Perspective

Environmental Biotechnology: Challenges and Opportunities for Chemical Engineers

Wilfred Chen, Ashok Mulchandani, and Marc A. Deshusses
Dept. of Chemical and Environmental Engineering, University of California, Riverside, CA 92521

DOI 10.1002/aic.10487
Published online in Wiley InterScience (www.interscience.wiley.com).

Introduction

Over the past few decades enormous quantities of industrial pollutants have been released into the environment. A large number of them, particularly those structurally related to natural compounds, are readily degraded or removed by microorganisms found in soil and water. However, superimposed on the wide variety of pollutants present in the environment is an increasing number of novel industrial compounds rarely found in nature. These xenobiotic compounds are usually removed slowly and tend to accumulate in the environment (Figure 1). Due to the high degree of toxicity, their accumulation can cause severe environmental problems.

Because of the problems associated with pollutant treatment by conventional methods, such as incineration or landfills, increasing consideration has been placed on the development of alternative, economical and reliable biological treatments. Although natural microorganisms collectively exhibit remarkable evolutionary capabilities to adapt to a wide range of chemicals, natural evolution occurs at a relatively slow rate, particularly when the acquisition of multiple catalytic activities is necessary (Figure 2). In these cases, the acceleration of these events via genetic engineering/processing engineering is helpful since the desirable traits can be carefully designed and controlled. The drive toward this goal represents the essence of environmental biotechnology.

A Chemical Engineering Perspective of Environmental Biotechnology

Environmental biotechnology refers to the utilization of microorganisms to improve environmental quality. Although the field of environmental biotechnology has been around for decades, starting with the use of activated sludge and anaerobic digestion in the early 20th century by civil engineers, the introduction of new technologies from modern microbiology and molecular biology has enabled engineers and scientists to tackle the more contemporary environmental problems such as detoxification of hazardous chemicals. Chemical engineers are uniquely poised to contribute in this emerging area since many of the potential solutions require a combined perspective from modern biology and process engineering, two areas where chemical engineers excel.

For example, the realization of environmental biotechnology into practical solutions requires the implementation of process design, which is the foundation of the chemical engineering discipline. The same is also true at the cellular level, where the functions of cells are determined primarily by networks of specific catalytic reactions. The nature and activities of these networks are dictated by the genetic information, thereby defining the ways in which engineers can influence cellular functions and metabolic capabilities toward the designed of improved biocatalysts for environmental remediation[1]. Although, superficially these strategies seem distant from traditional chemical engineering, a deeper inspection reveals that the design of biological catalysts, based on defined techniques from biochemistry and biology is indeed parallel to our understanding of chemical kinetics, transport, separation, and control. In addition, advances in genomics and proteomics are providing opportunities to predict, in a quantitative manner, the potential manipulations necessary.

Even though chemical engineers are well prepared to contribute new research directions in environmental biotechnology, only a few are working in this area today. Fortunately, the number of chemical engineers showing interest is growing every year and with the recent research emphasis on biotechnology, it is easy to envision that many others will join this exciting research area in the near future. In this article, we will attempt to highlight opportunities available for chemical engineers to make significant contributions and their future challenges.

Engineering Biosorbents for Heavy Metal Removal

Immobilization of heavy metals into biomass or precipitation through reduction to lesser bioactive metal species, such as metal sulfide are the major mechanisms employed by nature (microorganism, animals and plants) to counteract heavy metal toxicity. These natural mechanisms can be easily exploited to optimize biosorbents that are more efficient for heavy metal removal. In one example, a sulfide-dependent metal removal

Correspondence concerning this article should be addressed to W. Chen at wilfred@engr.ucr.edu.

