CELLULOSE-1-4-β- CELLOBIOSIDASE ACTIVITY

IN SOILS

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Abstract: Soil management practices such as crop rotation, tillage, organic matter input, and cover crops can greatly affect physical, chemical, and biological properties that affect soil microbial communities and extracellular enzyme activities through the alteration in soil organic carbon. There is an increasing demand for large scale biomass production, making the relationship between crop production and soil health more important. For sustainable healthy soils, the extracellular enzymes are of great importance because they play vital role in nutrient cycling through the decomposition of carbon compounds of plant cell wall and cellulose is the most abundant carbon molecule in the plant cell wall. As the most abundant biopolymer on earth, cellulose is broken down by an array of enzymes that have varied specificities and act in synergism. An assay protocol was developed to detect and quantify activity of a cellulose degrading enzyme, cellulose 1,4-*β*-cellobiosidase (EC 3.2.1.91) in soil. In addition to developing an assay method to quantify activities of cellobiosidase in soil, series of experiments were conducted to determine the effect of pH, storage temperature and time, and trace elements on stability and activity of cellobiosidase in soil. Moreover, the relationships among several cellulosic and carbon-transforming enzymes, and between their activities and microbial biomass and activities in soil cultivated with switch grass. Data shown that the optimum *β*-cellobiosidase activity in soil were approximately pH 5.5 and 60°C, respectively. The Michaelis constants (K_m) and maximum velocity (V_{max}) in the tested six soils ranged from 0.08 to 0.51 mM for K_m and from 71.5 to 318.1 µmol kg soil^{-1} h^{-1} for V_{max}. The temperature coefficient (Q_{10}) ranged from 1.72 to 1.99, and the activation energy (E_a) ranged from 42.5 to 53.7 kJ mol^{-1}. Soil pH, sample storage temperature and time, trace elements, and management practice affected stability and/or activity of cellobiosidase in soil. The tested enzyme activities had mostly positively correlated each other and with microbial biomass carbon content, with the exception of urease activities which was negatively correlated with microbial biomass. Enzyme activities were not significantly correlated with microbial respiration, suggesting that the detected activities are mostly originated from cell-free enzymes that have been stabilized by soil.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION ... 1

References ... 6

II. REVIEW OF LITERATURE ... 13

Properties and Structure of Cellulose ... 14
Biodegradation of Cellulose ... 19
Biological Distribution, States, and Sources of β-Cellobiosidase in soils ... 21
Mechanisms of Cellulase Activity ... 23
β-cellobiosidase Activity in Soils ... 27
Microorganisms involved in biodegradation of cellulose ... 28
Cellulolytic Enzymes ... 32
References ... 36

III. ASSAY OF β-CELLOBIOSIDASE ACTIVITY IN SOILS ... 57

Abstract ... 57
Introduction ... 58
Materials and methods ... 60
Soil ... 60
Reagents ... 61
Procedure ... 62
Results and Discussion ... 66
Effect of incubation pH ... 66
Effect of substrate concentration ... 68
Effect of amount of soil ... 70
Effect of incubation temperature ... 71
Enzyme kinetics ... 74
Conclusions ... 82
References ... 83

Chapter Page

IV. RELATIONSHIPS AMONG ENZYME ACTIVITIES, MICROBIAL RESPIRATION, AND MICROBIAL BIOMAS 90

Abstract 90
Introduction 91
Materials and methods 93
Site description 93
Sampling and analysis 93
Statistical methods 97
Results 97
Discussion 107
Conclusions 111
References 111

V. EFFECT OF pH, STORAGE TEMPERATURE AND TIME, AND TRACE ELEMENTS ON STABILITYAND ACTIVITY OF CELLOBIOSIDASE IN SOIL 118

V-1. Effect of Soil pH on Stability of Cellulose 1,4 - *β*- cellobiosidase 118

Introduction 118
Materials and methods 120
Results 122
Discussion 124
References 128

V-2. Effect of storage temperature and time on *β*-cellobiosidase (EC 3.2.1.91) activities in soils 131

Abstract 131
Introduction 132
Materials and methods 135
Results 139
Discussion 143
Conclusions 146
References 147

V-3. Effect of trace elements on cellulose 1,4-β-cellobiosidase activity in soil 153

