Trends in Biotechnology

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Review

Engineering Biocatalytic and Biosorptive Materials for Environmental Applications

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The challenges of increasing environmental contamination and scarcity of natural resources require innovative solutions to ensure a sustainable future. Recent breakthroughs in synthetic biology and protein engineering provide promising platform technologies to develop novel engineered biological materials for beneficial applications towards environmental sustainability. In particular, biocatalysis and biosorption are receiving increasing attention as sustainable approaches for environmental remediation and resource recovery from wastes. This review focuses on up-to-date advances in engineering biocatalytic and biosorptive materials that can degrade persistent organic contaminants of emerging concern, remove hazardous metal pollutants, and recover value-added metals. Opportunities and challenges for future research are also discussed.

Highlights

Advances in synthetic biology and protein engineering provide promising platform technologies for engineering renewable biomaterials with biocatalytic or biosorptive capabilities to address the crucial challenges of environmental pollution and natural resource scarcity.

Biocatalytic materials that harness the function of various biological enzymes to degrade recalcitrant contaminants to environmentally benign compounds can be developed using molecular biology techniques and physicochemical processes; these materials demonstrate great potential for environmental remediation applications.

Biosorptive materials engineered by decorating microbial cells with specific metal-binding proteins/peptides using genetic engineering can be used for hazardous metal removal and valuable metal recovery with high selectivity and binding efficiency.

Emerging Environmental Challenges

The pursuit of environmental sustainability is a key driving force for developing effective technological means to address the formidable challenges regarding the growing environmental pollution and natural resource scarcity. Recent literature has reported widespread detection of **contaminants of emerging concern** (see Glossary) in the environment. These contaminants tend to be persistent or only partially removed by conventional wastewater treatment and remediation processes, and they can pose severe risks to human health and the ecosystem even at trace concentrations. Therefore, there is a crucial need to develop advanced technologies for treating persistent contaminants in environmental remediation. Meanwhile, there is an ongoing paradigm-shift to view wastes as resources rather than economic burdens. Waste-based biorefining is of growing interest, and new technological development to this end is crucially important to recover valuable products while valorizing wastes and meeting environmental quality goals.

Synthetic biology and protein engineering technologies have been exploited for creating bioactive materials in a drive towards green chemistry for biomedical or industrial biotechnology applications, but the promise of these technologies in developing innovative strategies for beneficial environmental applications has only been recognized very recently. Particularly exciting research progress has been made in developing novel biocatalysts for efficient degradation of persistent organic contaminants [1,2]. In addition, engineered biosorptive materials have attracted significant interest as promising and cost-effective alternatives for removing and recovering metals from waste streams [3]. This review article will discuss up-to-date research efforts in engineering biocatalytic and biosorptive materials for environmental remediation and resource recovery applications, as well as some opportunities and challenges in this nascent field.

Engineering Biocatalytic Materials for Contaminant Remediation

Biocatalysis typically proceeds under ambient temperature and pressure, generates few toxic byproducts, has low energy requirements, and is an easier process to control. Notably,

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biocatalytic processes have been demonstrated for applications where no efficient synthetic chemical methods are yet to be developed [4]. Given these advantages, biocatalysis has been exploited for beneficial applications such as pharmaceutical synthesis and the production of value-added chemicals and renewable fuels in a drive towards green chemistry [5]. With recent progresses in discovering novel enzymatic functions and advances in technologies for engineering biocatalytic materials, biocatalysis receives increasing attention in environmental remediation. Particularly, among six classes of enzymes, oxidoreductases and hydrolases are of growing interest for degrading toxic compounds to environmentally benign products (Box 1).

Generation and Modification of Enzymes

Recombinant protein expression, where DNA encoding a target protein is cloned into a host organism such that it produces a large amount of the protein, has made it easier to generate enzymes for biotechnology applications. In the traditional approach, the enzyme of interest is purified from its original source such as bacteria, fungi, or plants, but such an approach may not be suitable for large-scale applications owing to low enzyme yield and cumbersome and costly purification procedures. By contrast, **recombinant DNA technology** facilitates the expression and purification of a desired enzyme in large quantities and allows redesign of a wild-type enzyme to enhance its stability, activity, or specificity through genetic modifications. Recent studies have

Box 1. Enzymes for Contaminant Degradation

Enzymes are highly efficient biological catalysts that can degrade toxic compounds to environmentally benign products, and they are divided into six major classes. Of these classes, oxidoreductases and hydrolases have been most studied for the degradation of toxic compounds to environmentally benign products owing to their high catalytic activity and ability to target a broad range of substrates including xenobiotic organic compounds. Other groups of enzymes also hold promise in the catalysis of contaminant transformation reactions and have demonstrated great potential in industrial and biotechnological applications.

Oxidoreductases catalyze the transfer of electrons from one molecule (the reductant) to another molecule (the oxidant). Oxidoreductases of biotechnological interest typically include laccases, peroxidases, and oxygenases. Laccases, which are multicopper-containing oxidases capable of performing one-electron oxidation of a broad range of substrates using oxygen as the electron acceptor, can transform various persistent organic contaminants such as endocrine disrupting compounds, pesticides, and pharmaceuticals [58]. Peroxidases catalyze oxidative transformation of organic pollutants through a free radical mechanism by using hydrogen peroxide as the electron acceptor. Horseradish peroxidases (HRPs), manganese peroxidases (MnPs), lignin peroxidases, and chloroperoxidases have been reported for remediation applications [59].

Hydrolases are hydrolytic enzymes that use water to catalyze the cleavage of chemical bonds, usually breaking a large molecule into two smaller molecules. Examples of hydrolases of biotechnological interest include esterases, proteases, lipases, amylases, acylases, and phosphatases. Lipases, cellulases, and haloalkane dehalogenases can hydrolytically degrade various persistent contaminants such as petroleum pollutants from oil spills, as well as organophosphates and carbamate insecticides [60].

Transferases catalyze the transfer of specific functional groups such as methyl, hydroxymethyl, phosphate, or sulfate from one molecule to another. Transferases are commonly used in industrial biotechnology for the synthesis of chemicals.

