

Enhancing Bioremediation with Enzymatic Processes: A Review

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Abstract: The use of extracellular enzymes has been standard in many industries for many years; only recently have they been studied as a means for enhancing bioremediation. Extracellular enzymes are either secreted from organisms such as white rot fungi or are produced during a fermentation process and possess the ability to break down bonds within organic compounds and/or catalyze their transformation into less toxic and more biodegradable forms. Unlike many microbes, enzymes remain effective in a wide range of pH and temperature ranges, particularly if they are immobilized on some carrier, and they can degrade a wide variety of compounds. Enzymes from white rot fungi have been shown to be effective degraders of TNT, phenols, PCBs, PAHs, and dyes; enzymes such as protease, lipase, and cellulase have demonstrated the ability to reduce pathogen counts, reduce the solids content, and increase deflocculation in sludge. Currently, high production costs inhibit the widespread use of extracellular enzymes for remediation, but bench studies and field studies have shown enzymatic treatment to be feasible options for bioremediation. This paper will explore the current state of use of extracellular enzymes in enhancing the bioremediation of recalcitrant substances and wastewater, as well as the benefits and disadvantages associated with the use of such enzymes.

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Introduction

The use of extracellular enzymes for industrial processes began in the 1960s and has since become a staple in such industries as textiles, food production, and detergent (Godfrey and Reichelt 1996; Uhlig 1998; Gianfeda and Rao 2004). The use of enzymes is desirable because they can perform the same function as many harsher chemicals, such as solvents, but at a neutral pH, a moderate temperature, and without production of hazardous waste. Although enzymes tend to be expensive due to the extraction and purification costs, they can be very cost effective because they minimize waste disposal and heating needs (Gianfeda and Rao 2004; Godfrey and Reichelt 1996). The use of extracellular enzymes is standard for various industries; however, research has just begun on how enzymes can be used to enhance bioremediation.

Bioremediation refers to the use of biological systems, such as bacteria, fungi, and enzymes, to degrade environmental pollutants. For passive approaches, the existing microbes naturally

attenuate the pollutants, but in active engineered bioremediation, the addition of specific enzymes or microbes to the contaminated area may be required. Such methods for remediation have the potential to be less expensive, less invasive, and more environmentally friendly than many chemical or physical remediation options (Baker and Herson 1994; Seah et al. 2001).

Extracellular enzymes refer to those enzymes that are either secreted by the microbes, such as lignin peroxidase (LiP) from white rot fungi, or those that enter the aqueous phase during an aerobic submerged fermentation process (Cheetham 1985; Milstein et al. 1994; Bhargava et al. 2003). Such enzymes are naturally produced by the microbes and then harvested. In most cases, each enzyme has only one specific function, such as to lower the activation energy for the degradation of an intramolecular bond, but some are able to affect a wide range of different substrates (Fullbrook 1996; Uhlig 1998; Gianfreda and Rao 2004). It is the latter type of enzyme that is most useful for bioremediation. Even though these enzymes were originally created for a substrate that the microbe would normally encounter in nature, they are also able to react with synthetic and xenobiotic compounds (Bollag 1992; Gianfreda and Rao 2004). Such reactions can transform a compound from a recalcitrant state to one that is more biodegradable (Gianfreda and Rao 2004). In other cases, extracellular enzymes are able to increase the degradation rate of already biodegradable substances, such as activated sludge, allowing for more efficient treatment processes (Whitely et al. 2002).

This paper explores the advantages and disadvantages of using extracellular enzymatic processes for enhancing bioremediation and the current state of laboratory and field research. This paper will also highlight areas where further research must be done before this technology can be used on a wide scale.

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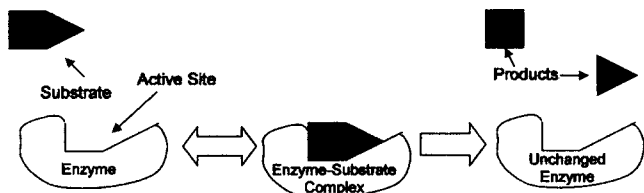


Fig. 1. Enzymatic reaction process

Enzymological Background

Enzymes are defined as substances that alter a reaction's rate and/or a reaction's activation energy without being present in the reaction products (Uhlig 1998). They are naturally produced by nearly every known organism in order to aid processes such as digestion, metabolism, and cell synthesis (Madigan et al. 2003). Most enzymes are made from only proteins, but some require a nonprotein prosthetic group in order to perform their functions (Uhlig 1998). Proteins are made from amino acid polymers connected by peptide bonds and may contain as many as 21 different types of amino acids. The order of the amino acids in the protein and the way that the protein is folded dictate what the function of the enzyme will be (Uhlig 1998; Madigan et al. 2003).

Each enzyme has defined active sites on its folded surface, and it is at these locations that the enzyme can bond to a substrate and catalyze a reaction (see Fig. 1) (Fullbrook 1996; Uhlig 1998). In the reaction, a substrate comes into contact with an active site and forms a temporary bond with the enzyme. This substrate-enzyme complex loosens the bonds that hold the substrate together, allowing the bonds to break. Once this occurs, the substrate pieces are released, and the enzyme is ready to catalyze another reaction (Uhlig 1998). The rate controlling step is the transformation of the substrate-enzyme complex to the separated enzyme and products. For optimal conditions, the substrate should saturate the enzyme, which minimizes the time between the completion of one reaction and the start of the next one (Uhlig 1998). The reaction velocity is always related to the enzyme concentration (Fullbrook 1996).

The strength and effectiveness of an enzyme is measured by its activity. One unit of activity of any enzyme is defined as the amount of the enzyme required to catalyze the transformation of 1 μmol of substrate per minute at 25°C and at the enzyme's optimal pH (Fullbrook 1996). This activity definition is cumbersome and inexact for most industries. Each enzyme provider and industry often has its own definitions for the activity of its products, which are not standardized (Fullbrook 1996; Duran and Esposito 2000). Between the temperatures of 10 and 40°C, the enzymatic activity tends to double for every 10°C increase. Also, the enzymatic activity will be the highest when the enzyme is working at its optimal pH, which is enzyme specific (Uhlig 1998). Enhancing bioremediation with enzymatic processes is most effective when the environmental conditions favor optimization. Providing such conditions is easy for ex situ bioremediation, but may be difficult for in situ bioremediation. Therefore, in situ enzymatic bioremediation may be less efficient and require more time and a higher enzyme concentration than ex situ bioremediation.

Advantages and Disadvantages of Extracellular Enzymes

Bioremediation carries with it many inherent difficulties, such as maintaining the microbial population necessary to degrade a cer-

tain compound, and the necessary growth conditions (i.e., proper temperature, oxygen availability, moisture levels, pollutant levels, and pH) for the required microbe (Baker and Herson 1994). Some microbes may require unique conditions difficult to optimize in the field. These factors can limit the overall success of bioremediation. Enzymes are able to act in a large range of environmental conditions and remain active even if these conditions quickly change (Ahuja et al. 2004; Gianfreda and Rao 2004). For example, protease is able to function effectively in pHs between 4 and 11 (the experiment stopped at pH 11, but the enzyme was still quite active at this time) and with temperatures less than 20°C and greater than 70°C (Whiteley et al. 2002). Enzymes often are able to work in multiple environments, especially if they are immobilized. This makes the enzymes even more resistant to harsh environments and enables the enzymes to be recovered and recycled after they are no longer needed (Gianfreda and Rao 2004).

