Methods for Measuring Hydrolase and Phenol Oxidase Activity

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Introduction

Wetlands and aquatic systems are furnished with intrinsic facets and processes that provide valuable functions, including water storage, water treatment, and wildlife habitat (Kadlec and Wallace, 2009, Reddy and DeLaune, 2008). Wetlands function to remove pollutants from point and nonpoint sources including organic matter, suspended solids, metals, and nutrient surplus (Kadlec and Wallace, 2009). Excessive nutrient inputs from urbanization and agriculture are a common threat to major ecosystems, which can lead to harmful eutrophic conditions. Therefore, removal of excess nutrients is essential to protect the ecological integrity of many ecosystems.

Microbial communities play a key role in ecosystem-level processes such as decomposition of organic matter, nutrient cycling (Wright and Reddy, 2001), and processes affecting the efficiency of nutrient cycling and ecosystem function (Yao et al., 2000). These microbial processes include the release of extracellular enzymes, which function to convert complex organic molecules to simple organic constituents during decomposition of organic material (Prenger, J. P. and K. R. Reddy, 2004). Soil enzymes are protein structured molecules that increase the reaction rate by catalyzing them without any permanent transformation (Dick and Kandeler, 2004). The substance acted upon by a soil enzyme is called a substrate. The enzymatic reaction cleaves the substrate and releases a product, which can be a nutrient contained in the substrate. Enzyme production is a function of microbial activity which is regulated in part by nutrient availability (Sinsabaugh, 1994), where microbes produce enzymes to mobilize resources from compound sources when nutrients are limited (Harder and Dijikhuizen, 1983). Microbial extracellular enzyme activity function as a bioindicator to characterize current wetland and aquatic ecosystem status, specifically nutrient shifts.

The objective of this publication is to describe the function and activity of microbial extracellular enzymes in wetland and aquatic ecosystems and to summarize standard techniques for enzymic assays

required to measure enzyme groups primarily involved in wetland and aquatic nutrient cycling – hydrolases and phenol oxidases.

Function and Significance of Extracellular Enzyme Activity in Wetlands and Aquatic Systems

Microbes release extracellular enzymes to access energy and nutrients present in complex substrates (Allison & Vitousek, 2005), which catalyze the initial, rate-limiting step of decomposition and nutrient mineralization (Asmar et al., 1994; Sinsabaugh, 1994). Most extracellular enzymes are hydrolytic, i.e., they involve the addition of a water molecule across the enzyme-susceptible linkage, which acts as a catalysis to facilitate the cleavage of bonds in molecules (Reddy, 2008) (Fig. 1). These complex molecules are typically broken down into simple and smaller subunits that can be taken into the cell.

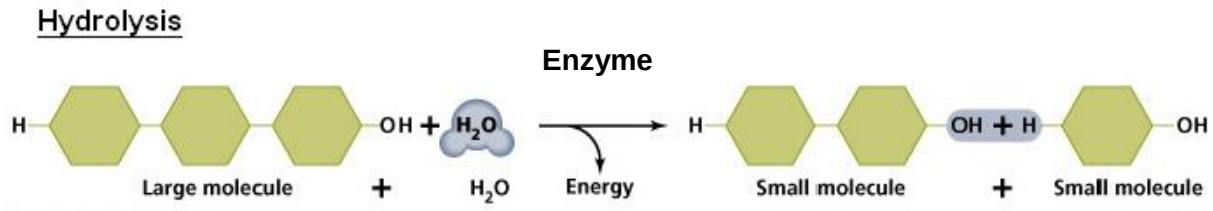

Figure 1. Hydrolytic enzyme activity function as a catalyst to breakdown complex substrates to smaller products by breaking the bond between water molecules. In this process, a hydrogen (H+) is added to one component and a hydroxide ion (OH-) is added to another one.

Hydrolase enzymes regulate the rate at which substrates are degraded to become available for microbial and plant uptake, making them the main mediators of soil biological processes, such as organic matter decomposition, mineralization and nutrient cycling (Marx et al., 2001).