Ligases catalyze the formation of chemical bonds between two large molecules, usually accompanied by hydrolysis of a pyrophosphate bond in ATP or another high-energy donor. Enzymes that catalyze joining of C–O, C–N, or C–S bonds belong to the class of ligases. An important example is the DNA ligase, an essential reagent for recombinant DNA technology.

Lyases catalyze the breaking of various chemical bonds other than hydrolysis or oxidation. This class of enzymes are often used as biocatalysts for chemical production. Isomerases catalyze the structural rearrangement of a molecule to convert it to its isomer, and are principally used in the manufacture of sugar compounds.

Glossary

Biocatalysis: the use of enzymes or microbial cells to facilitate a chemical transformation process.
Combinatorial library: collections of large numbers of DNA or protein variants that are produced by random mutagenesis in a combinatorial fashion.
Contaminants of emerging concern: synthetic or naturally occurring chemicals that have not been commonly monitored in the environment but that have potential to enter the environment and pose adverse effects to human health and ecosystems.
Curli amyloid fibers: proteinaceous component of the complex extracellular matrix produced by many *Enterobacteriaceae* that is responsible for surface adhesion, cell aggregation, and biofilm formation.
Directed evolution: a laboratory process used in protein engineering by which biological entities with desired traits are created through iterative rounds of genetic diversification and library screening or selection. Specifically, in directed evolution, a collection of random protein variants (a 'library') is generated by introducing multiple mutations into the gene encoding the target protein, and then proteins with improvements in desired properties can be obtained by screening/selection of the library.
Genetic engineering toolbox: the set of molecular techniques for engineering and manipulating genetic functions to achieve desired biological traits.
Recombinant DNA technology: a method that artificially brings together DNA molecules from two or more different species followed by transformation into a host organism to produce new genetic combinations of interest.
Lanthanide binding tags (LBTs): short peptide sequences comprising 15–20 amino acids that are specifically optimized to bind trivalent lanthanide ions (Ln^{3+}) with high affinity and selectivity.
Metal homeostasis and resistance: the ability of organisms to maintain levels of metal ions within physiological limits and detoxify excess metal ions inside the cells.

reported the production of recombinant laccases in various host organisms including bacteria, yeasts, and filamentous fungi [6]. In addition, horseradish peroxidases (HRPs) have been successfully expressed in the yeasts *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Cryptococcus* sp. S-2 [7]. The resulting recombinant HRPs were used to degrade synthetic dyes and estrogens. Production of recombinant hydrolases with great contaminant remediation potential, such as lipases, carboxylesterases and cutinases, has also been demonstrated [8–10].

The advances in protein engineering remarkably enable the development of biocatalytic materials with novel function and improved performance [11]. In general, protein engineering approaches include rational design (e.g., site-directed mutagenesis), random methods (e.g., random mutagenesis), and **directed evolution**. Rational design is a classical and effective approach when the structure and mechanism of the target protein are well known. For example, a very recent paper reported a novel fungal polyphenol oxidase that was engineered via site-directed mutagenesis to construct enzyme variants with increased activity and altered specificity in bioremediation of different chlorophenols [12]. By contrast, directed evolution can generate a remarkable range of new functional properties such as improved enzyme activity, stability, specificity, and selectivity in the absence of detailed knowledge of protein structure or catalytic mechanism [13]. As a powerful protein engineering technique, directed evolution mimics Darwinian evolution in the test tube to efficiently modify and evolve proteins, leading to the discovery of new reactivity that is extremely challenging to design [14]. Directed evolution has been effectively applied to evolving the microbial enzymes associated with broad-spectrum organophosphorus pesticide degradation, which resulted in a substantial enhancement in both the removal efficiency and stereoselectivity for the more toxic isomers of these compounds [15]. Recently, an ultrahigh-throughput screening method by embedding the enzyme in biomimetic gel-shell beads was developed [16], which considerably facilitated the directed evolution of a pesticide-degrading enzyme phosphotriesterase with 20-fold increase in enzyme activity. The platform technology holds great promise to modify various enzymes for enhancing their performance in environmental applications.

Metal transporter: proteins associated with uptake and efflux of metal ions across the plasma and organelle membranes.
Metalloregulatory proteins: a battery of regulatory proteins that can selectively recognize and coordinate with specific metal ions to physiologically mediate the metal homeostasis of organisms.
Metallothioneins (MTs): a group of cysteine-rich proteins that are able to bind heavy metal ions with high affinity via cysteine residues in Cys–Cys, Cys–X–Cys or Cys–X–X–Cys motifs.
PC synthase: the enzyme that catalyzes the synthesis of phytochelatin.
Phytochelatins (PCs): a family of small glutathione-based oligomers found in many plants which are capable of efficient sequestration of multiple types of metal and metalloid ions. The general structure for natural PCs is $(\gamma\text{-Glu–Cys})_n$–Gly and for synthetic PCs is $(\alpha\text{-Glu–Cys})_n$–Gly.
Rare earth elements (REEs): a set of 17 chemical elements in the periodic table, including 15 lanthanides, scandium, and yttrium.
S-layer: 2D monomolecular arrays consisting of one or more (glyco) proteins that are frequently found on the surface of prokaryotic cell envelope.

Immobilized Enzyme Materials for Contaminant Degradation

A successful system using enzyme biocatalysis must ensure high catalytic efficiency and enzyme stability while enabling the reuse and recycling of biocatalysts for efficient and cost-effective processes. Using free enzymes in solution for contaminant treatment is not suitable or economical owing to short enzyme lifetimes, non-reusability, and the high cost of single use. Immobilized enzymatic materials have received increasing attention as a strategy to improve enzyme stability and reusability. In general, immobilized enzymatic materials can be constructed by: (i) immobilizing enzymes through adsorption or covalent binding to a supporting matrix (e.g., porous glass beads, alginate–carbon composites, organic gels, and nanoparticles); (ii) entrapping or encapsulating enzymes within gels, fibers or semi-permeable membranes; and (iii) fabricating enzyme aggregates via crosslinking [17]. These strategies are illustrated in Figure 1A. The enzymes immobilized on the solid materials are more stable than free enzymes and can be readily recovered and reused. However, there are still limitations in these conventional immobilization methods, including impairment of enzyme activity owing to denaturation by chemical reagents used in covalent attachment and crosslinking processes, costly and time-consuming protein isolation and preparation, and mass-transfer limitations for entrapment and encapsulation systems.