Some extracellular enzymes are able to degrade only one specific substrate, while others can be used for many different substrates. Microbes will often first metabolize those compounds that are easiest for them to degrade before degrading more resilient compounds (Madigan et al. 2003). This works well if the microbes are well suited to degrade the compound of interest, but can be extremely detrimental to the project if the compound of interest is recalcitrant. This is not the case for enzymes with a high specificity. They will only degrade a specific target compound even if more easily degradable compounds are present (Fullbrook 1996; Uhlig 1998). If there are several different contaminants present, there is the potential for less specific enzymes to be used to degrade several contaminants concurrently (Gianfreda and Rao 2004). For example, laccase has been shown to degrade phenol (Ullah et al. 2000), polycyclic aromatic hydrocarbons (PAHs) (Dodor et al. 2004), polychlorinated biphenols (PCBs) (Novotny et al. 1997), herbicides (Mougin et al. 2000), and dyes (Mayer and Staples 2002). Although the enzymatic degradation of several compounds at once is feasible, it must also be noted that the degradation rate of the enzyme for each compound is reduced in such a system (Alvarez-Cohen and Speitel 2001). This means that in contaminated field sites, where there are often several different contaminants present, enzymatic treatment may be an attractive alternative to conventional treatments, but the treatment will probably take longer and require a higher enzyme dosage than what a bench study may suggest.

A very pragmatic benefit of enzymatic treatment is that the enzymes themselves are biodegradable proteins, meaning that the enzymes that are not recovered will degrade in the environment after they are no longer needed. Unlike other remediation methods, there is no buildup of biomass or chemicals that must be removed (Ahuja et al. 2004).

Although enzymatic technology is very promising, it has limitations. Microbes can reproduce and increase their population in order to consume a large amount of substrate, but extracellular enzymes cannot. Enzymes cannot reproduce themselves, meaning that any increase in enzyme population must come from outside of the system (i.e., humans adding more enzymes to the system). It has also been shown that enzymes may actually lose some reactivity after they interact with pollutants and could eventually become completely inactive (Gianfreda and Rao 2004). This means that the enzyme concentrations must be monitored and controlled in order to optimize enzyme kinetics for site-specific conditions. Since the enzymes are unable to reproduce, they also do not possess the adaptability that microbes possess through mutations. Mutations allow microbes to be able to metabolize new substrates and to survive in what were formerly considered harsh

environments (Heitkamp et al. 1988; Madigan et al. 2003; Singer et al. 2004; Somero 2004). Even though enzymes can survive in a wide range of environments, they are not able to adapt themselves to survive in environments that are outside this range.

The main disadvantage of using extracellular enzymes for bioremediation is the high cost of the enzymes themselves. For example, 100 units of LiP derived from *P. chrysosporium* costs \$100.00 (where 1 unit oxidizes 1 μmol veratryl alcohol per min/3 mL at pH 6.5 and 25°C), 5,000 units of purified horseradish peroxidase (HRP) costs \$4,000 (where 1 unit degrades 1 μmol of hydrogen peroxide at 25°C and a neutral pH using 4-aminoantipyrine/phenol assay at 510 nm), and 100,000 units of laccase C costs \$155,000 (activity undefined) (Duran and Esposito 2000). Much of the cost of producing enzymes comes from trying to make as pure an enzyme solution as possible, which performs only its proposed function and has no side activities. Crude enzyme solutions are cheaper to produce, but also tend to have side effects and side activities (Fullbrook 1996). The costs are expected to decrease as technology and techniques advance and as cheaper growth substrates are explored for the breeding of the parent bacteria and fungi (Ahuja et al. 2004; Gianfreda and Rao 2004; Ikehata et al. 2004).

Immobilized and Soluble Enzymes

When enzymes are harvested, they tend to be in the aqueous phase. For bioremediation purposes, enzymes can either remain in this phase or be immobilized onto some type of carrier. Each has its advantages and disadvantages. Mobile enzymes can be added at a single point and then spread due to diffusion, dispersion, and the flows of groundwater and surface water. This means that they will be able to impact a large contaminated area and be able to travel to where the contamination is (Gianfreda and Rao 2004). Due to sorption and retardation, it may take these enzymes a long time to travel through different media, but they still maintain some mobility. The main disadvantage of mobile enzymes is that these enzymes are much less robust than immobilized enzymes, making them more susceptible to temperature, substrate concentration, and pH swings and more likely to deactivate relatively quickly. Another disadvantage is that mobile enzymes cannot be recovered and reused after the remediation project is completed (Fullbrook 1996).

Enzymes can be immobilized onto a carrier, which can be granular, fibrous, a tube, or a membrane (Fullbrook 1996). Carriers include wood chips, granular clay, anthracite, and synthetic polymers, which can help the enzymes establish themselves in the soil matrix (Porta et al. 1994). Enzymes are either sorbed or covalently bonded to the surface of the carrier. Carriers may carry a charge on them, which could be transferred to the immobilized enzymes as well, changing their optimal pH by up to 2 pH units (Fullbrook 1996; Gianfreda and Rao 2004). In general, immobilization makes the enzyme more resistant to temperature, pH, and substrate concentration swings, giving it a longer lifetime and higher productivity per active unit (Fullbrook 1996; Russel et al. 2003; Gianfreda and Rao 2004; Kandelbauer et al. 2004). For example, Kandelbauer et al. (2004) found that immobilized laccase was able to decolorize 99% of the Lanazol yellow 4G dye and mobilized laccases was only able to decolorize 1% of this dye after the same contact time and under the same conditions. It has also been found that the immobilization of HRP onto CNBr-Sepharose made it 2.7 times more effective than mobile HRP (57 and 20%, respectively) in decolorizing Kraft effluent (Duran and

Esposito 2000). Since the enzymes are immobilized at known locations and have long lifespans, it is possible to recover the enzymes at the end of the project and reuse them at another site or for another purpose (Bollag 1992; Kandelbauer et al. 2004).

Kandelbauer et al. (2004) also noted that the manner in which the enzymes are immobilized affects their longevity. In an experiment using laccase to degrade industrial dye, it was noted that when the enzymes were immobilized on catalyst pellets and used in a mixer, the half-life for the enzymes was only 7 h. When the enzymes were immobilized in a batch reactor, no significant activity reduction could be noticed after 20 cycles.

In order for immobilized enzymes to be used in *in situ* bioremediation, the contaminants must be brought to the enzymes, perhaps by groundwater flow, but this may not be feasible. Immobilized enzymes may be much better suited for *ex situ* bioremediation. If immobilized enzymes are used for *ex situ* treatment instead of mobile enzymes, a smaller plant size is required, the amount of enzymes present in the effluent is drastically decreased, and product purification costs decrease (Fullbrook 1996; Russell et al. 2003). The reduction in product purification costs is due to the increased activity of the enzymes and the fact that the enzymes do not flow out of the system. If fewer enzymes are needed to do the same job, less money has to be spent on purification.