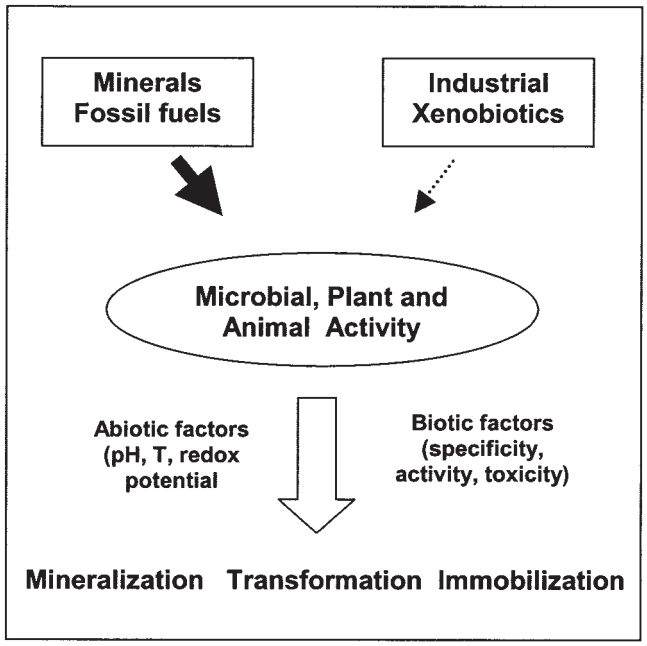

Figure 1. Main sources of environmental pollutants and factors influencing their nature removal from the environment.

strategy was developed by engineering the sulfate reduction pathway into a robust bacterium *E. coli*[2]. The resulting strains produced significantly more sulfide and removed more than 98% of the available cadmium under anaerobiosis. Further improvement in metal precipitation was achieved by engineering effective sulfate reduction under aerobic conditions. *E. coli* expressing both serine acetyltransferase and cysteine desulfhydrase overproduced cysteine and converted it to sulfide.[3] The resulting strain was effective in aerobically precipitating cadmium. This aerobic approach of metal precipitation is particularly attractive as large-scale processes could be implemented under aerobic conditions. The challenges are to incorporate these genetic modifications into a robust environmental microbe that could survive and thrive under the required operation conditions.

Similar success in engineering enhanced biosorbents has been achieved by displaying metal-binding peptides onto the cell surface. One example was recently reported by creating a repetitive metal-binding motif consisting of $(Glu\text{-}Cys)_nGly$.[4] These peptides emulate the structure of phytochelatins, metal-chelating molecules that play a major role in metal detoxification in plants and fungi. The phytochelatin analogs were presented on the bacterial surface, enhancing Cd^{2+} and Hg^{2+} bioaccumulation by 12-fold[4] and 20-fold,[5] respectively. Unlike nature metal-binding peptides, these "*de novo*" designed metal-binding peptides are attractive as they offer the potential of improved affinity and selectivity for heavy metals. To this end, the use of molecular modeling[6] and evolutionary strategies[7] may enable the rapid discovery of novel peptide sequences that are superior metal chelators.

In addition to peptides, metalloregulatory proteins are another group of useful metal-binding moiety with striking affinity and specificity. The highly specific nature of these proteins is the result of a cleverly designed genetic circuit that is tightly under their control.[8] Examples are MerR and ArsR, which are regulatory proteins used for controlling the expression of enzymes responsible for mercury and arsenic detoxification, respectively.[9-10] The high affinity and selectivity of MerR toward mercury has been exploited for the construction of microbial biosorbents specific for mercury removal.[11] Presence of surface-exposed MerR on an engineered strain enabled 6-fold higher Hg^{2+} biosorption. Hg^{2+} binding via MerR was very specific with no observable decline even in the presence of 100-fold excess Cd^{2+} and Zn^{2+}. Similarly, cells overexpressing ArsR accumulated 5- and 60-fold higher levels of arsenate and arsenite with no observable binding to other heavy metals.[12] As nature provides a wide range of metalloregulatory proteins for other important metal pollutants, this strategy may be used as a universal approach to enable selective binding of target metals of interest.