The selection of proper supporting matrix materials is crucially important for preparing functional and robust immobilized enzyme materials. Desirable characteristics of an ideal supporting material include non-toxicity, high capacity for enzyme loading, mechanical stability and

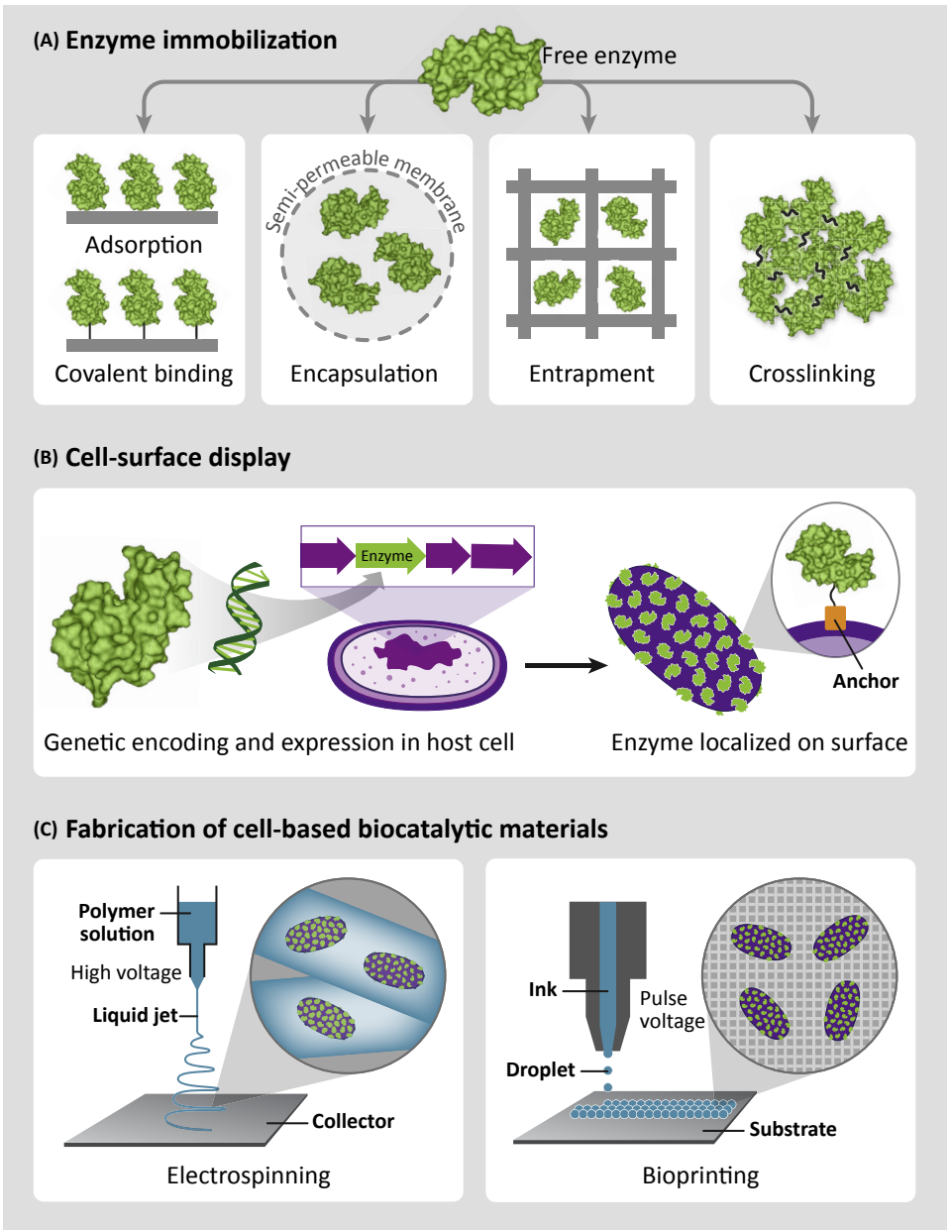

Figure 1. Schematic Illustration of Engineering Biocatalytic Materials through (A) Conventional Enzyme Immobilization, (B) Cell-Surface Display, and (C) Cell Immobilization by Electrospinning and Bioprinting.

rigidity, feasibility of regeneration, and economical accessibility. In recent years, various novel supporting materials have been developed for enzyme immobilization, including active membranes, vesicular silica, and nanomaterials (e.g., nanoparticles, nanofibers, nanotubes, and nanocomposites) [18]. These materials can be designed with new functionality to enhance enzyme stability, facilitate recycling, and increase catalytic efficiency. For example, using nanomaterials with superparamagnetic cores could facilitate material recycling via magnetic

separation [19,20]. In addition, enzyme immobilization on TiO_2 nanoparticles could allow synergistic enzymatic biocatalysis and TiO_2-mediated photocatalysis for efficient degradation of recalcitrant contaminants [21]. More examples of recent advances in developing immobilized enzyme biocatalytic materials for contaminant removal are given in Table 1.