Major Enzymes Used in Bioremediation

A wide variety of enzymes can be used for bioremediation, depending on what reactions need to occur and which contaminant needs to be degraded. Enzymes from white rot fungi have been found to be very capable of degrading a large number of different contaminants. In nature, white rot fungi degrade dead wood and other plants and are unique among eukaryotes because they are able to cleave the carbon-carbon bonds in contaminants such as PAHs. Before this discovery, it was believed that only bacterial processes could break this bond (Cremonesi et al. 1989). During the secondary metabolism of plant life, white rot fungi produce and secrete LiP, manganese peroxidase (MnP), and laccase. The amounts produced and strengths of these enzymes are different for each type of white rot fungi, resulting in different oxidative activities (Milstein et al. 1994; Ullah et al. 2000). Each of the enzymes can catalyze the one-electron oxidation of phenols and nonphenolic substrates. This results in the production of cation-radical intermediates, which can be used to further oxidize nonphenolic substrates (Kirk and Farrell 1987; Milstein et al. 1994; Novotny et al. 2004). Such enzymes are found naturally in the soil system, indicating that they are able to be used in field studies as well as on the bench (Wang et al. 2002).

LiPs, like all other peroxidases, are hemoproteins which catalyze reactions when in the presence of hydrogen peroxide (Duran and Esposito 2000). Its natural function is the degradation of lignin, which is a compound found mainly in the cell walls of higher plants and acts as the glue that holds the cells together (Kirk and Farrell 1987). LiP is capable of attacking other types of compounds, creating cation radicals, which can further degrade the target compound (Hammerli et al. 1986; Gianfreda and Rao 2004; Novotny et al. 2004). After LiP has been oxidized by hydrogen peroxide, it can be reduced again by veratryl alcohol. Though this cycle, hydrogen peroxide is actually removed from the solution, keeping its concentration within a tolerable zone for the LiP. If the hydrogen peroxide concentration is too low, the LiP is not able to catalyze any reactions, and if its concentration is too high,

Table 1. Summary of Enzymes Used to Degrade PAHs

Contaminant	Enzyme	Parent microbe	Contact time	Contaminant decrease	References
Pyrene	LiP	<i>Phanerochaete chrysosporium</i>	NA ^a	High	Hammel et al. (1986)
Benzo[a]pyrene	LiP	NA ^a	2 h	High	Haemmerli et al. (1986)
Anthracene and Benzo[a]pyrene	Laccase	<i>Trametes versicolor</i>	24 h	80–90%	Dodor et al. (2004)
Anthracene and Benzo[a]pyrene	Laccase	<i>Trametes versicolor</i>	24 h	90–100%	Collins et al. (1996)
Anthracene, Phenanthrene, Benzo[a]anthracene, Benzo[a]pyrene	MnP	<i>Phanerochaete laevis</i>	5 weeks	17–33%	Bogan and Lamar (1996)
Fluorene	MnP	<i>Phanerochaete laevis</i>	6 days	94%	Bogan et al. (1996)

^aNot available.

then the LiP is overoxidized and inactivated (Haemmerli et al. 1986).

It has been found that a single enzyme, even LiP, is incapable of degrading lignin entirely, which is why the white rot fungi need the two other enzymes, MnP and laccase, in order to break down the wood (Kirk and Farrell 1987). MnP is also a hydrogen peroxide dependent enzyme, but it can only oxidize organics when in the presence of Mn(II). MnP oxidizes Mn(II) to Mn(III), which acts as an obligatory oxidation intermediate for the oxidation of various compounds (Glenn et al. 1986; Kirk and Farrell 1987; Cremonisi et al. 1989; Lackner et al. 1991; Field et al. 1994; Cameron et al. 2000). The Mn(III) ions migrate away from the enzyme and start the oxidation of the lignin and other compounds before they even reach the enzyme. This reaction means that MnP has a high potential for penetrating deep into the soil fines (Field et al. 1994).

The last enzyme produced by white rot fungi is laccase. Laccase is a copper containing oxidase that is able to react with a large number of aromatic alcohols and amines and substrates, only needing oxygen as a cosubstrate. In the process of oxidizing the substrate, laccases reduce this oxygen to water (Duran and Esposito 2000; Kandelbauer et al. 2004; Keum and Li 2004). It has been found that contaminants with a high ionization potential are resistant to such laccase-induced redox reactions (Keum and Li 2004).

In addition to enzymes produced by white rot fungi, there are a large number of other extracellular enzymes that hold potential for bioremediation. HRP is a peroxidase that is secreted by the root hairs of the horseradish plant and can catalyze the oxidation of compounds such as phenols, biphenols, anilines, and benzidines over a large range of pHs and temperatures (Duran and Esposito 2000). Due to their hydrogen peroxide requirement, peroxidases can only be used in certain environments (Duran and Esposito 2000). Environments such as wastewater require a different type of enzyme. These extracellular enzymes, such as proteases, amylases, and lipases, are produced during the aerobic fermentation of organic matter by yeast or other microbes (Cheetham 1985). Rather than catalyzing the oxidation of recalcitrant compounds, these enzymes catalyze the degradation of organic matter (Parmar et al. 2001a, b; Whiteley et al. 2002). Each of these enzymes fulfills a specific niche in the natural world, and it may be possible to utilize their abilities for remediation purposes.

Enzymatic Biodegradation Studies

Currently, engineers and scientists are examining the ability of extracellular enzymes to bioremediate a large number of compounds. Because enzymatic bioremediation is still a new field, almost all of the work thus far has been limited to bench studies. To date, very few field studies have yet been attempted. However, the results suggest that enzymatic processes can be an effective means for enhancing bioremediation, but also that further studies must be done under field conditions before large scale implementation can begin.

Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are among the most common industrial pollutants in the world, resulting from such processes as coal gasification, coking, and wood preservation (Mahaffey et al. 1988; Field et al. 1994; Dodor et al. 2004). Lower molecular weight (MW) PAHs are readily biodegradable by many microbes, such as *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Micrococcus*, *Beijerinckia*, *Nocardia*, *Vibrio*, and *Flavobacterium* (Guerin and Jones 1988), but higher MW PAHs, such as those with four or more aromatic rings, tend to be quite recalcitrant and toxic (Heitkamp et al. 1988; Wammer and Peters 2005). The ability of these normally recalcitrant compounds to be degraded is significantly increased with the addition of LiP, MnP, and laccase (see Table 1).

LiP is very effective in the bioremediation of PAHs. LiP from *Phanerochaete chrysosporium*, for example, is able to degrade PAHs with ionization potentials up to 7.55 eV (Hammel et al. 1986, 1992; Bogan et al. 1996). LiP was able to carry out the first degradation step of pyrene, a four ring PAH, with 84% of the products consisting of pyrene-1,6-dione and pyrene-1,8-dione and water accounting for 90% of the quinone oxygen. These two products cannot be further degraded by LiP, but are more readily degradable than pyrene by certain microbes. Although pyrene is not mutagenic, these two products are (Hammel et al. 1986). In the field, these products must be mineralized as quickly as possible after they are formed in order to reduce any risk to human health. Haemmerli et al. (1986) showed that LiP was also capable of degrading benzo[a]pyrene into 52% 1,6-quinone, 25% 3,6-quinone, and 23% 6,12 quinone. These product ratios are very similar to those found from the degradation of benzo[a]pyrene using chemical and electrochemical means. This suggests that

Table 2. Summary of Enzymes Used to Degrade TNT

Contaminant	Enzyme	Parent microbe	Contact time	Contaminant decrease	References
TNT	MnP	<i>Phanerochaete chrysosporium</i>	NA ^a	Low	Cameron et al. (2000)
Aminodinitrotoluenes	LiP	<i>Phanerochaete chrysosporium</i>	NA ^a	Medium	Cameron et al. (2000)
TNT	MnP	<i>Nematoloma frowardii</i> and <i>Stropharia rugosoannulata</i>	72 h	0–3%	Scheibner and Hofrichter (1998)
4-AmDNT	MnP	<i>Nematoloma frowardii</i> and <i>Stropharia rugosoannulata</i>	72 h	32–36%, 100% in presence of co-substrate	Scheibner and Hofrichter (1998)
TNT	Laccase	<i>Trametes villosa</i>	48 h	30%, 100% in the presence of catechol	Wang et al. (2002)
4-AmDNT	Laccase	<i>Trametes villosa</i>	48 h	0%, 80% in the presence of catechol	Wang et al. (2002)

^aNot available.

certain chemicals will degrade in specific pathways no matter what the degradation catalyst may be.