Designer Strains for Enhanced Biodegradation

Using well-established tools from metabolic engineering and biochemistry, efforts have been made on engineering microbes to function as "designer biocatalysts,"[13-14] in which certain desirable traits are brought together with the aim of optimizing the rate and specificity of biodegradation pathways. One common bottleneck is the transport of pollutants across the cell membrane, which limits the overall rate in many microbial biodegradation. An example is for a class of neurotoxic organophospates, which are used extensively as pesticides and chemical warfare agents. Although an enzyme, organophosphorus hydrolase (OPH), has been shown to degrade these pesticides effectively, the use of whole cell detoxification is limited by the transport barrier of substrates across the cell membrane. Display of OPH onto the cell surface has been employed to bypass this transport barrier, resulting in 7-fold faster degradation compared to whole cells expressing OPH intracellularly.[15] This simple approach typified the unique combination of chemical engineering principle with modern genetics, and has been similarly employed for other useful environmental applications such as the display of metal-binding proteins described earlier. Although only fairly simple enzymes or peptides are successfully displayed so far, continued development in this area should pave the way for the successful display of more complex enzymes, such as dioxygenases or monooxygenases, enabling a broader class of pollutants to be targeted.

Recruiting different pathways into a designer microbe is another powerful approach to enhance biodegradation. Very often, these pathways are combined with other existing pathways to enable complete biodegradation. For example, construction of a hybrid strain which is capable of mineralizing components of a benzene, toluene, and *p*-xylene mixture simultaneously was attempted by redesigning the metabolic pathway of *Pseudomonas putida*.[13] A hybrid strain carrying both the *tod* and the *tol* pathways was constructed and was found to mineralize a benzene, toluene, and *p*-xylene mixture without accumulation of any metabolic intermediate. Since the number of known biodegradation pathways is increasing everyday, this in combination with the increasing number of genome sequence elucidated for environmental microbes, should allow us to rationally combine useful pathways across species into any desirable combinations using tools available from metabolic engineering. In this respect, it will also be interesting to see whether multiple enzymes can also be dis-