Introduction 153
Materials and methods 154
Results and Discussion 155

References 161

VI. EFFECT OF CROP ROTATION AND RESIDUES MANAGEMENT ON CELLULASE, β -CELLOBIOSIDASE, β-GLUCOSIDASE, α- and β-GALACTOSIDASE, β-D-FUCOSIDASE, AND INVERTASE ACTIVITIES IN SOILS 164

Abstract 164
Introduction 165
Materials and methods 168
Results 171
Discussion 183
Conclusions 185
References 185

VII. CONCLUSION 198

LIST OF TABLES

Table Page

2.1 Cellulolytic and xylanolytic enzymes producing microorganism 31
3.1 Classification, vegetation, and basic properties of soils used..........................61
3.2 Precision of the method 74
3.3 Enzyme kinetic parameters, K_m and V_{max} values, of β-cellobiosidase in six soils calculated from three linear transformation of the Michaelis- Menten equation 76
3.4 Temperature coefficient (Q_{10}) and activation energy (E_a) of β-cellobiosidase activity in soils tested in the non-denaturing temperatures ranging from 10 to 50°C 80
4.1 Classification, vegetation, and basic properties of soils used.........................94
4.2 Correlation matrix (r) of soil microbial biomass, respiration, and microbial enzyme activities across the locations, management, and the depths in 54 soils 104
4.3 Principal Component loadings of soil enzyme activities, microbial biomass carbon and respiration under native mixed vegetation and switch grass cultivated system of three different soils 105
5.1 Characteristics of soils used...121
5.2 Physicochemical characteristics of sol used in the experiment 138
5.3 Inhibition of β-cellobiosidase activity in soils by 25 µmol g $^{-1}$soil of trace elements 157
6.1 Characteristics of soils used...170
6.2 Principal Component scores of soil enzyme activities under different cropping systems..180

LIST OF FIGURES

Figure Page

2.1a Cellulose fiber 15
2.1b Cellulose structure 16
2.2 Decomposition of cellulose into glucose by the synergistic action of different enzymes 20
3.1 Effect of pH on *β*-cellobiosidase activity in soils 67
3.2 Effect of substrate concentration on *β*-cellobiosidase activity in soils 69
3.3 Effect of amount of soil on *β*-cellobiosidase activity in the assays 71
3.4 Effect of incubation time on *β*-cellobiosidase activity in the tested soils........72
3.5 Effect of incubation temperature on *β*-cellobiosidase activity in soils 73
3.6 Three linear transformation plots of the Michaelis-Menten equation for *β*-cellobiosidase activity in Kirkland 1 soil as an example how K_m and V_{max} values were calculated. The slope and intercept of the linear regressions were used to calculate the kinetic parameters. A, Lineweaver – Burk plot, B, Eadie – Hofstee plot, and C, Hanes – Woolf plot. Velocity (V) was expressed as µmol ρ-nitrophenol released kg^{-1} soil h^{-1} Substrate concentration (S) was in mM 75
3.7 Linear transformation plots of the Arrhenius equation for *β*-cellobiosidase activity in the selected six soils at temperatures ranging from 10 to 50ºC 82
4.1 Distribution of C-transforming enzymes with 0-30 cm soil depths................99
4.2 Microbial Biomass carbon and microbial respiration in soils of three depths at the studied locations (CC, EF, and 4N are locations as described above) separated by unmanaged mixed grass (UNM) and switchgrass (SW). Different letters on bars indicate significantly different means according to the least significant difference test as P< 0.05. Error bars indicate standard deviation of averages samples 102
4.3 Factor scores of soil enzyme activities, microbial biomass carbon and respiration under native mixed vegetation and switch grass cultivated system of three different soils 106
5.1 pH stability of soil *β*-cellobiosidase 123
5.2 Effect of storage temperature and time on *β*-cellobiosidase (EC 3.2.1.91) activities in soils...140
5.3 Effect of trace element concentrations on *β*-cellobiosidase activity in six soils 159
6.1 Effect of cropping system on the activity of *β*-cellobiosidase.......................172

6.2 Effect of cropping system on the activity of β-glucosidase 173
6.3 Effect of cropping system on the activity of β-galactosidase 174
6.4 Effect of cropping system on the activity of α-galactosidase 175
6.5 Effect of cropping system on the activity of cellulase 178
6.6 Effect of cropping system on the activity of invertase 178
6.7 Effect of cropping system on the activity of β-D- Fucosidase 179
6.8 Factor scores of different cropping systems regarding soil enzyme activities 181