Dodor et al. (2004) examined the ability of immobilized and free laccase from *Trametes versicolor* to degrade anthracene and benzo[a]pyrene, two PAHs. Both forms of this enzyme were able to degrade between 80 and 90% of each of these compounds when in the presence of the mediator 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and between 5 and 20% in the absence of this mediator. The major difference between immobilized and free laccase is its ability to withstand pH extremes. In very acidic or alkaline environments, free laccase loses 97% of its activity, and the immobilized laccase loses only 60%. This demonstrates how immobilized enzymes are more robust than their mobile counterparts. Collins et al. (1996) also found laccase from *Trametes versicolor* to be effective in degrading anthracene and benzo[a]pyrene, leading to the degradation of 90–100% of these contaminants in 24 h. In this study, both crude and refined enzymes were shown to be equally effective as catalysts for this reaction, indicating that costly purification processes may be able to be bypassed for enzymes such as laccase. This information may make large scale use of laccases more cost effective in the field.

The ability of MnP derived from *Phanerochaete laevis* to degrade PAHs was also examined. This study showed that this enzyme was able to degrade 33.2% of the initial anthracene concentration, 28.3% of the initial phenanthrene concentration, 26.7% of the initial benzo[a]anthracene concentration, and 17.3% of the initial benzo[a]pyrene concentration. The researchers stated that these numbers are quite low due to experimental errors and do not necessarily indicate the true extent of the remediation (Bogan and Lamar 1996). Another study showed MnP from *P. chrysosporium* to be quite effective in the degradation of PAHs, leading to a 94% decrease in fluorene bound to sandy loam after 6 days (Bogan et al. 1996). The differences in these results may be attributed to the different parent fungi, the different ionization potentials for these two compounds, or simply lab error. The second study indicates that MnP treatment may be a viable option for remediation, if only for the less recalcitrant PAHs.

2,4,6-Trinitrotoluene (TNT)

TNT is perhaps the most well known explosive in the world and a common nitroaromatic recalcitrant pollutant. The use of white rot fungi to remediate TNT has been well documented (Bumpus and Tatatko 1994; Donnelly et al. 1997). Studies have also been done to examine how effective their enzymes are at enhancing the bioremediation of TNT and its intermediate compounds (see Table 2). MnP from *Phanerochaete chrysosporium* was found to be capable of degrading TNT, and LiP from the same fungus is capable of degrading aminodinitrotoluenes into its intermediates (Cameron et al. 2000). Scheibner and Hofrichter (1998) used MnP from *Nematoloma frowardii* and *Stropharia rugosoannulata* to degrade TNT and 4-amino-2,6-dinitrotoluene (4-AmDNT). The addition of only MnP to TNT yielded no degradation of the pollutant, but the addition of MnP from *N. frowardii* and *S. rugosoannulata* to 4-AmDNT resulted in a 36 and 32% decrease in the 4-AmDNT concentration, respectively, after 72 h.

When the reaction was performed in the presence of 1 mM reduced glutathione, all of the 4-AmDNA was reduced by MnP from both parent fungi, but only 3% of the TNT was reduced. In addition to this experiment, a mixture containing 22.9% hydroxylaminodinitrotoluenes, 31.4% 2-AmDNT, 31.6% 4-AmDNT, 3.3% residual TNT, and 10.8% unknown products was created. When MnP from *N. frowardii* and *S. rugosoannulata* was added to the mixture in the presence of 10 mM reduced glutathione, 15 and 17.5% of the mixture, respectively, was mineralized (Scheibner and Hofrichter 1998). It is not practical for all of the intermediates to be able to be degraded when the initial substrate cannot be degraded, as occurred in the presence of glutathione, unless there is another enzyme or microbe present that can start the degradation process. As seen in the second experiment, it is more desirable because this allows for degradation of TNT to continue through to mineralization. Although the extent of the mineralization was limited, this study demonstrated that MnP is capable of mineralizing TNT and its intermediates concurrently. This is encouraging news for the use of MnP to degrade TNT in the field. In the field, the intermediates can accumulate once the

initial substrate is degraded. These intermediates can also be toxic, especially if they are in high enough concentrations. The fact that MnP is able to carry the degradation of TNT to mineralization holds promise for future field application. It should also be noted that changing the concentration of enzymes used or adding a cosubstrate may increase these mineralization rates.

One such cosubstrate may be catechol. When laccase from *Trametes villosa* was added to a solution containing 4AmDNT and TNT, only 30% of the AmDNT and none of the TNT was transformed. When the same experiment was done in the presence of humic monomer catechol, 100% of the 4AmDNT and up to 80% of the TNT was degraded. The optimal pH for this reaction was close to neutral (Wang et al. 2002), which is beneficial to natural environments. The use of enzymatic remediation must be reconsidered if the enzymatic activity is affected by the presence of the substrate. Nepovim et al. (2004) found that the presence of TNT generally reduced the activity of the peroxidases in the solution. The presence of diaminitrotoluenes decreased peroxidase activity after 6 h, but then resulted in an overall increase in activity after 27 h. Substrates impact enzymes differently, which must be accounted for in the field, particularly if multiple substrates are present. Although it would be impossible to test how every combination of nitroaromatic compounds and their intermediates affect enzymatic activity, some knowledge must be attained in order to determine how effective these enzymes will be in the field.

Phenols

Several extracellular enzymes are able to remediate phenol contamination. Phenols are aromatic compounds created during coal refining, oil refining, plastic production, and resin production. Many phenols are toxic and suspected carcinogens (Flock et al. 1999). HRP was able to remove about 60% of the aqueous chlorophenol in a solution and 50% of aqueous phenol in a separate solution in a stirred membrane reactor (Flock et al. 1999). Soybean peroxidase (SP) was expected to yield similar results, but only reduced phenol and chlorophenol concentrations by 10%. This experiment was done in the presence of hydrogen peroxide and an abundance of free radicals, which was believed to have inhibited the SP. It was shown, however, that the addition of detergent increased the production of these enzymes by up to 350% when present in small amounts (less than 0.6% w/v) and did not have any adverse effects on their activities when present in larger concentrations (Flock et al. 1999).