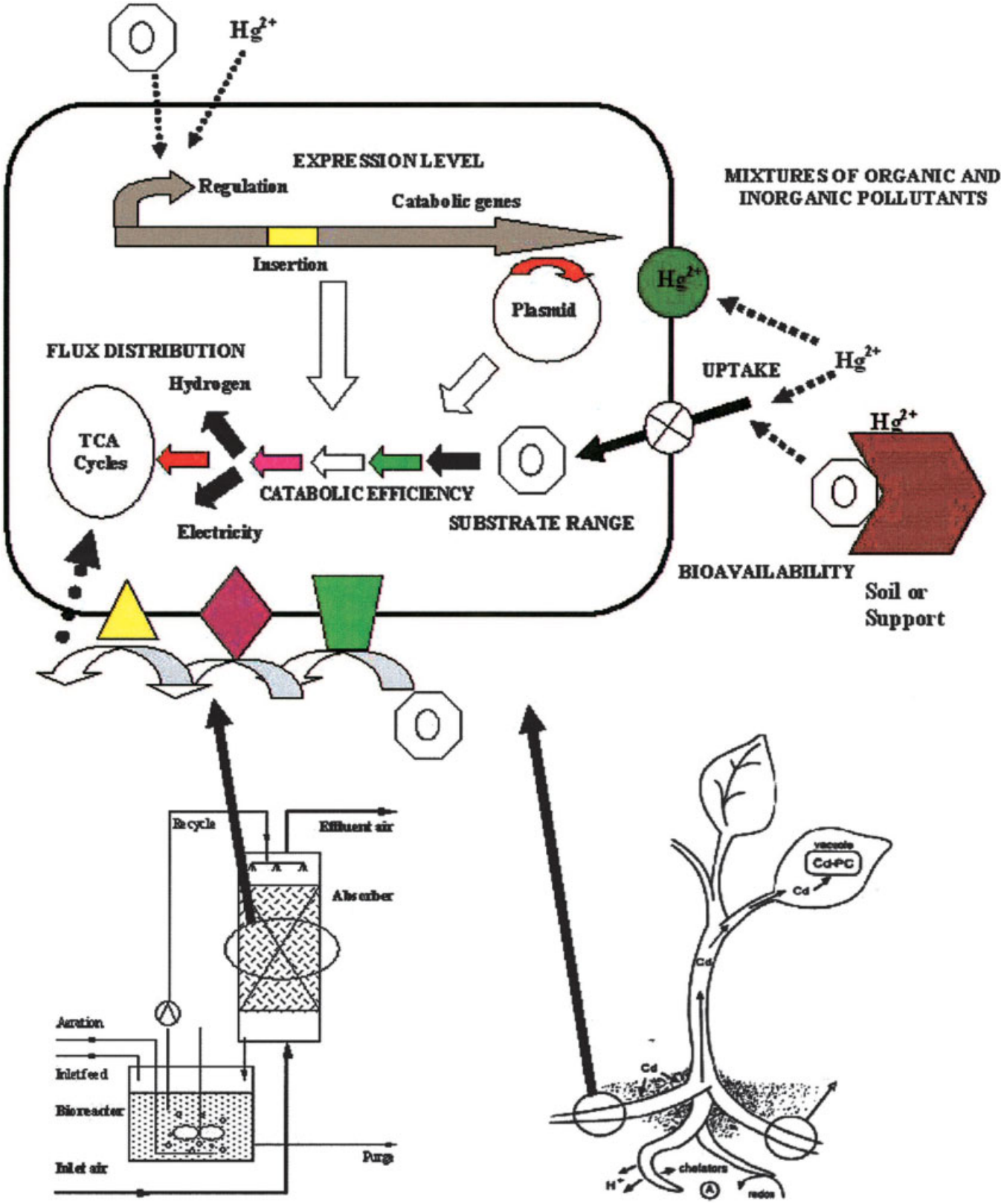

Figure 2. Potential opportunities and strategies in environmental biotechnology.

The major bottlenecks for *ex-situ* or *in-situ* applications are capitalized.

played onto the surface, allowing sequential degradation to occur without any uptake limitation. The challenges here are to devise strategies that will allow not only multiple enzyme display, but also the display of complex enzymes without compromising integrity and viability.

Another promising approach for success remediation is the introduction of biodegradation pathways into microbes that thrive in the contaminated environment. *Deinococcus radiodurans* is a soil bacterium that can survive acute exposures to ionizing radiation of 15,000 Gy without lethality.[16] A recombinant *D. radiodurans* strain expressing toluene dioxygenase was shown to effectively oxidize toluene, chlorobenzene, and TCE in a highly irradiating environment.[17] The recombinant strains were also tolerant to the solvent effects of toluene and TCE at levels exceeding those of many radioactive waste sites. The prospect of using this strategy to alleviate the toxicity of radionuclides and heavy metals, and to provide efficient treatment for a variety of organic wastes is very promising.

Similarly selective advantages can be achieved by exploiting the synergistic plant-microbe relationship in a rhizoshere. This strategy was recently reported using a wheat rhizosphere system for the detoxification of soil-borne trichloroethylene (TCE).[18] The toluene *o*-monooxygenase (Tom) gene was introduced into *Pseudomonas fluorescents* 2-79, a bacterium that colonizes the wheat root, enabling the establishment of a bacterium-plant-soil microcosm. Treatment of TCE-contaminated surface and near-surface soil was demonstrated, with more than 63% of the initial TCE removed within 4 days. The most attractive aspect of this technology is the low cost associated since only expenses required for planting is necessary. Our group is actively pursuing this strategy by engineering both metal-binding and TCE capabilities into a single rhizobacterium, allowing it to retain TCE degradation in the presence of high level of metal contamination. Since over 40% of all superfund sites in the U.S. are co-contaminated with organic pollutants and heavy metals, the use of plant-microbe rhizoremediation will provide an ecologically sound and safe method for restoration and remediation. This will also represent an excellent opportunity for chemical engineers working primarily with microorganisms to collaborate with others focusing on plant, combining the unique features of rhizoremediation with phytoremediation.

