A more informed and controlled approach to pathway engineering is required to maximise the potential of a pathway. The toluate *meta*-cleavage pathway encoded in the *Pseudomonas putida* mt-2 TOL plasmid pWWO is an example of a pathway that has biodegredative potential and produces industrially interesting intermediates as well as toxic intermediates. As an essentially linear metabolic pathway, this pathway was chosen as a model system to test a pathway simulation program developed by Regan *et al.* (1993). Toluate dioxygenase (*xylXYZ*) or 4-oxalocrotonate decarboxylase (*xylI*) was cloned into the broad host range vector pMMB66EH forming pQR189 and pQR194 respectively. Each was introduced into *Escherichia coli* JM107 harbouring a plasmid (pQR150) encoding the entire *meta*-cleavage pathway, forming two double plasmid systems: pQR189 + pQR150 and pQR194 + pQR150. The effect of the amplification of these enzymes, on metabolite concentrations and the flux through the pathway, were determined experimentally and will be discussed with reference to model predictions and data obtained with pQR150 alone.

X2-128 THE KINETICS OF METHANE OXIDATION BY A MARINE METHANOTROPHIC POPULATION AND THE USE OF GENE PROBING TO ASSESS POPULATIONS OF MARINE METHANOTROPHS FOR IN-SITU BIOREMEDIATION OF TCE, Kelly S. Smith and Mary E. Lidstrom, Environmental Engineering Science 138-78, California Institute of Technology, Pasadena, CA 91125.

Bacteria that grow on methane as their sole carbon and energy source (methanotrophs) are capable of degrading a variety of halogenated methanes and ethylenes, including trichloroethylene (TCE). These compounds are common pollutants in a variety of coastal and nearshore marine environments, and therefore, marine methanotrophs are attractive candidates for bioremediation of these contaminants. Detection and identification of these methanotrophs, as well as characterization of their rates of oxidation of methane and TCE, is vital to our understanding of their response to biostimulation protocols for in-situ bioremediation of TCE . Additionally, this information is necessary to the development of predictive models for in-situ bioremediation processes. We have measured the rate of methane oxidation by the native methanotrophic population of an estuarine sediment and determined the Michaelis-Menten parameters K_s and V_{max} for methane. We have isolated several methanotrophs from enrichments of this estuarine sediment, and we have used gene probes based on 16S rRNA sequences to identify the genera of these isolates. These same probes have been used to characterize the methanotrophic genera in sediment when it is enriched under conditions similar to those which might be used for in-situ bioremediation of marine environments.

X2-129 *Abstract Withdrawn*

X2-130 NUCLEOTIDE SEQUENCE AND BIOCHEMICAL PROPERTIES OF A NICOTINAMIDE ADENINE DINUCLEOTIDE-LINKED DEHYDROGENASE ASSOCIATED WITH BENZENE DIOXYGENASE IN *PSEUDOMONAS PUTIDA* ML2, Hai-Meng Tan, Christopher B.H. Goh, Karen P.Y. Fong, Department of Microbiology, National University of Singapore, Lower Kent Ridge Road, Singapore 0511.

Pseudomonas putida ML2 (NCIB 12190) is able to utilise benzene as a sole source of carbon and energy through the *ortho* pathway. Benzene is first converted by the enzyme benzene dioxygenase to *cis*-benzene dihydrodiol. The structural genes for the individual components of benzene dioxygenase have been designated *bedC1C2BA*. Further catabolism of *cis*-benzene dihydrodiol involves an NAD$^+$-dependent dehydrogenation reaction mediated by the enzyme *cis*-benzene dihydrodiol dehydrogenase to form catechol.

P. putida ML2 harbours a catabolic plasmid, pHMT112, which has been reported to contain the *bed* gene cluster encoding benzene dioxygenase and a dehydrogenase for the conversion of benzene to catechol. The nucleotide sequence of the DNA upstream of *bedC1C2BA* on pHMT112 was determined and revealed the presence of a 1098-bp ORF. Both the nucleotide sequence and predicted 365-amino acid sequence exhibited significant homology to glycerol dehydrogenases from *Escherichia coli, Citrobacter fruendii* and *Bacillus stearothermophilus* when searched against the database. *In vitro* translation of the DNA showed a protein of ca. 39 kDa. A DNA fragment containing the dehydrogenase gene (*bedD*) when introduced into *P. putida* 39/D deficient in *cis*-toluene dihydrodiol dehydrogenase restored growth of Pp 39/D with benzene as sole carbon and energy source. The gene was placed under the control of the *tac* promoter and cloned into *E. coli* DH5α. Cell extracts of the transformed strain were subjected to ammonium sulphate precipitation, gel-filtration (Sepharose CL-6B) and ion-exchange chromatography (DE52). Polyacrylamide gel electrophoresis in the presence of SDS showed a subunit of 39 kDa. The purified protein was found to require monovalent cations for activity. It had a rather broad substrate range being able to dehydrogenate glycerol, 1,2-propanediol and *cis*-benzene glycol. The enzyme was specific for NAD$^+$ as cofactor but not NADP$^+$. The association of such a dehydrogenase with an aromatic ring-hydroxylating dioxygenase in *P. putida* ML2 appears rather unique among *Pseudomonas* species capable of growth on aromatic hydrocarbons.

X2-131 **GENETIC DESIGN OF YEAST CYTOCHROME C AS BIOCATALYST FOR POLYCYCLIC HYDROCARBON OXIDATION.** Rafael Vazquez-Duhalt[1], Eduardo Torres[1], J. Victor Sandoval[1], Federico I. Rosell[2] and Grant Mauk[2]. [1]Laboratory of Environmental Biotechnology, Instituto de Biotecnología-UNAM. Apartado Postal 510-3. Cuernavaca, Morelos. 62271 México. [2]Department of Biochemistry, University of British Columbia. Vancouver, B.C. Canada V6T 1Z3.