Ullah et al. (2000) discovered that laccase from white rot fungus was capable of remediating chlorophenols. The laccase was extracted from *Cotriolus versicolor* grown on wheat husk and wheat bran cultures that were incubated for 20–30 days. The aqueous laccase was able to completely degrade 2,4-dichlorophenol with an initial concentration of 50 ppm in 5 h. The same enzyme removed 75–80% of the pentachlorophenol (50 ppm initial concentration) from solution within 24 h. Leontievsky et al. (2000) determined that laccase, LiP, and MnP from *Cotriolus versicolor* were also able to transform trichlorophenol into the less toxic compounds of 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone. The same results were true when the same enzymes were extracted from *Panus tigrinus*. Laccase appeared to dominate the transformation when the enzymes were from *Cotriolus versicolor*, and MnP dominated when the enzymes came from *Panus tigrinus*, indicating that two different degradation pathways were present. In another study, immobilized laccase from *Pleurotus ostreatus* in a packed-bed reactor

demonstrated continuous elimination of 2,6-dimethoxyphenol by causing it to precipitate out of solution (Hublik and Schinner 2000). Immobilized LiP was able to remove 55% of the total phenol from paper mill effluent while losing almost no activity during the process (Peralta-Zamora et al. 1998).

Mozhaev et al. (2002) found that arylsulfotransferase (AST) isolated from *Clostridium innocuum* is capable of sulfating many phenol containing compounds. It uses arylsulfates as a sulfate donor and can use numerous substances, such as Tyramine, 4-Methylbelliferone, phenol, p-Cresol, and m-Cresol, as sulfate acceptors. When in the presence of phenol, AST maintains an activity level of 84% of its maximum activity. This indicates a strong potential for the remediation of phenol using AST.

Extracellular enzymes have been shown to be very effective in transforming and removing phenol in a solution (see Table 3). The ability of these phenol degraders to retain their activity over a long period of time means that they could be left in the field with minimal upkeep for days or weeks. Enzymes are also able to be quite effective with contact times on the order of a few hours or days. This means that it may be possible for enzymes to effectively pretreat the phenol in a liquid effluent, such as from a paper plant, before it is sent to a wastewater treatment plant. Enzymes may also effectively enhance the treatment of phenol contamination in soils.

In 1990, a field study was conducted that examined the ability of two white rot fungi, *Phanerochaete chrysosporium* and *P. sordida*, to degrade pentachlorophenol (PCP) in a soil. Although these fungi were operating under suboptimum conditions, they still were able to degrade 88–91% of the PCP in 6.5 weeks. Very little of the PCP was mineralized; instead, its intermediate products bonded to soil particles and were not available for further degradation (Lamar and Dietrich 1990). Ricotta et al. (1996) also noticed that PCP was readily degradable by a white rot fungus, but was very slow to mineralize, even under laboratory conditions. This is quite problematic because the intermediates may be more toxic than the original compound. These intermediates must also be degradable if this treatment option is to be feasible. This field study focused only on the ability of white rot fungi to degrade PCP, but did not focus on the ability of their enzymes to enhance this degradation. It does show that white rot fungi are capable of degrading a recalcitrant phenol, indicating that white rot fungal extracellular enzymes are also capable of degrading this compound. Field studies should be done to examine the ability of these enzymes to enhance this degradation process.

Polychlorinated Biphenyls (PCBs)

In addition to degrading phenols, enzymes from white rot fungi have also shown the ability to degrade PCBs (see Table 4). PCBs are synthetic compounds that have between one and ten chlorine atoms attached to an aromatic biphenol frame, creating 209 theoretical congeners. Between 20 and 60 of these congeners are found in commercial products (Ohtsubo et al. 2004; Pieper 2005). Extracellular MnP, LiP, and laccase produced by fungi such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Coriolopsis polyzona*, and *Pleurotus ostreatus* are capable of degrading various PCBs. They are relatively stable and maintain relatively high activity levels, but the ability of the enzymes to degrade PCBs is determined by their parent fungus (Novotny et al. 1997). For example, MnP, LiP, and laccase produced by *Trametes versicolor* were able to reduce 50% of a Delor 106 PCB mixture after 3 weeks, but the same enzymes produced by *Pleurotus ostreatus* were unable to reduce the mixture (Novotny et al. 2004). Such

Table 3. Summary of Enzymes Used to Degrade Phenols

Contaminant	Enzyme	Parent microbe	Contact time	Contaminant decrease	References
Chlorophenol	HRP	Horseradish	1 h	50%	Flock et al. (1999)
Phenol	HRP	Horseradish	1 h	60%	Flock et al. (1999)
Phenol, Chlorophenol	SP	Soybean	1 h	10%	Flock et al. (1999)
2,4-dichlorophenol	Laccase	<i>Cotiolus versicolor</i>	5 h	100%	Ullah et al. (2000)
Pentachlorophenol	Laccase	<i>Cotiolus versicolor</i>	24 h	75–80%	Ullah et al. (2000)
Trichlorophenol	Laccase, MnP, LiP	<i>Cotiolus versicolor</i> and <i>Panus tigris</i>	NA ^a	High	Leontievsky et al. (2000)
2,6-dimethoxyphenol	Laccase	<i>Pleurotus ostreatus</i>	Continuous degradation	100%	Hublick and Schinner (2000)
Total phenol	LiP	NA ^a	3 h	55%	Peralta-Zamora et al. (1998)
Phenols	AST	<i>Clostridium innocuum</i>	NA ^a	High	Mozhaev et al. (2002)

^aNot available.

results indicate that the same enzymes from different fungi possess different remediative properties and that these differences can completely change their function.

Keum and Li (2004) determined that laccase from *Trametes versicolor* and *Pleurotus ostreatus* were both capable of degrading a large range of hydroxyl PCBs, which are toxic metabolites of PCBs and are endocrine disrupting compounds. For example, laccase from *Trametes versicolor* was capable of degrading over 65% of the 4-hydroxybiphenyl in a solution in 3 h and at a pH of 4.0, and laccase from *Pleurotus ostreatus* was capable of degrading over 50% of the same compound under the same conditions. A small potential for degradation by laccases was even found to exist for 4-hydroxy-PCB 26, a hydroxy PCB with 3 chlorine atoms and an ionization potential of over 9, meaning that laccases may be able to degrade even very recalcitrant compounds. For PCBs, the amount of chlorination is what decides its biodegradability. The larger the number of chlorine atoms in the molecule, the more inhibited the enzymes are (Keum and Li 2004; Ohtsubo et al. 2004; Pieper 2005). The ionization potential increases with the amount of chlorination, and there has been found to be an inverse linear correlation between the ionization potential and the enzymatic removal rate coefficients for PCBs (Keum and Li 2004). It should be noted that the ionization potential for this compound is 8.6440 eV, which is much higher than the 7.55 eV ceiling found for PAHs. This suggests that the use of ionization potential alone may not be an effective measure of a compound's

enzymatic biodegradability, but the type and structure of the compound must also be considered.

Dyes

Although enzymes have been used for decades in the textile industry as detergents, only recently have extracellular enzymes been examined for their ability to decolor and degrade dyes (Gianfreda and Rao 2004). In order for dyes to be degraded in a wastewater treatment plant, the chromophores in the dyes must be oxidized and cleaved (Kandelbauer et al. 2004). Two enzymes, laccase and MnP, are proven to be quite effective in this function. Laccase produced by *Pycnoporus sanguineus* in liquid cultures can completely decolor bromophenol blue and malachite green (both triphenylmethane dyes) and partially decolor orange G and amaranth (both azo dyes). Immobilization of the enzyme on alumina increased its thermal stability and made it less affected by inhibitors, such as halides and dye additives. Immobilized laccase was also able to decrease the toxicity of the dyes by up to 80% (Mayer and Staples 2002). Furthermore, laccases from a variety of parent fungi, including *Trametes versicolor*, *Trametes hirsute*, *Pleurotus ostreatus*, and *Phlebia tremellosa*, have been found to be effective decolorizers for a wide variety of structurally different dyes (Kandelbauer et al. 2004).