A notable opportunity that has been so far overlooked by most chemical engineers is the production of valuable products and energy directly from wastewater.[19] Of particular interest is the possibility of biohydrogen and bioelectricity production. In most cases, only natural microorganisms are exploited, resulting in fairly modest yields. However, this poor conversion also represents an excellent opportunity to employ the tools from metabolic engineering, protein engineering, molecular evolution, and system biology for the discovery of novel microorganisms with significantly improved efficiencies.

Enzyme Engineering for Improved Biodegradation

The ever-increasing information regarding the structure and function of enzymes and pathways involved in biodegradation of recalcitrant pollutants offers opportunities for improving enzymes or entire pathways by genetic engineering. Control mechanism and enzyme properties can be tailored by site directed mutagenesis, which is often guided by computer-assisted modeling of the three-dimensional(3-D) protein structures. For example, site directed approaches have been applied to enlarge the binding pocket of haloalkane dehalogenase,[20] resulting in several-fold faster dechlorination of dichlorohexane. However, no mutant tested could utilize the more bulky substrates, such as TCE, suggesting limitations using this structural based approach. Perhaps the use of computational methods to predict subtle and distal changes in the protein backbone without perturbing the overall protein structure could be used to further improve enzyme function and stability.

Site-directed or rational approaches can often fail because it is known that mutations far from the active site can modulate catalytic activity or substrate recognition but are difficult to predict *a priori.* These methods are also restrictive because they allow the exploration of only a very limited sequence space at a time. This is clearly indicated by the creation of several chimeric enzymes guided by sequence comparison between two similar biphenyl dioxygenases.[21] Although the resulting variants were capable of hydroxylating both double *ortho*- and *para*-substituted PCBs, combining the substrate range of the two parental enzymes, no new activity was observed. In this case, irrational approaches such as DNA shuffling,[22] which allow the cross-breeding of genes between diverse classes of species, can be a preferable alternative to direct the evolution of enzymes or pathways with highly specialized traits. In two independent studies, the substrate range of biphenyl dioxygenases toward PCBs has been successfully extended using directed evolution.[23-24] Variants were obtained by random shuffling of DNA segments between the large subunit of two wild type biphenyl dixoxygenases. Several variants had extended substrate ranges for PCBs exceeding those of the two parental enzymes.

Similar attempts to extend the substrate specificity of toluene *ortho*-monooxygenase (TOM)[25] and OPH[26-27] have been successful. In both cases, DNA shuffling was combined with simple plate screening assays, resulting in rapidly degradation of virtually nondegradable substrates. These examples are perhaps the best reminder, suggesting that other important biodegradation enzymes could be similarly improved with this strategy since the number of related dioxygenases, monoxygenases, and hydrolases for different pollutants are virtually unlimited. Molecular evolution is probably the most useful way for evolving biodegradation enzymes for extended substrate specificities since microbial degradation of xenobiotics is usually by cometabolism and does not exert a natural selective pressure on bacteria. Computational methods that are useful to guide experimental design for directed evolution may be used to predict the optimal number of mutants that must be screened. Moreover, an optimal design of the parental DNA sequence set will allow a more focused probing of sequence space in only those regions that are likely to yield functional hybrids and should lead to a more efficient utilization of experimental resources, saving time and effort by reducing the number of evolutionary cycles.