Cell Surface-Display Enzyme Materials

Cell surface-display enzymes are a new class of biocatalysts where the target enzyme is expressed and automatically localized on the surface of host cells through engineered biological machinery by using synthetic biology techniques (Figure 1B). Notably, cell surface display, where the host cell serves as the supporting platform for enzyme expression and immobilization, can dramatically enhance enzyme stability and enable reuse while ensuring functionality of the target enzyme. Furthermore, cell surface-display enzyme biocatalysts are superior to conventional immobilized enzyme biocatalysts in the following aspects: (i) the enzymes are produced and automatically immobilized on the cell surface in one step, circumventing time-consuming and expensive protein purification; (ii) enzyme functionality can be largely reserved because there is no use of harmful chemical reagents; and (iii) the biocatalyst can be easily regenerated through cell cultivation. The most widely used host cells for enzyme surface display include *Escherichia coli*, *Pseudomonas putida*, and the yeast *S. cerevisiae* because these organisms have established **genetic engineering toolboxes** and surface-display systems. The details of the surface-display platform technology have been reviewed well elsewhere [22]. While prior research efforts using the cell-surface display technology have mostly focused on biomedical and industrial biotechnology applications, developing surface-display enzyme biocatalysts for contaminant degradation is an attractive trend of growing interest. Functional enzymes such as diisopropylfluorophosphatase [23], laccase [24], nitrilase [25], and triphenylmethane reductase [26] have been displayed on the cell surface of *E. coli*, *P. putida*, or *S. cerevisiae*. The engineered biocatalysts could effectively degrade persistent contaminants including toxic organophosphorus compounds, chlorpyrifos, herbicides, industrial dyes, pharmaceuticals, and endocrine disrupting compounds.

Furthermore, immobilization of cells on solid materials has been explored as a strategy to minimize cell washout and maintain high concentration of the biocatalyst in a bioreactor for efficient contaminant treatment. Immobilization of functionalized cells could also improve the recovery and reuse of the biocatalysts relative to the suspended form. Recent advances in electrospinning and bioprinting technologies have enabled fabrication of biocatalytic materials that immobilize engineered cells on porous substrates (Figure 1C). Electrospinning is a versatile method for fabricating polymer nanofibers with diameters ranging from several micrometers to several tens of nanometers by creating an electrically charged jet of polymer solution at a pendent droplet [27]. The inherently high surface-to-volume ratio of electrospun fibers could enhance cell attachment, loading, and mass transfer properties [27]. As such, electrospinning is attractive as an emerging method for cell immobilization. A recent study demonstrated that yeast surface display of enhanced green fluorescent protein could maintain its viability and correct structural conformation on immobilization within nanofibers via electrospinning [28]. This study provided a powerful method to directly immobilize functionalized cells with potential applications in biocatalysis. Another promising technology for direct cell immobilization is inkjet bioprinting, which allows high-throughput deposition of biological agents at predefined locations [29]. The ability of inkjet printing to deposit droplets with picoliter volumes at micrometer-length accuracy makes it a promising method to immobilize biological agents on supporting substrates in a straightforward and systematic manner. One recent study fabricated novel biocatalytic membranes using inkjet printing and demonstrated their efficient performance in the degradation of emerging contaminants bisphenol A and acetaminophen [30]. The

Table 1. Immobilized Enzyme Materials for Contaminant Remediation

Enzyme	Supporting material	Immobilization method	Contaminants	Proposed degradation mechanism	Refs
Oxidoreductases					
Laccase from *Trametes versicolor*	Composite nanofibers	Crosslinking	Crystal violet, triclosan	Crystal violet, N/A[a]; triclosan oxidation, dechlorination, and oligomerization by interacting with the copper cluster in laccase	[62,63]
Laccase from *Trametes versicolor*	Magnetic mesoporous silica microbeads	Crosslinking	Acetaminophen and non-phenolic pharmaceuticals such as mefenamic acid, fenofibrate, and indomethacin	Direct oxidation of acetaminophen; the resulting radicals can interact with electron donor groups in non-phenolic pharmaceuticals	[64]
Laccase from *Trametes versicolor*	Halloysite nanotubes tuned with Fe_3O_4 and functionalized with γ-aminopropyltriethoxysilane (A-M-HNTs)	Covalent binding	Sulfamethoxazole	Oxidation by interacting with a redox mediator of laccase	[65]
Laccase from *Trametes versicolor* or *Cerrena unicolor*	Magnetic nanoparticles	Crosslinking	Recalcitrant dyes such as remazol brilliant blue R, tetracycline, and a mixture of 13 selected pharmaceuticals	Dyes, N/A; direct oxidation of tetracycline and pharmaceuticals by laccase	[19,20,66]
Laccase from *Trametes versicolor* or *Pleurotus ostreatus*	TiO_2 nanoparticles	Covalent binding	Bisphenol A and carbamazepine	Direct oxidation of bisphenol A; resulting radicals can react with carbamazepine	[67,68]
Laccase from *Cerrena* sp. HYB07	Alginate, chitosan, carrier-free, or magnetic nanoparticles	Entrapment, covalent binding, crosslinking	Malachite green	Demethylation	[69]
Horseradish peroxidase from *Armoracia rusticana*	Crosslinked enzyme aggregates	Crosslinking	Recalcitrant dyes including methyl orange dye, indigo, rhodamine B, and rhodamine 6G	N/A	[70]
Horseradish peroxidase	Chitosan–halloysite hybrid nanotubes	Crosslinking	Phenol	N/A	[71]
Horseradish peroxidase	TiO_2 hollow nanofibers	Encapsulation	2,4-Dichlorophenol	Photochemical/ enzymatic oxidation	[2]
Horseradish peroxidase	Silica-coated magnetic nanoparticles, graphene oxide nanosheets, graphene oxide/Fe_3O_4, NH_2-modified magnetic $Fe3O_4/SiO_2$	Adsorption	Tetramethylbenzidine, phenol, 2,4-dichlorophenol	Direct oxidation by horseradish peroxidase in the presence of H_2O_2	[72,73]
Soybean peroxidase	Silica-coated magnetic nanoparticles	Crosslinking	Ferulic acid	Oxidation of phenolic compounds in the presence of H_2O_2	[74]
Soybean peroxidase	Poly(styrene-co-maleic anhydride) (SMA) nanofiber	Adsorption	Diclofenac, naproxen, iopamidol, imidacloprid, bisphenol A, and 2,4-dichlorophenol	Photocatalytic and enzymatic oxidation	[75]

Table 1. (continued)