Less common in wetlands and aquatic systems, but of great importance, is a group of aerobic enzymes known as phenol oxidase, which involve the breakdown of aromatic ring structure in phenolic compounds to polyphenols (Duran et al. 2002). This enzyme is severely limited in wetlands largely due to anaerobic conditions, as phenol oxidase requires biomolecular oxygen for its activity (Fig. 2) (Freeman et al., 2004).

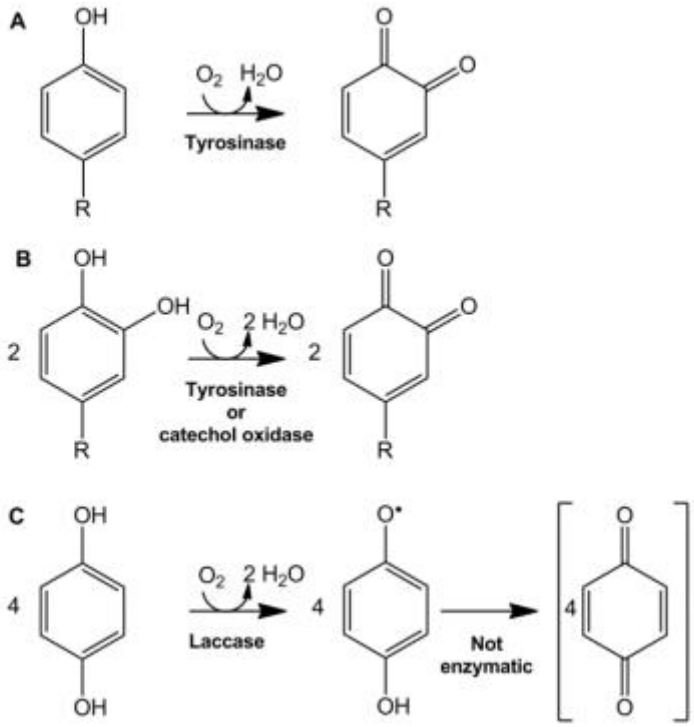

Figure 2. Phenol oxidase is mainly characterized into three groups known as laccases, tyrosinase, and catechol oxidases. This image illustrates the oxidation of phenolic compounds catalyzed by tyrosinase (A and B), catechol oxidase (B), and laccase (C).

The outcomes of this process range from partial oxidation and the release of oxidative intermediates, to complete breakdown and formation of non-phenolic product, such as CO_2 (Duran et al., 2002). Phenol oxidase is one of the few enzymes capable of degrading recalcitrant phenolic material such as lignin (McLatchey and Reddy, 1998). In addition, phenol oxidase aggregate activity mediates key ecosystem functions of lignin degradation, humification, carbon mineralization and dissolved organic carbon export (Sinsabaugh, 2010).

Hydrolase Enzyme Activity Assays

In wetland science, a suite of hydrolase enzymes – produced by a variety of bacteria, fungi, microorganisms and even plant cells – are regularly analyzed due to their role in key elements involved in nutrient cycling, notably; Phosphatase, Leucine aminopeptidase, N-acetyl-β-D-glucosaminidase, β-D-xylosidase, β-D-Glucosidase and Arylsulphatase (Table 1). Many methodologies have been developed for a wide range of soil enzymes; they vary in substrate, assay conditions, incubation time and detection methods (e.g. colorimetric, fluorometric, radiolabeled). When utilizing colorimetric method, species of interest undergo a chemical reaction with a specific reagent to produce a colored product which then can be measured using a colorimeter. Colorimetric assay is dependent on the release of p-nitrophenol, a commonly used substrate, resulting in a distinct yellow color that is indicative of enzymatic reaction and can be measured with a spectrophotometer and a standardized calibration curve (Tabatabai, 1994; Deng et al., 2013). Methods currently used are described by Alef and Nannipieri, 1995, Burns, 1978, Gianfreda and Bollag, 1996, Ladd, 1985, Manafi et al., 1991, Roberge, 1978, Schinner et al., 1996, Tabatabai, 1994, Wilson and Goulding, 1986.