Evolutionary and Genomic Approaches to Biodegradation

Evolutionary approaches are extremely useful for optimization of an entire biodegradation pathway comparing to step-by-step modifications offered by rational design. This was recently demonstrated by the modification of an arsenic resistance operon using DNA shuffling.[28] Cells expressing the op-

timized operon grew in up to 0.5 M arsenate, a 40-fold increase in resistance. Moreover, a 12-fold increase in the activity of one of the gene products (arsC) was observed in the absence of any physical modification to the gene itself. The authors speculate that modifications to other genes in the operon effect the function of the *arsC* gene product. Such unexpected but exciting results are more likely to be realized using irrational approaches. This strategy is particularly attractive since the ultimate goal of many remediation approaches is for complete mineralization of the pollutants, and the concurrent optimization of an entire pathway will allow the efficient search for the correct coordination between a complex set of biodegradation reactions. Along the same line, recent advances in genome shuffling between species, which allow the exchange and recombination of diverse pathways into a single species,[29] will further accelerate the discovery of novel microbes that are useful for the remediation of even a complex mixture of pollutants.

The availability of bacterial genomes relevant to biodegradation in recent years has allowed the feasibility to study the complex interactions between cellular reactions from a genomic[30] and proteomic[31] level. A quantitative understanding of how cells function requires every gene and protein to be placed in their dynamic context, which entails the integrated consideration of many interacting components. From this perspective, a system biology approach is necessary to predict the functioning of an organism in a complex environment and to describe the outcome of the thousands of individual reactions that are simultaneously taking place in a microbial cell. So far, such prokaryotic models have been limited primarily to *E. coli* and a few pathogens. However, similar modeling approaches should be able to predict contaminant bioremediation by microorganisms that are known to predominate in polluted environments. Recently, de Lorenzo and coworkers[32] presented a pioneering study on the characteristics of the "global biodegradation network", in which they considered the global pool of known chemical reactions implicated in biodegradation regardless of their microbial hosts. The characteristics of this network support an evolutionary scenario in which the reactions evolved from the central metabolism toward more diversified reactions, allowing us to understand the evolution of new pathways for the degradation of xenobiotics and provide the basis for predicting the abilities of chemicals to undergo biological degradation, and for quantifying the evolutionary rate for their elimination in the future. This type of analysis, when coupled with the predictive approach for microbial catabolism using the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD) as a knowledge base and various sets of heuristic rules,[33] will lead to untapped and improved strategies for bioremediation. This represents an excellent opportunity for chemical engineers who are already involved with system biology, and will undoubtedly evolve into an important research direction within the next 5 years.

Process Engineering for Improved Biodegradation

Scientists have generally considered biological treatment processes too inefficient to challenge chemical treatment processes, particularly in treating large volumes of waste. The typically long contact time of 10 s or even longer between the pollutants and microbes would require an impractically large process for a practical treatment plant. However, it is well known that biological processes are generally safer, greener, and cheaper to run. Recently, advances in process design have brought this dream into reality by converting a chemical scrubber that removes hydrogen sulfide (a gas that smells like rotten-egg) into a biotrickling filter, which reduces hydrogen sulfide into non-smelly sulfate.[34] To achieve a gas contact time that is comparable with that of a chemical scrubber (1.6-2.3 s), the researchers passed contaminated air through the biofilter at a high velocity using a packing material with a high surface area. The unusually high air velocity resulted in a higher gas-film mass-transfer coefficient and outstanding H_2S removal. After almost 2 years of continuous operation, the bioscrubbers still retained H_2S removal efficiencies of more than 98%. This finding is important as it suggests that substantial improvement in process performance could be achieved using a very simple design improvement, based on chemical engineering principles. The obvious challenges are whether similar conversions of chemical treatment processes into biological processes could be achieved, offering the same treatment capacity that are much cheaper and safer. Considering the cost benefit and environmental impact of switching to biological treatment processes, this is one opportunity that chemical engineers cannot afford to miss.