Enzyme	Supporting material	Immobilization method	Contaminants	Proposed degradation mechanism	Refs
Tyrosinase	Graphene oxide	Crosslinking	Phenol and bisphenol A	Hydroxylation of monophenols to *o*-diphenols and subsequent oxidation of *o*-diphenols to *o*-quinines	[76]
Hydrolases					
Organophosphorus hydrolase OpdA	Nonwoven fabrics	Crosslinking	Methyl parathion	Cleave the P–O bond of methyl parathion to generate *p*-nitrophenol	[77]
Organophosphorus hydrolase	Carbon nanotube paper	Covalent binding	Methyl paraoxon	Cleave the P–O bond of methyl parathion to generate *p*-nitrophenol	[78]
Lipase from *Alcaligenes* sp.	Zeolite imidazolate framework-8	Entrapment	*p*-Nitrophenyl caprylate	Hydrolysis to produce *p*-nitrophenyl	[79]
Multienzymes					
Cyanase rTI-Cyn and carbonic anhydrase rTI-CA	Magnetic nanoparticles	Crosslinking	Cyanate	Transformation of cyanate to ammonia and carbon dioxide in a bicarbonate-dependent reaction	[80]
Haloalkane dehalogenase and epoxide hydrolase	PVA particles, lentiKats	Encapsulation	1,2,3-Trichloropropane	Dehalogenation and hydrolysis to produce glycerol	[81]
Monooxygenase CphC-I and dioxygenase CphA-I	Fulvic acid-activated montmorillonite	Adsorption	4-Chlorophenol	Oxidative transformation of 4-chlorophenol to hydroxyquinol followed by ring fission of hydroxyquinol	[82]
Laccase from *Tinea versicolor* and peroxidase from *Bjerkandera adusta*	Magnetic silica microspheres	Adsorption	Phenolic contaminants in biorefinery wastewater	Breakdown of phenolic ring structures into simpler molecules by free radical chain reactions	[83]
Formate dehydrogenase, NADH oxidase, and peroxidase	Agarose beads	Adsorption	Phenol, 4-aminophenol, 2,4-dichlorophenol, or α-naphthol	H_2O_2 produced from the oxidation of formic acid by formate dehydrogenase and NADH oxidase was used to oxidize phenolic compounds in the presence of peroxidases	[84]

[a]N/A, data not available.

biocatalytic membranes developed could be easily recovered from the reaction solution and maintained stability in reuse.

Engineering Biosorptive Materials for Removal and Recovery of Metals

Biosorption is a physicochemical process that removes substances from the aqueous phase by biological materials (i.e., biosorbents) based on a range of mechanisms such as absorption,

adsorption, ion exchange, surface complexation, and accumulation. Any biological materials capable of binding target chemical substances into its cellular structure can be used as biosorbents, and these include microbial, plant, and animal biomass, as well as their derived products [31]. Among the various types of biosorbents, microbial cells have been extensively investigated for developing efficient and cost-effective biosorption processes given their availability in large amounts by simple cultivation and the versatile binding sites and functional groups in their cell-wall constituents towards a broad spectrum of target chemicals, especially metal ions [31]. However, the use of natural microbial cells as biosorbents suffers from: (i) poor specificity for target chemicals (e.g., metal ions) in the presence of other coexisting ions; and (ii) relatively low binding capacity owing to the limited number of binding sites on the cell surface [32]. Therefore, there is a growing interest in engineering microbial cells through genetic manipulation to construct innovative biosorbents with improved binding properties for metal removal and recovery.

Biosorptive materials with improved metal ion selectivity and increased binding capacity can be developed by arming microbial cells with metal-binding proteins or peptides. Organisms in nature have evolved diverse systems for regulating metal uptake, transport, storage, and excretion to maintain **metal homeostasis and resistance**, providing a rich reservoir of biological macromolecules for highly efficient metal binding. Common metal-binding proteins and peptides derived from organisms are summarized in Box 2. Advances in genetic engineering have made it possible to manipulate microbial cells to overexpress desirable metal-binding proteins/peptides in the cytoplasm or on the cell wall. *E. coli* and *S. cerevisiae* are the most widely used microorganisms as host cells due to their well-established genetic engineering toolboxes, fast cell growth, and potential to achieve a high level of heterologous protein expression. Figure 2 illustrates the two common strategies for engineering microbial cells as biosorptive materials for metal removal and recovery. Intracellular expression of a specific metal-binding protein/peptide in the cytoplasm is an effective method for removing metal ions that can be readily imported into microbial cells. Extracellular display of a metal-binding protein/peptide on the cell surface is an approach of increasing interest because extracellular expression minimizes disturbance of the intracellular metabolic and biochemical processes while making the binding sites more accessible to metal ions in the external environment. Moreover, as for metal recovery applications, biosorbent cells developed through intracellular protein expression need to be destroyed to release the sequestrated metals, while cells with extracellular protein expression could be reused after desorbing metal ions bound outside the cells (Figure 2). The following sections describe current progress in developing engineered microbial cells as biosorptive materials for removing hazardous metal pollutants and recovering valued metals from waste streams.

Removal of Hazardous Metal Pollutants by Engineered Biosorbents

Recent research efforts have made significant progress in engineering microbial biosorbents for removing hazardous metal pollutants such as heavy metals and radioactive metals in wastewater and aquatic environments. The **metallothioneins** (MTs), **phytochelatins** (PCs), and **metalloregulatory proteins** originating from various organisms including bacteria, fungi, plants, animals, and human have been functionally expressed in host microbial cells to improve the removal of different metal ions, as summarized in Table 2.

Engineering host cells with MTs could considerably increase the binding capacity for a wide range of heavy metal ions. For example, a recent study reported that an engineered *S. cerevisiae* expressing MTs from *Arabidopsis thaliana* and *Noccaea caerulescens* in the inner side of plasma membrane exhibited enhanced uptake and accumulation of Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Cd^{2+}, by 12–147-fold [33]. Although expressing MTs could provide superior binding capacity, one

Box 2. Metal-Binding Proteins and Peptides Commonly Used in Biosorbent Engineering

Over billions of years, organisms have evolved diverse and sophisticated systems for the uptake, transport, storage, and excretion of different metal ions to control metal homeostasis. In these systems, proteins capable of specifically recognizing and binding metal ions play crucial roles in the assimilation of essential metals and the elimination of toxic metals. Among the proteins associated with metal homeostasis, MTs, PCs, and metalloregulatory proteins are frequently exploited to engineer biosorptive materials with superior selectivity and binding efficiency.