Most soil enzyme studies have utilized fluorometric assay methods, specifically fluorogenic 4-methylumbelliferone (MUF) labeled substrate, to measure enzyme activity (Kang et al., 2013). Substrates like MUF contain an artificial fluorescent molecule and one or more natural molecules (e.g.

glucose, amino acids), linked by a specific binding (e.g. peptide binding, ester binding); fluorescence is observed after enzymatic splitting of the complex molecule (Hoppe, 1993) (Fig. 3).

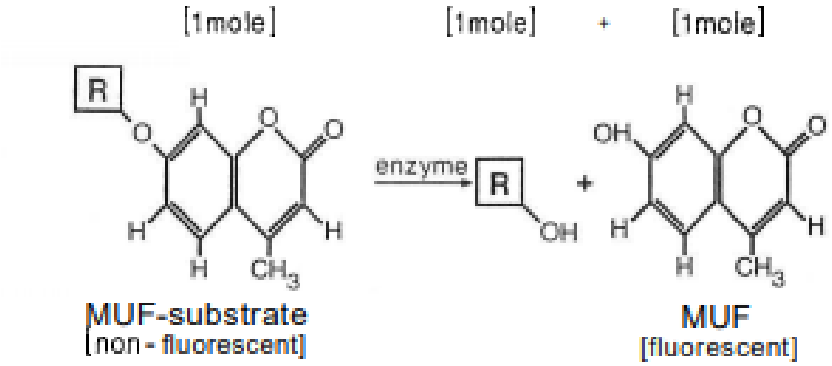

Figure 3. Molecular structure and enzymatic hydrolysis of 4-methylumbelliferyl (MUF) substrates.

This method is particularly well-suited for wetland soil samples because it allows for easy correction of interferences caused by highly colored phenolic compounds, which are commonly found in wetland soils (Freeman et al.1995). MUF is highly fluorescent and thus can easily detect small quantities of hydrolyzed substrate which makes it significantly more sensitive than conventional colorimetric techniques, which is especially effective for measuring low activity in peatland samples (Freeman et al.1995). No side effects of MUF on enzyme activity has yet been identified (Marx et al., 2001) and, as stated by Hoppe (1983), measurements of enzyme activity using MUF substrate have a close relationship with naturally occurring processes. Enzyme activity rates are typically expressed as millimoles (mmol) or micromoles (µmol) of substrate hydrolyzed per mass (g^{-1} or kg^{-1}) of material per hour (h^{-1}). In addition, abundance of microbial biomass can be related to the enzyme activities in soils to determine the approximate induction or stimulation of enzyme activities under given conditions (Sinsabaugh et al., 2008, Nemergut et al., 2010, Leff et al. 2012). For this reason, enzyme activities can

be expressed on both a mass basis (µmol substrate converted (MUF) per gram dry weight per hour (µmol MUF g $DW^{-1}h^{-1}$), and per gram microbial biomass C (µmol MUF g^{-1} MBC h^{-1}).

Phenol Oxidase Activity Assay

In soil biochemistry, enzymes classified as phenol oxidase are often designated in relation to a particular substrate (e.g. monophenol oxidase, tyrosinase, catechol oxidase, diphenol oxidase) even though the individual enzyme generally show activity to varying extents, against a wide range of molecules (Baldrian, 2006). Because environmental assays may capture activity from some or all enzymes, most assays do not discriminate individual enzymes within this group, so the generic term "phenol oxidase" is used to describe the activity of enzymes that oxidize phenolic groups and consume oxygen (Sinsabaugh, 2010). Phenol oxidase is mainly characterized into three groups known as laccases, tyrosinases and catechol oxidases, with subtle differences within their structure and oxidative potential.