MnP proved highly effective at reducing dyes as well. The amount of decolorization was done by comparing the dye's ab-

Table 4. Summary of Enzymes Used to Degrade PCBs

Contaminant	Enzyme	Parent microbe	Contact time	Contaminant decrease	References
Delor 106 PCB	MnP, LiP, laccase	<i>Trametes versicolor</i>	3 weeks	50%	Novotny et al. (2004)
4-hydroxybiphenyl	Laccase	<i>Trametes versicolor</i>	3 h	65%	Keum and Li (2004)
4-hydroxybiphenyl	Laccase	<i>Pleurotus ostreatus</i>	3 h	50%	Keum and Li (2004)

Table 5. Summary of Enzymes Used to Degrade Dyes

Contaminant	Enzyme	Parent microbe	Contact time	Contaminant decrease	References
Bromophenol Blue Malachite Green	Laccase	<i>Pycnoporus sanguineus</i>	NA ^a	100%	Mayer and Staples (2002)
Orange G Amatanth	Laccase	<i>Pycnoporus sanguineus</i>	NA ^a	High	Mayer and Staples (2002)
Reactive Orange 16 Remazol Brilliant Blue R	MnP	NA ^a	7 days	85–100%	Novotny et al. (2004)

^aNot available.

sorbance before the enzyme was added and 7 days after the enzyme was added. MnP in a stationary culture was able to decolor approximately 85% of the Reactive Orange 16 dye and 99.7% of the Remazol Brilliant Blue R dye in that time period. In a submerged culture with 1 g/L of the inorganic surfactant Tween 80 added, MnP was able to decolor approximately 87% of the Reactive Orange 16 dye and 100% of the Remazol Brilliant Blue R dye. Significant decolorization was also shown for the addition of MnP to Drimaren Blue, Acid Black, and Drimaren Red in a polyurethane foam reactor (Novotny et al. 2004).

A wide variety of physical and chemical procedures are currently in use to decolorize industrial dyes and to cleave their chromophores. The use of microbes for these purposes is just beginning (see Table 5). Field studies have been done using white rot fungi and bacteria for this purpose, but not their enzymes by themselves (Kandelbauer et al. 2004). A major obstacle that will have to be overcome is the long contact time required for decolorization to occur. The 7 day contact time that Novotny et al. (2004) used is not feasible for a large scale treatment facility, and this will have to be significantly shortened before a field study can be done.

Organophosphates (OPs)

OPs are toxic substances that are used in herbicides, pesticides, and nerve gas. Qiao et al. (2003) developed a genetically engineered form of carboxylesterase that will degrade and detoxify the OP wastes malathion, parathion, and monocrotophos. The enzyme degraded about 80% of the malathion in 90 min, about 85% of the parathion in 6 h, and about 20% of the monocrotophos in 9 h. The reason why malathion and parathion degraded faster and

more completely than monocrotophos is that these two compounds contain carboxylester bonds, which are readily broken down by a carboxylesterase, while the other compound does not. These contact times appear to be quite feasible for a field implementation. Zhang et al. (2004) also studied the impact of a recombinant carboxylesterase derived from an insecticide-resistant mosquito on OPs. They discovered that 0.1 nmol of the enzyme was able to neutralize 1 nmol of chlorpyrifos and 2 nmol of paraoxon individually (see Table 6).

Laccase derived from *Phanerochaete chrysosporium* and *T. versicolor*, as well as purified laccase, were found to be able to inactivate the herbicide diketonitrile by converting it into a benzoic acid analogue. The purified laccase requires the presence of 2 mM 2,2'-azinobis(3-ethyl-benzthiazoline-6-sulfonic acid) and at a temperature of 30–50°C and a pH of 3 in order for this reaction to occur (Mougin et al. 2000). Such extreme conditions are not feasible for field implementation, but this study is able to illustrate that laccase can catalyze the degradation of diketonitrile, which further demonstrates its versatility.

A field-scale remediation study utilizing primarily enzymatic processes for the remediation of an OP occurred in 2000, and it was the first of its type in the United States. A site was contaminated by an accidental atrazine spill, resulting in concentrations of up to 29,000 ppm in the soil. Atrazine chlorohyrolase (AtzA), which is nontoxic to plants, was encapsulated in dead *E. coli* cells and dispersed throughout a test bed at a concentration of 0.5% (w/w). After 8 weeks, the atrazine level in the test bed with the AtzA declined by 52% and by 77% in the test bed with the AtzA and additional phosphate, whereas the concentration in the control did not decrease significantly (Strong et al. 2000). The

Table 6. Summary of Enzymes Used to Degrade OPs

Contaminant	Enzyme	Parent microbe	Contact time	Contaminant decrease	References
Malathion	Carboxylesterase	NA ^a	90 min	80%	Qiao et al. (2003)
Parathion	Carboxylesterase	NA ^a	6 h	85%	Qiao et al. (2003)
Monocrotophos	Carboxylesterase	NA ^a	9 h	20%	Qiao et al. (2003)
Chlorpyrifos	Carboxylesterase	<i>Insecticide resistant mosquito</i>	4 h	100%	Zhang et al. (2004)
Diketonitrile	Laccase	<i>Phanerochaete chrysosporium</i> <i>Trametes versicolor</i>	12–15 days	High	Mougin et al. (2000)
Atrazine	AtzA	<i>E. coli</i>	8 week	52%, 77%	Strong et al. (2000)

^aNot available.

Table 7. Summary of Enzymes Used in Wastewater Treatment

Function	Enzyme	Contact time	Results	References
Disinfection	Protease	48 h, 96 h	6 order of magnitude reduction (OMR) in fecal coliform population, 5 OMR in <i>Salmonella</i> population	Parmar et al. (2001a)
Disinfection	<i>Culvularia</i> haloperoxidase	10 min, 20 min	6 OMR in <i>E. coli</i> population, 5 OMR in <i>S. epidermidis</i> population	Hansen et al. (2003)
Deflocculation	Protease, Cellulase, and β -glucosidase	24 h	Large decrease in floc sizes	Watson et al. (2004)
COD removal	Microcat-XPCW	48 h	40% increase in COD removal	Bioscience (2003)
Solids reduction	Alcalase, Cellulase, Fungal Protease, Lipase	96 h	46–53.8% reduction in solids content	Parmar et al. (2001b)
Sewerline degreasing	Protease, Lipase, Amylase	Field implementation	Grease clogging in sewerlines has stopped	Fisher, private communication (2005)
Lift station degreasing	Oleozyme	Field implementation	Grease clogging in lift station has stopped	Aster Bio (2005a)
Municipal wastewater treatment enhancement	Munizyme	Field implementation	5% increase in TSS removal, 5% increase in BOD removal, 22% increase in ammonia removal	Aster Bio (2005b)
Mixed industrial and municipal wastewater treatment enhancement	Munizyme and Phenozyme	Field implementation	36.3% increase in TSS removal, 32% increase in BOD removal, 24.1% increase in COD removal	Aster Bio (2005c)

dead *E. coli* cells were used in order to stabilize the enzyme during storage, even though it results in a 35% initial drop in enzymatic activity. The study was performed in autumn, when soil temperatures descended to 7 °C, which is much lower than the optimum temperature for enzymatic activity (Strong et al. 2000). This study indicates that enzymatic treatment can still be effective in degrading recalcitrant compounds under less than optimal conditions in real world settings and that such a treatment can function even in a heavily contaminated site.