CHAPTER I

INTRODUCTION

The world's human population has increased four times in the last century alone. This population boom, in part a result of improved agricultural and industrial techniques, places continued pressure on food production in order to feed the growing numbers. Intensified food production over the years has taken a toll on the health of agricultural soils (FAO, 2017) as well as their quality (Verhulst et al., 2010). Soil quality for sustainable crop production is related to soil health. As a living system the soil consists of microbial communities whose activities include nutrient cycling, symbiotic relationships with plant roots, pest, weed and disease control, and soil aggregate formation and aeration which influence susceptibility to erosion and water infiltration. Therefore, exploring the role of the soil microorganisms and their extracellular enzymes is gaining attention for agricultural sustainability. A healthy soil is rich in organic matter which act as a reservoir of soil nutrients and moisture and allows a high diversity of soil microorganisms to flourish. The activities of the soil microorganism are affected by many factors including soil management practices (Anna et al., 2017; Maharjan et al., 2017; Xue et al., 2018; Babin et al., 2019; Saikia et al., 2020; Nazaries et al., 2021; Muhammad et al., 2021). Soil management practices such as crop rotation, tillage, fertilizer, and organic matter input, use

of pesticides, cover crops and stover removal, can greatly affect physical, chemical, and biological properties that affect soil microbial communities and extracellular enzyme activities through the alteration in soil organic carbon (SOC) (Nautiyal et al., 2010; Lombard et al., 2011; Xun et al., 2015). For a given soil under a specified climate regime, SOC is largely controlled by the amount of residue inputs, the drivers of residue decomposition, and SOC stabilization and turnover. Soil properties, such as parent material, soil organic matter, pH and clay content influence soil microbial biomass and functional diversity (Nautiyal et al., 2010; Lombard et al., 2011; Xun et al., 2015). Soil microorganisms drive the turnover of exogenous organic materials into soil organic matter (Matulich and Martiny, 2015). The quality of applied organic materials can regulate microbial abundance and function (Zhang et al., 2012; Song et al., 2014; Liu et al., 2014). Soil organic matter provides energy to microbes, and soil with higher content of SOM generally has higher microbial biomass and functional diversity and shows higher extracellular activities (Staddon et al., 1997; Bending et al., 2002; Malosso et al., 2005; Hao et al., 2008; Luo et al., 2015; Jian et al., 2016). Organic amendment addition may increase the content of SOC via mineralization in agricultural ecosystems (Grunwald et al., 2016; Ribeiro et al., 2010).

Major components of SOC subjected to microbial decomposition include cellulose, hemicelluloses, and lignin. Cellulose can account for between 30 and 60% of plant material and its decomposition is of major importance to the biogeochemical cycling of carbon (C) and essential plant nutrients (Paul and Clark, 1996). In structure, cellulose is a carbohydrate composed of glucose units bound together in a long, linear chain by β-linkages at C atoms 1 and 4 of the sugar molecule. The degradation of cellulose is a complex process requiring

the participation of many enzymes, known as cellulases, which act in synergism and yield readily available carbon to microorganisms in the form of glucose and cellobiose. Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials (Ma et al., 2013; Quintanilla et al., 2015). These microorganisms can be aerobic, anaerobic, mesophilic, or thermophilic. Among them, the genera of *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Trichoderma*, and *Aspergillus* are the most extensively studied cellulose producers (Kuhad et al., 2011). Structurally fungal cellulases are simpler as compared to bacterial cellulase systems, cellulosomes (Bayer et al., 1994; Bayer et al., 1998; Zhang et al., 2006). Fungal cellulases typically have two separate domains: a catalytic domain (CD) and a cellulose binding module (CBM), which is joined by a short polylinker region to the catalytic domain at the N-terminal. The CBM is comprised of approximately 35 amino acids, and the linker region is rich in serine and threonine. The main difference between cellulosomes and free cellulase enzyme is in the component of cellulosomes-cohesin containing scaffolding and dockerin containing enzyme. The free cellulase contains cellulose binding domains (CBMs), which are replaced by a dockerin in cellulosomal complex, and a single scaffolding-born CBM directs the entire cellulosomes complex to cellulosic biomass (Carvalho et al., 2003; Bayer et al., 2004).