Methods for determining oxidative activity began to take root in the early to mid-twentieth century, with analyses measuring oxygen consumption and later by using l-3,4-dihydroxyphenylalanine (DOPA) as a substrate for phenol oxidases (Sinsabaugh and Linkins, 1988; Pind et al., 1994; Sinsabaugh et al., 2008). Additional methods have been proposed that include guaiacol (Nannipieri et al., 1991), catechol (Perucci et al., 2000; Benitez et al., 2006), ABTS (laccase substrate) (Luis et al., 2005; Floch et al., 2007), pyrogallol (Allison and Vitousek, 2004), or o-toluidine (Fioretto et al., 2000; Di Nardo et al., 2004).

Methods using DOPA substrate are commonly used to measure phenol oxidase activity. Pind et al. (1994) based method is frequently used in soil enzymology and specifically wetland soils, in which the determination of phenol oxidase activity uses phenolic amino acid L-3,4-dihydroxy phenylalanine (L-DOPA) as the model substrate. L-DOPA is oxidized by all three major types of phenol oxidases, and it produces dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone), which presents as a red pigmented

color (Mason 1948, Pomerantz and Murthy 1974). The absorbance of this color is measured on a spectrophotometer with higher absorbance readings corresponding to greater activities of phenol oxidases.

Conclusion

Recently, there has been an increasing appeal towards utilizing fluorogenic substrates to measure soil enzyme activities largely due to their high sensitivity compared to other common methods like a standard colorimetric enzyme assay. The high sensitivity of fluorometric enzyme assays provides the ability to detect enzyme activities in small samples (e.g. microaggregates and rhizosphere samples) and/or low activity samples (subsoil, peat, and soil solutions) (Kandeler, 2007). Colorimetric enzyme assays are generally labor intensive, time consuming and constrained to one enzyme per sample run (Deng et al., 2011). However, the colorimetric method has the advantage of measuring a wide range of substances and has relatively low susceptibility against disturbances, so these assays are applied as far as possible and therefore utilize on a variety of different soil samples (Bisswanger, 2014).

In order to conduct an enzyme based analysis based on these methods it is important to: acquire reagents (enzyme target, substrate, co-factors etc.), setup instrumentation (calibration performance testing), establish assay concept validation experiments (establish preliminary assay parameters), determine optimization requirements, validation experiments (robustness verification), and prepare method documentation (Acker et al., 2014).

Table 1.

Hydrolase Enzymes	EC	Substrate	Element	Description
Phosphatase	EC 3.1.3.1	4-MUF phosphatase	P	Catalyze the breakdown of organic ester phosphates to an inorganic phosphate ion (Chrost, 1991).
Leucine aminopeptidase	EC 3.4.11.1	L-Leucine-AMC hydrochloride	N	Catalyze the release of amino acids from complex proteins and peptides (Sinsabaugh et al, 1993).
N-acetyl-β-D-glucosaminidase	EC 3.2.1.96	4-MUF-N-acetyl-ß-D-glucosaminide	N	Involved in the breakdown of chitin and peptidoglycan to amino sugars (Hall et al. 2014).
β-D-xylosidase	EC 3.2.1.37	4-MUF- β-Dxylopyranoside	C	Hemicellulase (xylan) catalyzing the breakdown of hemicelluloses yielding xylose monomers (Dunn et al., 2014)
β-D-Glucosidase	EC 3.2.1.21	4-MUF β-D-glucopyranoside	C	Catalyzes the hydrolysis of glycosides, resulting in the release of β-linked monosaccharide (Eivazi and Tabatabai, 1988).
Arylsulphatase	EC 3.1.6.1	MUF-Sulfate	S	Catalyzes the hydrolysis of sulfate esters resulting in the release of SO_4^{2-} (Tabatabai and Bremner, 1970).

EC, enzyme commission classification; MUF, Methylumbelliferone; AMC, 7-Amino-4-methylcoumarin

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