Wastewater

Enzymatic treatment of wastewater can reduce many of its harmful effects for the environment and human health. Wastewater contains a variety of fecal-borne pathogens, such as *Salmonella*, *Vibrio cholerae*, *Salmonella typhi*, and *Giradia*, that can lead to severe gastroenteritis, cholera, typhoid, and other water-borne diseases (Metcalf and Eddy 2003). It has been shown that enzymes are able to kill such pathogens through the degradation of their cell walls (Uhlig 1998; Parmar et al. 2001b; Whiteley et al. 2003) (see Table 7). The rigidity of the cell wall comes from a peptidoglycan layer, which is primary composed of amino acids, various sugars, and teichoic acid. In Gram positive cells, the peptidoglycan layer is thick and rigid and surrounded by a lipopolysaccharide membrane on the outside. Gram negative cells have a thin layer of peptidoglycan surrounded by a lipopolysaccharide membrane on the inside and the outside of this layer

(Uhlig 1998; Madigan et al. 2003). The outer membrane must be degraded by a lysozyme, ethylenediamine tetraacetic acid (EDTA), or similar enzyme before the proteases, lipases, and amylases can degrade the peptidoglycan layer (Uhlig 1998). A hole in the cell wall is eventually created. Since the pressure inside the cell may be as much as 2 atm, the cells burst without the rigidity of the cell wall (see Fig. 2) (Madigan et al. 2003).

For Gram positive cells, only lysozyme is needed for the degradation of the cell walls. Lysozyme is an enzyme that is derived from the whites of chicken eggs and catalyzes the hydrolysis of β -1-4-glycosidic bonds that hold the proteins and sugars in the peptidoglycan layer together (Scawen and Melling 1985; Hu and Lu 2004; Moak and Molineux 2004). This is not the case for a Gram negative cell, which cannot be degraded by lysozyme alone (Scawen and Melling 1985; Gill and Holley 2003). Both lysozyme and EDTA, which causes the membranes to release lipopolysaccharide molecules into the solution, are required for degradation of the Gram negative cells (Scawen and Melling 1985). This combination of enzymes is very effective in the lysis of Gram negative cells while the cell is in a buffer solution, but is not as effective when the cell is in a nutrient rich solution (Gill and Holley 2003). The difference is that cells are able to repair some of the damage that the EDTA does to the outer membrane when there are sufficient nutrients present in the solution. Such repairs cannot occur in a buffer solution, allowing the EDTA to more effectively degrade the outer membrane. In conventional

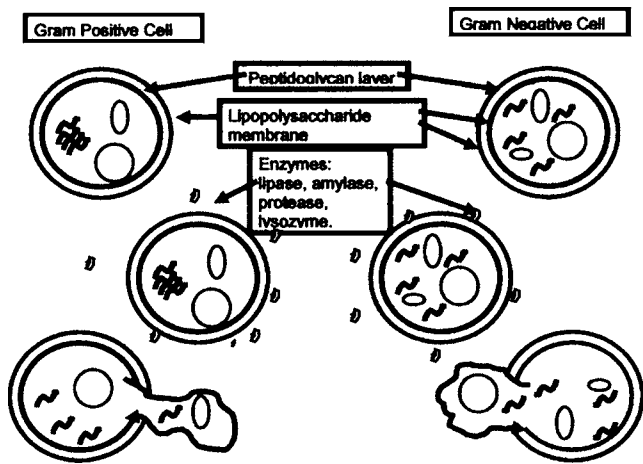


Fig. 2. Lysis of Gram positive and Gram negative cells due to addition of extracellular enzymes

wastewater treatment, disinfection is the last step before the wastewater is released. At this point in the process, most of the nutrients in the wastewater have been removed, making it akin to the buffer solution. The combination of lysozyme and EDTA may be an effective means of cell lysis in this situation. On the other hand, raw influent may more closely resemble the nutrient rich solution, where this combination is not as effective. Studies must be done to determine how well the combination of EDTA and lysozyme works in real life systems such as wastewater.

Situations such as this one demonstrate that there is not one specific enzyme that works the best in all cases. In nature, there is often a wide variety of both Gram positive and Gram negative bacteria coexisting in wastewater, meaning that adding one type of enzyme may not be an effective means of killing all of the microbes. A mixture containing a variety of enzymes may be the most effective means of wastewater disinfection. One of the potential problems that could develop from using a mixture of enzymes is that the enzymes degrade each other in addition to the cell walls. For example, a protease could catalyze the degradation of the amino acids that make up another enzyme. A minimization of such interactions would be required.

Parmar et al. (2001a) observed that the addition of alkaline protease to wastewater greatly decreased its coliform and *Salmonella* counts. Protease at a concentration of 0.03% was added to a solution containing 4×10^6 colony forming units/mL (cfu/mL) coliforms and 1.5×10^7 cfu/mL *Salmonella* and kept at 40°C. After 2 days, coliform levels were below detection limits when the solution was kept at pH 10 and pH 12. After 4 days, the *Salmonella* concentrations fell to 7×10^3 cfu/mL when the pH was 7, 3×10^3 cfu/mL when the pH was 10, and 4×10^2 cfu/mL when the pH was 12. The high pH required for maximum efficiency cannot be reached under real-world, in situ requirements, but a 4 order of magnitude drop in the *Salmonella* concentration was still able to occur at a neutral pH. Even though the enzyme system is not at its peak efficiency under real-world conditions, they can still be quite an effective disinfectant. When looking at the microscale, it has been estimated that the lysis of a bacterial cell in suspension can take up to 1 h in a solution containing 250 mg/L of ovalyszyme with an activity of about 20,000 units/g protein (Uhlig 1998). Both instances proved that extracellular enzymes are capable of killing pathogens and other bacteria, but the long contact time required for disinfection may be problematic if this technology were ever to be scaled up for

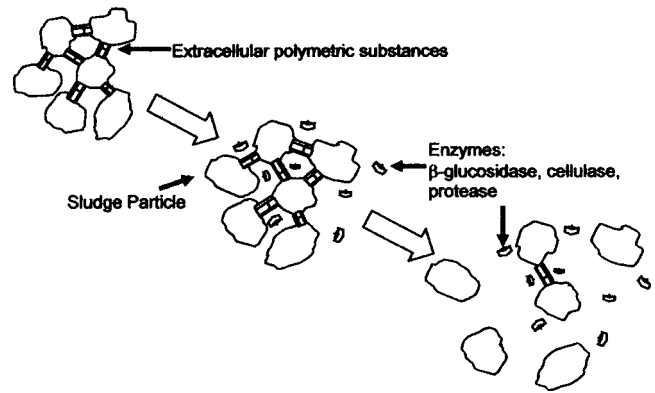


Fig. 3. Addition of enzymes to activated sludge has been shown to deflocculate activated sludge particles by degrading extracellular polymeric bonds that bind sludge particles together

use in a wastewater treatment plant. However, not all enzymatic disinfection systems requires a long contact time. *Culvularia* haloperoxidase kills microbes very quickly by oxidating halides, such as iodine and chloride, to antimicrobial compounds. This system results in a 6 order of magnitude reduction in the *E. coli* population in 10 min and a 5 order of magnitude reduction in the *S. epidermidis* population in 20 min, and the treatment caused large population reductions for many other microbial species as well (Hansen et al. 2003). This study indicates that there is more than one mechanism by which enzymatic activities lead to disinfection, but this latter mechanism has only been examined for the disinfection of surfaces in the presence of halides. It could potentially be used to enhance chlorine disinfection methods for wastewater, but further studies must be done to prove this.