Iso-1-cytochrome c from *Saccharomyces cerevisieae* is able to oxidize polycyclic aromatic hydrocarbons (PAHs) in presence of hydrogen peroxide. Anthracene and pyrene are oxidized by yeast cytochrome c to form anthraquinone and 1,8-pyrenedione respectively. Seven other hemoproteins (chloroperoxidase, horseradish peroxidase, microperoxidase, horse heart cytochrome c, cytochrome c_{551}, hemoglobin, and lignin peroxidase) and hemin were also tested for the oxidation of pyrene. Chloroperoxidase, lignin peroxidase, hemoglobin and three type-c cytochromes are able to oxidize pyrene in a medium containing 10% of acetonitrile and hydrogen peroxide. Iso-1-cytochrome c from *Saccharomyces cerevisieae* was modified by site-directed mutagenesis in the Phe82 and Cys102 positions. The Phe82 substitution alters significantly the kinetic behavior of the protein, while the Cys102 modification does affect neither kinetic nor stability constants. The Gly82;Thr102 variant is 10 times more active and shows a catalytic efficiency 10-folds higher than the wild-type iso-1-cytochrome c. However, Phe82 variants showed lower stability against inactivation by hydrogen peroxide than the wild-type protein. These site-directed mutations do not alter significantly the stability and activity of the hemoprotein in increasing concentrations of tetrahydrofuran.

X2-132 MOLECULAR GENETIC OF AN ANAEROBIC BACTERIAL STRAIN RESPONSIBLE OF PHENOL TRANSFORMATION TO BENZOATE. Richard Villemur, Tong Li and Jean-Guy Bisaillon. Institut Armand-Frappier, Univ. du Québec, Laval, Canada.

A consortium of bacteria was selected for its capacity to degrade phenol under methanogenic conditions. The consortium was shown to degrade phenol via its carboxylation in the para-position to form 4-hydroxybenzoate which is dehydroxylated to benzoate. Bacteria responsible for the transformation of phenol to benzoate are present in high concentration in the consortium. These phenol-carboxylating microorganisms are nonsyntrophic since inhibition of methanogenesis and presence of hydrogen in the gaseous phase did not prevent the carboxylation of phenol. However, in these latter cultures, the degradation of benzoate is not observed. This suggest that methanogens are required in syntrophy to degrade benzoate further. After heat treatment at 80°C for 15 min., the consortium retained its carboxylating activity, suggesting that spore-forming bacteria such as *Clostridium* are implicated in the first degradation steps of phenol (carboxylation, dehydroxylation) to benzoate. We isolated the bacterial strain directly involved in these steps. We amplified by PCR and sequenced its ribosomal 16S gene. Sequence analysis of this gene revealed that we might be in presence of a new species closely related to *Clostridium* species. Phylogenetic studies are underway as the microbiological identification.

In other anaerobic systems, coA ligase was shown to be involved in degradation of phenolic compounds. Two degenerated oligonucleotides were generated representing respectively a coA ligase consensus sequence. Using the touchdown PCR strategy, we were able to amplify one DNA fragment from the genomic DNA of the carboxylating strain. Sequence analysis from the cloned amplicon revealed that the coA ligase consensus sequences were present. Experiments are underway to confirm a coA ligase activity and the corresponding gene in that bacterial strain.

Late Abstract

LIGNIN PEROXIDASE IS THE KEY ENZYME IN THE DECOLORIZATION OF OLIVE MILL WASTE-WATERS BY PHANEROCHAETE CHRYSOSPORIUM. Sami Sayadi, Fathi Zorgani, Marc Labat, Mohamèd Jaoua, Sami Aifa, Ali Gargouri, Sonia Zekri, and Radhouane Ellouz, Centre de Biotechnologie de Sfax, B.P. "W" 3038 Sfax, Tunisia.

Pollution by olive mill waste-waters (OMW) is becoming a crucial problem in theMediterranean area, particularly with the main producers of oil, Italy, Spain, Greece and Tunisia. This effluent is black and highly toxic due to its high concentration of aromatic compounds. The treatment of OMW by *Phanerochaete chrysosporium* was investigated.

The effect of various physiological parameters such as the nature and concentration of the nitrogen or carbon sources and aeration, on OMW clean up was developed. In high nitrogen/glycerol medium under oxigen, *P. chrysosporium* is able to remove more than 75% of the color and 80% of the chemical oxigen demand (COD). The decolorization of OMW corresponds to a depolymerization of high molecular weight aromatics combined with mineralization of a wide range of monoaromatic compounds.

The relative contribution of lignin peroxidase (LiP) versus manganese peroxidase (MnP) to the decolorization of olive mill waste-waters (OMW) by *Phanerochaete chrysosporium* was investigated. A relatively low level (25%) of OMW decolorization was found when *P. chrysosporium* was grown in a high MnII concentration and in which a high level of MnP (38 nKat/ml) was produced. In contrast, a high degree of OMW decolorization (more than 70 %) was observed when *P. chrysosporium* was grown in low MnII concentration but which resulted in a high level of LiP activity (8 nKat/ml). In this culture medium, increasing the MnII concentration resulted in decreased OMW decolorization and LiP activity. The highest rates of OMW decolorization level were obtained at low initial chemical oxygen demand (COD) combined with high extracellular LiP. This Data, plus the positive effect of veratryl alcohol on OMW decolorization and LiP activity, indicate that LiP plays a more important role than MnP in OMW decolorization.