MTs, a family of low molecular weight (6–10 kDa) proteins, are found in a wide range of organisms including humans, animals, plants, and diverse eukaryotic and prokaryotic microorganisms. MTs have a cysteine-rich (~30% of total amino acids) structure that lacks aromatic amino acids and histidine. The main function of MTs in biological systems is to detoxify excess heavy metal ions, protect against oxidative stress, and maintain metal homeostasis. Cys–Cys, Cys–X–Cys, or Cys–X–X–Cys (where X is an amino acid different from cysteine) motifs shared among MTs underlie their outstanding ability to sequester heavy metal ions. All MTs have high binding affinity for a broad spectrum of heavy metals, including Cd, Pb, Zn, Hg, Cu, Ni, As, and Ag [61].

PCs are a family of short cysteine-rich peptides that are responsible for metal sequestration and detoxification to maintain intracellular metal homeostasis in many plant species and in some groups of microorganisms. The general structure of PCs comprises tandem repeats of a Glu–Cys dipeptide terminated by a Gly residue: $(\gamma\text{-Glu–Cys})_n$–Gly for natural PCs and $(\alpha\text{-Glu–Cys})_n$–Gly for synthetic PCs (i.e., ECs). PCs are capable of binding various heavy metals, such as Cd, Hg, As, and Pb, and display highest affinity for Cd through the formation of thiolate complexes [37].

Metalloregulatory proteins (also known as metal sensor proteins) are specialized regulatory proteins that control the expression of genes associated with metallochaperones, metal importers, and metal efflux transporters that regulate metal bioavailability. In comparison with MTs and PCs, metalloregulatory proteins exhibit high selectivity by specifically recognizing only one or a particular subgroup of metal ions. Furthermore, metalloregulatory proteins are highly sensitive for their target metal ion(s) and their transcriptional responses can be triggered by strikingly low metal concentrations (at the femtomolar level or lower) [39].

major intrinsic shortcoming of using MTs is the lack of high selectivity owing to their low specificity for binding different metal ions. Coexpression of MTs with a specific **metal transporter** is a way to improve the specificity towards the target metal ion. Such a strategy has been implemented to construct highly selective biosorbents for nickel, mercury and arsenite by overexpressing MTs together with the metal transporter genes *nixA* (for nickel), *merT* and *merP* (for mercury), and *glpF* (for arsenite), respectively [34]. For example, recombinant *E. coli* strains expressing pea MT with either the NiCoT (a nickel transporter protein encoded by *nixA* gene) from *Helicobacter pylori* or the

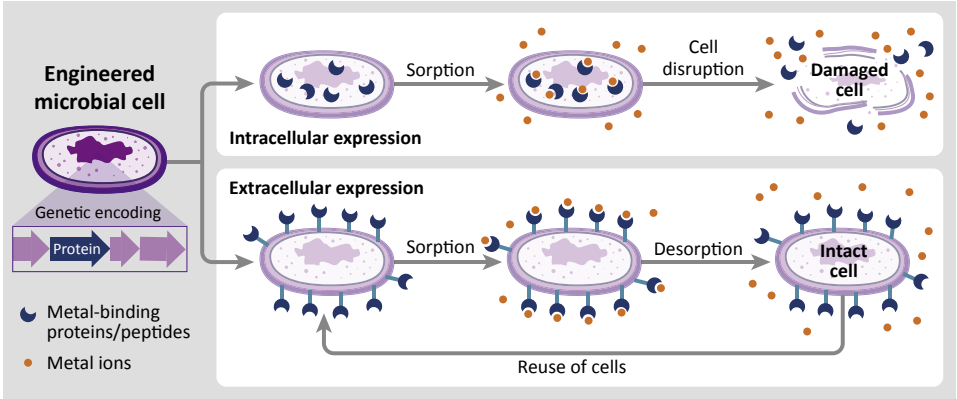

Figure 2. Engineering Microbial Biosorbents with Intracellular Expression or Extracellular Display of Metal-Binding Proteins/Peptides for Metal Removal and Recovery.

Table 2. Recent Advances in Engineering Biosorbents for Hazardous Metal Removal

Host microorganism	Expression site	Metal-binding peptides/proteins	Target metal	Refs
Escherichia coli	Intracellular	Phytochelatin from *Pyrus calleryana*	Cu^{2+}, Cd^{2+}, and Hg^{2+}	[35]
	Intracellular	Phytochelatin from *Thlaspi caerulescens*	Cd^{2+}	[36]
	Intracellular	*Corynebacterium glutamicum* metallothionein	Pb^{2+}, Zn^{2+}, and Cd^{2+}	[85]
	Intracellular	Phytochelatin from *Ceratophyllum demersum*	Cd^{2+}, As^{3+}, and As^{5+}	[86]
	Intracellular	Pea metallothionein and Ni/Co transporter (NiCoT) from *Helocobacter pylori* and *Staphylococcus aureus*	Ni^{2+}	[34]
	Intracellular	Ni/Co transporter (NiCoT) from *Novosphingobium aromaticivorans*	Ni^{2+}, and Co^{2+}	[87]
	Intracellular	Crab metallothionein	Zn^{2+}, Cu^{2+}, and Cd^{2+}	[88]
	Intracellular	Rice metallothionein isoforms fused with glutathione-*S*-transferase (GST)	Hg^{2+}	[89]
	Cell surface	Full-length and truncated CadR from *Pseudomonas putida*	Cd^{2+}	[41]
	Cell surface	PbrR from *Cupriavidus metallidurans* CH34	Pb^{2+}	[42]
	Cell surface	Full-length PbrR and a putative Pb^{2+}-binding domain (PbBD) derived from PbrR	Pb^{2+}	[43]
	Cell surface	Cadmium-binding motif (cbm) and cadmium-binding β motif (cbβm)	Cd^{2+}	[90]
Rhodopseudomonas palustris	Intracellular	MerP–MerT and pea metallothionein	Hg^{2+}	[91]
Deinococcus radiodurans	Intracellular	A synthetic phytochelatin analog (EC20)	Cd^{2+}	[38]
	Intracellular	Ni/Co transporter proteins	^{60}Co	[49]
Pseudomonas aeruginosa	Cell surface	Cd^{2+}-responsive transcriptional regulator protein CadR from *Pseudomonas syringae*	Cd^{2+}	[44]
Pseudomonas putida	Cell surface	Cyanobacterial metallothionein	Cu^{2+}	[92]
Stenotrophomonas sp.	Cell surface	Synthetic phytochelatin	Cd^{2+}	[93]
Sphingobium japonicum	Cell surface	Synthetic phytochelatin	Cd^{2+}	[94]
Saccharomyces cerevisiae	Intracellular	Human metallothionein	Cu^{2+}	[95]
	Intracellular	Synthetic phytochelatin	Pb^{2+} and Cd^{2+}	[96]
	Intracellular	Metallothioneins from *Arabidopsis thaliana* and *Noccaea caerulescens*	Cu^{2+}, Zn^{2+}, and Cd^{2+}	[33]
	Intracellular	Human hepatic metallothionein	Pb^{2+}	[97]
	Cell surface	*Solanum nigrum* metallothionein	Cd^{2+}	[98]
	Cell surface	N- and C-terminal regions of CadR from *Pseudomonas putida*	Cd^{2+}	[47]
	Cell surface	Mercuric ion resistance operon regulatory protein MerR from *Pseudomonas aeruginosa*	Hg^{2+}	[40]
	Cell surface	Ni^{2+}-dependent transcriptional repressor mutant protein	UO_2^{2+}	[46]