Enzymes are able to break apart large sludge particles, creating more surface area for microbes to attack. This allows for a more complete and more efficient degradation of the sludge particles (Whiteley et al. 2002). Such particles are held together by extracellular polymeric substances that come from cell autolysis, bacterial metabolic reactions, and the wastewater itself (see Fig. 3). These substances consist of primarily polysaccharides and proteins. When in the presence of free β -glucosidase, cellulase, and protease, these bonds break apart, and the particles deflocculate. A sludge solution was studied that had floc sizes ranging from 20 to 99 μm with 39% of the flocs being between 50 and 69 μm . When protease (10% w/v) was added, 60% of flocs had a size less than 29 μm after 24 h. The addition of β -glucosidase and cellulase yielded similar results (Watson et al. 2004). Making the wastewater more biodegradable, however, also increases the amount of oxygen that the microbes consume. When the Microcat-XPCW mixture from Bioscience, Inc. was added to the effluent from a pulp and paper plant, the chemical oxygen demand (COD) removal rate increased to almost 2.5 times that found in the normal treatment system. However, the amount of oxygen consumed also increased by 477% (Bioscience 2003). The higher oxygen consumption rate indicates higher microbial activity, leading to higher removal rates. The enzyme mixture makes the wastewater more biodegradable, allowing the microbes to work more effectively. The demand for more oxygen means that the treatment system must be aerated to prevent the wastewater from becoming anoxic or anaerobic. This cost may act as a deterrent from using enzyme mixtures on a large scale, so further studies must be done to reduce the ensuing oxygen consumption.

Parmar et al. (2001b) did an extensive study on the ability of

various extracellular enzymes to reduce the solids content in sewage. The organic matter in sewage contains 40–60% protein, 25–50% carbohydrate, and 8–12% fats (Metcalf and Eddy 2003), which can be degraded by protease, amylase, and lipase, respectively. They added various concentrations of alcalase (alkaline protease), esperase, econase, lipase EX, protease, Septizyme, fungal protease, and cellulase to a sludge solution both individually and as mixtures at different pHs. All of the enzymes produced at least a 20% reduction after being incubated for 96 h at 40°C, while the control had only an 8% reduction after the incubation. A 53.8% reduction occurred when alcalase was added to the sludge at a concentration of 0.03% and a pH of 9. Cellulase, fungal protease, and lipase were all also highly effective, providing reductions of 46–51% when under acidic conditions. If such technology is going to be implemented on a large scale, the enzymes must be proven to be effective at more moderate temperatures and more neutral pHs.

Several field studies have been done using commercial enzyme mixtures to enhance wastewater treatment. An enzyme mixture from Enzyme Solutions, Inc. containing a protease, a lipase, an amylase, and other enzymes mixed with surfactants has been implemented to degrease sewers in Fort Wayne, Ind. (Fisher, private communication 2005). The enzymes solubilize the greases and fats in the wastewater, preventing it from clogging the sewer lines. The greases and fats are then removed from the wastewater at the wastewater treatment plant. A similar mixture, Oleozyme from Aster Bio, was also used to reduce grease clogs in a wastewater lift station (Aster Bio 2005a). In both cases, grease clogs were eliminated, and the accumulation of grease in the sewer lines was greatly decreased.

Enzymes can also be used at municipal wastewater treatment plants. Munizyme, an enzyme/microbial mixture from Aster Bio, was added to municipal wastewater before it entered a conventional wastewater treatment plant. The addition of this enzyme mixture increased the total suspended solids (TSS) removal from 79 to 84%, the biochemical oxygen demand (BOD) removal from 83 to 88%, and the ammonia removal from 65 to 87% (Aster Bio 2005b). Aster Bio (2005c) did another field study adding a mixture of Munizyme and Phenozyyme, a phenol degrading enzyme mixture, to mixed municipal and industrial wastewater going through a trickling filter treatment system. On average, the addition of the enzymes improved TSS removal by 36.3%, BOD removal by 32%, and COD removal by 24.1%. These studies show that enzyme mixtures can be an effective additive to wastewater treatment processes that improves the efficiency of an existing plant.

Conclusion

The use of extracellular enzymes has been proven to be an effective means of enhancing bioremediation for a variety of compounds, including PAHs, PCBs, OPs, dyes, phenols, and wastewater. MnP, LiP, and laccase produced from various white rot fungi are especially effective at degrading TNT, phenols, PCBs, PAHs, and dyes, although their ability to degrade these compounds appears to be determined by their parent fungus. OPs appear to be easily degraded by carboxylesterase, regardless of the origin of the enzyme. Protease possesses the capacity to reduce pathogen counts, reduce solids concentration, and increase deflocculation in sludge. β -glucosidase and cellulase are also able to deflocculate sludge, and the addition of lipase and cellulase to a sludge mixture yields a large reduction in the solids content.

Before enzymatic technology can be used for bioremediation on a large scale, further studies must be done. A vast majority of the studies that have already been completed focus on using one specific enzyme to degrade one specific compound at a time. Such a scenario rarely occurs in the field. A given field site may contain any number of different compounds ranging from the easily biodegradable compounds to extremely recalcitrant ones, and using one type of enzyme may not be effective for the whole system. Further studies must be done using multiple enzymes to degrade many different compounds concurrently. The use of appropriate cosubstrates must also be examined as a possible means in enhancing the degradation efficiency of the remediation system. Furthermore, many lab studies thus far have been performed using sterile soil as the reaction media. This also does not model what actually occurs in the field. In the field, the enzymatic reactions take place in the presence of ambient bacteria, which may help or hinder the process. Either way, they may be a pivotal factor in the success of the remediation plan. Such simplifications of natural systems is necessary in determining if enzymatic treatment works or not, but now that such validation has occurred, the focus must change to how well such technology can be scaled up.

In order to provide a proof of concept for enzymatic bioremediation, many studies were done at high temperatures and at the enzyme's optimum pH, which may be quite acidic or basic. What are needed are studies that determine enzymatic efficiency under more moderate temperatures and neutral pHs, like those found in the field. Because field conditions vary from hot and arid to cold and wet, studies must also be done to determine how well enzymatic bioremediation works in a variety of field conditions. Factors such as moisture, soil composition, and diurnal temperature variances should also be included in these studies.

Most importantly, more field studies must be done in order to prove how effective enzymes are in enhancing bioremediation. This is the only way to prove that enzymatic bioremediation is an effective and desirable alternative to current remediation strategies. In general, studies have shown that extracellular enzymes can be a valuable option for the remediation of certain recalcitrant compounds and sludge, but this technology must be validated in the field.

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