In nature, complete cellulose hydrolysis is mediated by a combination of three main types of cellulases (Juturu and Wu, 2014; Kuhad et al., 2011; Sukumaran et al., 2005; Yang et al., 2013). These are endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and *β*-glucosidases (EC 3.2.1.21). All these enzymes belong to glycosyl hydrolase (GH) family (Henrissat and Davies, 1997). Exoglucanases, are also known as cellobiohydrolases

(CBH), hydrolyze glycosidic bonds at the amorphous regions of the cellulose generating long chain oligomers (nonreducing ends) for the action of exoglucanases or cellobiohydrolases, which cleave the long chain oligosaccharides generated by the action of endoglucanases into short chain oligosaccharides. Among the 128 GH families, the CBH can be found in GH families 5, 6, 7, 9, 48, and 74 (Annamalai et al., 2016). There are two types of exoglucanases (CBHI and CBHII), acting unidirectionally on the long chain oligomers either from the reducing (CBHI, EC 3.2.1.176) or nonreducing ends (CBHII, EC 3.2.1.91) liberating cellobiose, which is further hydrolyzed to glucose by β-glucosidases (EC 3.2.1.21) (Juturu and Wu, 2014). The crystal structures of several thermostable GH 7 CBHs, have been solved, both in complex with unmodified substrates and in the uncomplexed form (Divne et al., 1998, 1994; Grassick et al., 2004; Munoz et al., 2001; Parkkinen et al., 2008; Stahlberg et al., 1996). The general structure consists of a β-sandwich, with loops extending from this structure to form an enclosed cellulose-binding tunnel. The three-dimensional structures of the catalytic domains of CBHI and CBHII revealed that their active sites are situated in tunnels formed by long loops on the enzymes surface. The active sites of homologous endoglucanases lack these loops and have more open active sites permitting catalytic activity in the internal positions of cellulose chains. Site-directed mutagenesis and structural studies have identified the key catalytic residues of both CBHI and CBHII. Similarly, the primary interaction surface of the cellulose-binding domain has been defined and residues responsible for its tight binding to cellulose identified.

Cellulose substrates isolated from different sources, though all composed of linear β-1,4-linked glucose polymers, differ significantly in structure as measured in terms of

amorphous content, crystallinity, and size and shape of crystallites (Montanari et al., 2005); these structural differences may substantially affect susceptibility to cellulase action. In addition, depending on variations of the patterns of inter- and intrachain hydrogen bonds, cellulose may form different crystalline allomorphs (e.g., cellulose Iα, Iβ, II, III, and IV), upon which the binding and activities of cellulases may be different. Endoglucanases acting alone may attack the non-crystalline regions effectively, but effective attack on the crystalline portion of cellulose requires in addition the synergistic action of the exoglucanases (CBH).

Various bacteria and fungi are known to secrete endo or exo-acting cellulases that act on cellulose, resulting in the release of glucose and cellobiose. There have been extensive studies into the cellulolytic system of Trichoderma reesei, which is composed of two cellobiohydrolases (CBHI and CBHII) (Miettinen-Oinonen and Suominen, 2002; Geng, 2014). Miettinen-Oinonen and Suominen (2002) quantified the CBHI and CBHII in T. reesei by double-antibody sandwich enzymelinked immunosorbent assay (ELISA). However, Brook et al. (1990) isolated three cellobiohydrolases named CBHI-A, CBHI-B, and CBHII from crude extracts of Talaromyces emersonii liquid cultures. Recently, four cellobiohydrolase I enzymes named as CBHI-A, CBHI-B, CBHI-C, and CBHI-D have been purified from the growth of Penicillium decumbens JU-A10. The enzyme activity was tested against p-nitrophenyl- β-D-cellobioside (pNP) (Gao et al., 2012). The cellodextrin and cellobiose have their inhibitory activities during cellulose hydrolysis, thus β-glucosidase is essential to break the final glycosidic bonds of cellobiose so as to produce sufficient glucose molecules and reduce or eliminate cellobiose inhibition (Maki et al., 2009; Dashtban et al., 2010). The CBH assay is more difficult than endoglucanase and β-