NiCoTs from *Staphylococcus aureus* showed significant increase in Ni^{2+} accumulation in the presence of competitor ions Na^{+}, Co^{2+}, and Cd^{2+} [34].

Engineered microbial cells that overexpress PCs have also been demonstrated to enhance biosorption of various heavy metals. Recently, recombinant *E. coli* cells that overexpressed the *PcPCS1* gene from *Pyrus calleryana* (which encodes **PC synthase**) was constructed and the increased accumulation of Cd^{2+}, Cu^{2+}, and Hg^{2+} was achieved by using this engineered biosorbent [35]. Functional expression of the PCs from *Thlaspi caerulescens* and *Schizosaccharomyces pombe* in *E. coli* was also reported to enhance specific binding of Cd^{2+} [36]. PCs are usually preferred over MTs as expression targets because their unique structural characteristics (namely the continuously repeating γ-Glu–Cys unit that is responsible for metal binding) enable higher affinity and binding capacity [37]. In addition to natural PCs, synthetic PCs (termed ECs) with desirable binding capacity can be created by tuning the number of metal-binding motifs [i.e., *n* in $(\alpha\text{-Glu–Cys})_n$–Gly]. The longest EC ever reported for engineering the microbial cell biosorbent is EC20, namely $(\alpha\text{-Glu–Cys})_{20}$–Gly, which resulted in superior cadmium-binding capacity [38].

Metalloregulatory proteins are usually used as the expression target when a high specificity for target metal adsorption is required. The unique structures of these metalloregulatory proteins can effectively recognize and bind the target metal ions by complexing the metal ions in specific coordination geometries [39]. Several studies have demonstrated that overexpression of metalloregulatory proteins could considerably improve the host cell selectivity for the metal ions of interest [40–42]. For example, engineered *E. coli* cells expressing a putative Pb^{2+}-binding domain (PbBD) derived from the PbrR (Pb^{2+}-sensing transcriptional factor) on the cell surface could selectively adsorb Pb^{2+} in the presence of Cd^{2+} and Zn^{2+} [43]. In addition, MerR, the mercuric ion resistance operon regulatory protein, was recently reported to be displayed on the *S. cerevisiae* cell surface for the first time, resulting in higher adsorption capacity for Hg^{2+} as well as excellent selectivity against the coexistence of Cd^{2+} and Cu^{2+} [40]. As another example, *Pseudomonas aeruginosa* cells engineered with CadR, a cadmium-induced regulator protein, on their surface showed improved adsorption of Cd^{2+}, and Ca^{2+} and Mg^{2+} ions had no significant effects on the biosorption efficiency [44]. Efforts in engineering biosorbent cells via overexpression of other metalloregulatory proteins, such as CueR, CadR, GolS, and Nik, have also been made to improve the binding capacity and selectivity for the corresponding heavy metals (e.g., Ag^{+}, Cd^{2+}, and Au^{3+}) [41,42,44–48]. In addition to heavy metals, biosorbents engineered with metalloregulatory proteins have also been reported to remove radionuclides. Yeast cells with surface-displayed tandem metal-binding domains of NikRm, a NikR mutant protein that was designed for uranyl ion (UO_2^{2+}) binding, could selectively remove UO_2^{2+} from contaminated water in the presence of other interfering metal ions [46].

Other proteins/peptides in addition to MTs, PCs, and metalloregulatory proteins can also be designed and used for engineering microbial cell biosorbents for metal removal. Metal transporter proteins are one alternative expression target. For example, expression of the Ni/Co transporter proteins (NiCoTs) from *Rhodopseudomonas palustris* and *Novosphingobium aromaticivorans* in *Deinococcus radiodurans* could effectively and selectively remove radioactive cobalt (^{60}Co) from a solution containing other metal ions such as Fe^{2+} and Cr^{3+} [49]. Moreover, rational design of metal-binding peptides can be exploited for biosorbent engineering [50,51]. A recent study used a strategy that combined bioinformatics and genetic engineering techniques to design high-affinity metal-binding peptides *in silico* and to engineer *E. coli* cells with these peptides on the cell surface for enhanced Cd^{2+} adsorption. The engineered cells showed greater affinity for Cd^{2+} versus other coexisting metal ions as well [50].