Conclusions

Undoubtedly, the numbers of problems that define environmental biotechnology are not restricted to those discussed in this article. However, it is our opinion that these perhaps represent the best opportunities for chemical engineers to make significant contributions, because of the unique intertwining of molecular biology, microbiology, reaction engineering, transport phenomena, and process design. Because of the interdisciplinary nature of this research area, a successful chemical engineer in the field must receive training in an interdisciplinary environment where expertise from different areas is available, a trend that is consistent with the evolving nature of chemical engineering education.

Although the ability to predictively design microbes or enzymes for any given remediation remains an overwhelming task, the increasing understanding of fundamental mechanistic principles generated from both genomic research or directed evolution will likely lead to the emergence of novel solutions for improved bioremediation. Chemical engineers must embrace this important opportunity in a fashion similar to the efforts invested on the early evolution of metabolic engineering and tissue engineering, two important areas that are now partially defined by chemical engineers.

Acknowledgment

The financial supports from NSF (BES0422791, BES0329482, and BES0331416) and U.S. EPA (R829606) are gratefully acknowledged.

Literature Cited

1. Bailey JE. Toward a science of metabolic engineering. *Science.* 1991;252:1668-1691.
2. Bang SW, Clark DS, Keasling JD. Engineering hydrogen

sulfide production and cadmium removal by expression of the thiosulfate reductase gene (phsABC) from Salmonella enterica serovar typhimurium in *Escherichia coli*. *Appl Environ Microbiol*. 2000;66:3939-3944.
3. Wang CL, Maratukulam PDL, Clark DS, Keasling JD. Metabolic engineering of an aerobic sulfate reduction pathway and its application to precipitation of cadmium on the cell surface. *Appl Environ Microbiol*. 2000;66:4497-4502.
4. Bae W, Chen W, Mulchandani A, Mehra R. Enhanced bioaccumulation of heavy metals by bacterial cells displaying synthetic phytochelatins. *Biotechnol Bioeng*. 2000; 70:518-524.
5. Bae W, Mehra R, Mulchandani A, Chen W. Genetic engineering of *Escherichia coli* for enhanced uptake and bioaccumulation of mercury. *Appl Environ Microbiol*. 2001;67:5335-5338.
6. Klemba M, Gardner KH, Marino S, Clarke KD, Regan L. Novel metal-binding proteins by design. *Nat Struct Biol.* 1994; 2:368-373.
7. Mejare M, Ljung S, Bulow L. Selection of cadmium specific hexapeptides and their expression as OmpA fusion proteins in *Escherichia coli. Protein Eng*. 1998;**11:**489-494.
8. Bontidean I, Berggren C, Johansson G, Csorgi E, Mattiasson B, Lloyd JR, Jakeman KJ, Brown NL. Detection of heavy metal ions at femtomolar levels using protein-based biosensors. *Anal Chem*. 1998;70:4162-4169.
9. O'Halloran TV, Frantz B, Shin MK, Ralston DM, Wright JG. The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. *Cell*. 1989;56:119-129.
10. Diorio C, Cai J, Marmor J Shinder R, DuBow MS. An *Escherichia coli* chromosomal *ars* operon homolog is functional in arsenic detoxification and is conserved in gram-negative bacteria. *J Bacteriol*. 1995;177:2050-2056.
11. Bae W, Wu C, Kostal J, Mulchandai A, Chen W. Enhanced mercury biosorption by bacterial cells with surface-displayed MerR. *Appl Environ Microbiol*. 2003;69: 3176-3180.
12. Kostal J, Yang R, Wu CH, Mulchandani A, Chen W. Enhanced arsenic accumulation in engineered bacterial cells expressing ArsR. *Appl Environ Microbiol*. 2004;70**:** 4582-4587.