glucosidase assays due to lack of proper substrates and hindrances from cellulase components (Zhang et al., 2009). Although there is no such single standard assay method for CBH activity, the Avicel method (Dashtban et al., 2010; Zhang et al., 2009) has been repeatedly used. Because of this, very scanty information is available regarding the activity of CBH in complex system like soil. However, a little effort was made to study the activity of CBH in soil using methods developed for the assay of purified enzymes (McCleary et al., 2012) based on a protocol suggested by Deshpande et al. (1984). This method employed the use of *p*-nitrophenyl cellobiose and p-nitrophenyl lactoside, to selectively measure the activity of cellulose-β-1-4- cellobiosidase in the presence of other cellulolytic enzymes. To the best of knowledge, there are no systematic evaluation and development of methods for the detection and quantification of CBH activity in complex media such as soil.

Keeping in view the above discussion, a series of experiments were carried out with following objectives:

1. To develop an assay method to quantify activities of β-cellobiosidase in soil.
2. To determine effect of pH, storage temperature and time, and trace elements on stability and activity of β-cellobiosidase in soil.
3. To evaluate the relationships among several cellulosic enzymes, and between their activities and microbial biomass and activities in soil; and
4. To assess the effect of crop rotation and residues management on activities of β-cellobiosidase and carbon-transforming enzymes in soils.

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CHAPTER II

LITERATURE REVIEW

In the world, the research work in the agriculture sector mainly focuses on the sustainable production of crops. Food security is the main concern because the population boom has led to the scarcity of available food (Mehrabi et al., 2018; Fróna et al., 2019). It is expected that growth and yield will fulfill the future demands of fiber and food production rather than the increase in agricultural land (Kopittke et al., 2019). It can be attained by exploring the role of soil microbes and their enzyme systems, which are involved in the maintenance of the soil fertility via nutrient cycling. The soil biota helps to maintain the fertility of soil via organic matter mineralization and biogeochemical cycling of necessary nutrients of plants (Turner, 2010). Several soil microorganisms decompose the organic carbon-based materials present in soil (Romaní et al., 2006; Sabir et al., 2017).

The lignin, hemicellulose, and cellulose are carbonaceous materials decomposed by the microbes. The plant consists of 30-60% (dry weight) of cellulose and the degradation of cellulose affects the biogeochemical cycles of carbon (C) and other nutrients of plants (Acosta-Martínez et al., 2019; Basu et al., 2020). Degradation of cellulose releases glucose

as the end product that may also be used as an energy source of microbial growth (Eriksson, 1978; Coughlan, 1992; Lynd et al., 2002; Jain et al., 2021). The process cellulose decomposition involves numerous extra-cellular enzymes including cellobiohydrolases, endoglucanases, and β-glucosidases (Sukumaran et al., 2005; Kuhad et al., 2011; Yang et al., 2013; Juturu and Wu, 2014). The presence and effectiveness of these enzymes are directly associated with the type of soil microbial communities, and it is indispensable to understand mutual interaction in the action of these enzymes and their production in response to different soil management practices under diverse conditions (Nautiyal et al., 2010; Lombard et al., 2011; Xun et al., 2015; Thapa et al., 2020). Moreover, the development of easy and reliable assays for the determination of soil enzymes will pave the way for a better understanding of the role of these enzymes in the decomposition of cellulose in soil. Therefore, it is necessary to comprehend the properties, forms, and structure of cellulose in nature.

Properties and structure of Cellulose

The bacterial cells and cell walls of plants are rich in cellulose. It is an extremely insoluble carbohydrate and humans cannot digest it. Cellulose is essential for the strength and structure of plants. It also has numerous industrial applications. Generally, the number of cellulose ranges between 90 to 33% (by weight). The wood consists of 40-44% (dry weight) of cellulose (Jayasekara, and Ratnayake, 2019). Cellulose made by living organisms is known as “native cellulose” (Hon, 1994). Cellulose, a long-chain polymer, is made up of d-glucose (6-carbon containing sugar) and its general formula is (C6H10O5) n (Basu, 2010; Chibbar et al., 2016). It has a high molecular weight (<500,000) and the degree of polymerization (DP) value can exceed 10,000 (Shibazaki et al., 1995; Brown,

1996). Its crystalline form comprises thousands of glucose molecule units, but eight monomeric units of glucose are requisite to make an insoluble product (Brown, 2004). In native cellulose, the number of glucose units varies depending on the source.