Recovery of Precious Metals and Rare Earth Elements by Engineered Biosorbents

The recovery of precious metals and **rare earth elements** (REEs) from waste streams is of particular significance to resolve the conflict between their diminishing supplies and growing demands for socioeconomic development. A crucial challenge in metal recovery via biosorption processes is how to distinguish the high-value target metal ion from other coexisting ions in the solution such that it can be selectively separated and recovered with high purity. As such, a major trend of recent research in developing microbial biosorbents for valued metal recovery applications is to focus on the criterion of high selectivity and specificity a well as enhanced binding capacity [52].

Advances in engineering highly selective microbial cell biosorbents for recovering precious metals including gold, silver, and platinum have been reported. For example, expression of GolB, a putative gold-chaperone from *Salmonella* that can effectively recognize gold ion, on the cell surface of *E. coli* enabled selective adsorption of Au^{3+} in the presence of Cu^{2+}, Cd^{2+}, Zn^{2+}, or Ni^{2+}. Furthermore, the Au^{3+} adsorbed onto cells could be easily and selectively recovered by cleavage of the cysteine residues on GolB using papain, a cysteine-specific protease, and the engineered *E. coli* cell with GolB were readily reusable after regeneration [48]. In addition, expression of the Ag-binding domain of the CueR from *P. putida* on the *S. cerevisiae* cell surface achieved enhanced adsorption capacity and higher selectivity towards Ag^{+} in the presence of other competing metal ions [45].

Attempts have also been made to engineer microbial cell biosorbents for recovering REEs. Particularly, **lanthanide binding tags** (LBTs), originally developed by combinatorial peptide synthesis and screening together with explicit tactics of peptide design, are of great potential as the expression targets for engineering microbial biosorbents to recover REEs with high affinity and selectivity. Engineered *Caulobacter crescentus* cells that displayed LBTs at high density on the **S-layer** showed improved biosorption to REEs compared to the control cells without LBTs. The REEs could be recovered with an excellent efficiency (>90%) after treating the cells with citrate as the desorbing agent [53]. A follow-up study constructed a recombinant *E. coli* strain expressing eight tandem copies of double-LBTs on the cell surface. The engineered *E. coli* strain exhibited enhanced Tb^{3+} biosorption capacity which was twofold higher than cells without LBTs. When this engineered strain was applied to recover REEs in leachates from metal-mine tailings and rare earth deposits, it showed enhanced efficiency of REE adsorption from all leachate samples, increased selectivity and specificity for REEs over other interfering metal ions, and greater affinity to REEs with smaller radii (e.g., Tb, Dy, Er, Yb) than those with larger radii (e.g., La, Pr, Ce) [3]. In a more recent study, *E. coli* cells were genetically modified to express the **curli amyloid fibers** that displayed LBTs (curli-LBT) [54]. The curli-LBT filter made by immobilizing the curli-LBT onto a polycarbonate membrane could preferentially bind several high-value heavy REEs with high specificity in the presence of other coexisting metals. This type of filter could be potentially used as a rapid, selective, and scalable biosorptive material for recovering REEs.

Concluding Remarks and Future Perspectives

Current progress in synthetic biology and protein engineering is opening up tremendous opportunities to advance the application of biological processes at the molecular level to develop innovative solutions to the emerging problems facing environmental sustainability. These advances have enabled the development of biocatalytic and biosorptive materials with novel functionality, providing a renewable and cost-effective strategy to treat recalcitrant contaminants of emerging concern and to recover valuable products from wastes. Although recent research efforts have made significant and exciting progress towards this end, crucial challenges remain to be resolved before engineered biological materials can be used to solve environmental problems in practice (see Outstanding Questions).

Outstanding Questions

How to improve the robustness of engineered biocatalytic or biosorptive materials under environmentally relevant conditions?

How can biomaterials be engineered and used so as to minimize the potential environmental and ecological risks?

What is the fate, transport, and transformation of engineered biological materials in the environment?

First, it is highly desirable that the biocatalytic or biosorptive material is robust under environmentally relevant conditions. Robustness here is collectively referred to as the material functionality and stability against a number of important parameters relevant to complex wastewater and polluted environments (e.g., pH, salinity, biomolecules, natural organic matters, and toxic contaminants). Current research efforts in developing biocatalytic or biosorptive materials normally focus on characterizing the functionality of the material under ideal laboratory conditions, whereas the real-world application-relevant environmental conditions are more complex. Therefore, it will be important to rigorously characterize and enhance the robustness of the engineered material such that it could become a viable option for contaminant treatment. Promising strategies for improving the robustness of engineered biological materials include rational design based on understanding structure–activity relationships at the molecular level, directed evolution to optimize protein stability under environmental conditions of interest, and the **combinatorial library** approach to discover proteins/peptides with distinct properties that can function under anticipated environmental conditions.

In addition, a major concern about using genetically engineered biological materials for environmental applications lies in their potential ecological risks. Concerns primarily arise from possibility of introducing mobile genetic elements and antibiotic resistance marker genes into the environment. These concerns could be addressed by using advanced genome-editing techniques which can create stable and marker-free engineered cells [55]. Designing well-controlled reactor systems and treatment processes is also important to minimize the release of engineered biological materials into the environment. On the other hand, more research will be necessary to advance our understanding of the environmental fate, transport, and transformation of genetically engineered materials. Research to this end will provide a scientific basis for risk management while new technological breakthroughs are being made in developing engineered biological materials to address crucial environmental problems.

Finally, for practical implementation of engineered biocatalytic and biosorptive materials, a crucial factor to consider is the cost. In conventional approaches that immobilize the target protein on supporting matrix, protein extraction and purification are time-consuming and expensive steps. By contrast, engineered cell-based materials with intracellular expression or extracellular display of the target protein would be advantageous in terms of cost-effectiveness because the strategy bypasses the need for protein extraction and purification. In addition, efficient large-scale preparation of the engineered biological materials will be necessary for real-world applications. Cell-based materials hold great promise for scaling up, as evidenced by existing applications in the field of industrial biotechnology where large amounts of host microbial cells (e.g., *E. coli* and *S. cerevisiae*) are cultivated on a routine basis to produce commercial products [56,57]. Future research to address logistic challenges such as generating the engineered biocatalytic/biosorptive materials onsite at scale, or enhancing stability and longevity during transportation and storage, would greatly benefit practical applications.

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