13. Lee JY, Roh JR, Kim HS. Metabolic engineering of *Pseudomonas putida* for the simultaneous biodegradation of benzene, toluene, and *p*-xylene mixture. *Biotechnol Bioeng*. 1994;4:1146-1152.
14. Wackett LP, Sadowsky MJ, Newman LM, Hur HG, Li S. Metabolism of polyhalogenated compounds by a genetically engineered bacterium. *Nature*. 1994;368:627-629.
15. Richins R, Kaneva I, Mulchandani A, Chen W. Biodegradation of organophosphorus pesticides by surface-expressed organophosphorus hydrolase. *Nature Biotechnol*. 1997;15:984-987.
16. Daly MJ, Ouyang L, Minton KW. *In vitro* damage and *recA*-dependent repair of plasmid and chromosomal DNA in the radioresistant bacterium *Dinococcus radiodurans***.** *J Bacteriol*. 1994**;**176:3508-3517.
17. Lange CC, Wackett LP, Minton KW, Daly MJ. Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments. *Nat Biotechnol*. 1998;16:929-933.
18. Yee DC, Maynard JA, Wood TK. Rhizoremediation of trichloroethylene by a recombinant root-colonizing *Pseudomonas fluorescens* strain expressing toluene *ortho*-monooxygenase constitutively. *Appl Environ Microbial*. 1998;64:112-118.
19. Angenent LT, Karim1 K, Al-Dahhan1 MH, Wrenn BH, Domý′guez-Espinosa R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends Biotechnol*. 2004; 22:477-482.
20. Holloway P, Knoke KL, Trevors JT, Lee H. Alteration of the substrate range of haloalkane dehalogenase by site-directed mutagenesis. *Biotechnol Bioeng*. 1998;59**:**520-523.
21. Kimura N, Nishi A, Goto M, Furukawa K. Functional analysis of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. *J Bacteriol* 1997;179:3996-3943.
22. Stemmer WPC. Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature*. 1994;370**:**389-391.
23. Kumamaru T, Suenaga H, Mitsuoka M, Watanabe M, Furukawa K. Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase**.** *Nat Biotechnol*. 1998;16**:**663-666.
24. Brühlmann F, Chen W. Tuning biphenyl dioxygenase for extended substrate specificity. *Biotechnol Bioeng*. 1999; 63:544-551.
25. Canada KA, Iwashita S, Shim H, Wood TK. Directed evolution of toluene ortho monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *J Bacteriol.* 2002;184:344-349.
26. Cho CM-H, Mulchandani A, Chen W. Bacterial cell surface display of organophosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. *Appl Environ Microbiol*. 2002;68:2026-2030.
27. Cho C.M-H, Mulchandani A, Chen W. Altering the substrate specificity of organophosphorus hydrolase for enhanced hydrolysis of chlorpyrifos. *Appl Environ Microbiol.* 2004;70**:**4681-4685.
28. Crameri A, Dawes G, Rodriguez E Jr, Silver S, Stemmer WPC. Molecular evolution of an arsenate detoxification pathway by DNA shuffling. *Nat Biotechnol*. 1997;15:436-438.
29. Dai MH, Copley SD. Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum ATCC 39723*. *Appl Environ Microbiol.* 2004;70:2391-2397.
30. Jimenez JI, Minambres B, Garcia JL, Diaz E. Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ Microbiol*. 2002**;**4:824-841.
31. Reardon KF, Kim KH. Two-dimensional electrphoresis analysis of protein production during growth of *Pseudomonas putida* F1 on toluene, phenol, and their mixture. *Electrophoresis*. 2002;23:2233-2241.
32. Pazos F, Valencia A, de Lorenzo V. The organization of the microbial biodegradation network from a systems-biology perspective. *EMBO Rep.* 2003;4:994-999.
33. Hou BK, Wackett LP, Ellis LB. Microbial pathway prediction: a functional group approach. *J Chem. Inf. Comput. Sci*. 2003;43:1051-1057.
34. Gabriel D, Deshusses MA. Retrofitting existing chemical scrubbers to biotrickling filters for H_2S emission control. *Proc Natl Acad Sci USA*. 2003;100:6308-6312.