The per chain glucose units in the cellulose of the primary cell wall are 8000 (dp 8000) while the secondary wall contains 15,000 glucose units (Brown, 2004). Most of the terrestrial biomass contain cellulose a component of their skeletal structure (Klass, 1998).

The glucose subunits in cellulose are linked via beta 1-4 glycosidic bonds (Figure 2.1.b). The alignment of glucose is reversed in cellulose as compared to other polysaccharides (Brett, 2000). The glucose molecules exhibit the β-orientation. All the hydroxyl (OH) groups of glucose rings are oriented below the plane of the ring except the OH-group of carbon 1 that is directed above the ring plane (Rowland and Roberts, 1972; Henriksson and Lennholm, 2009). To make β-1-4 glycosidic bonds, every alternate glucose molecule in cellulose is inverted (Carpita, 2011). The hydroxyl group of carbon 1 is directed upwards, and that of carbon 4 is directed downward. Now, to make a β-1-4 glycosidic bond, one of these molecules should be inverted so that both the hydroxyl groups come in the same plane. This is the reason for the inversion of every alternate glucose molecule in cellulose (Berg et al., 2002).

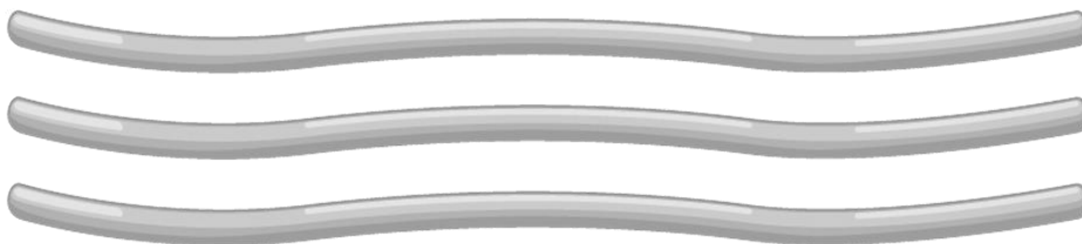


Figure 2.1.a. Cellulose Fiber

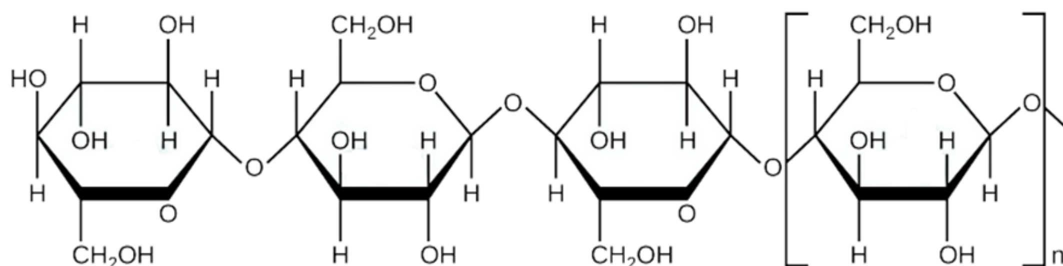


Figure 2.1.b. Cellulose structure

Cellulose is an unbranched polymer of glucose in which the arrangement of glucose chains is linear and parallel to each other. Contrary to glycogen and starch, no helix, coil, or branch is present in these chains (Brown, 2004). Association of 60-70 chains through Van der Waals forces and inter-chain hydrogen bonds resulted in the formation of micro-fibrils (Blackwell, 1982; Rees et al., 1982; Brown, 2004). The highly ordered crystalline structure of chains is maintained by the hydrogen bonding and consequently, the strong and stiff micro-fibrils are formed which are further arranged to make cellulose of plants cell wall (Colvin and Beer, 1960; Itoh and Brown, 1984; Revol et al., 1992; Brett, 2000). In this way, the cellulose maintains the structural stability in plants (Brigham, 2018). In vascular plants, the DP value for long chains of cellulose varies from 7000-14,000 or more for secondary walls, whereas the DP value for primary walls is 500 (Navarro et al., 2019). Cellulose exists in six crystalline forms (Cellulose I, II, IIII, IIIII, IVI, and IVII) which are different in their electron and/or x-ray diffraction patterns Fan et al., 1980; Nevell and Zeronian, 1985).

Only two allomorphs of native cellulose i.e., cellulose I and II are present in nature but cellulose I makes the most of native cellulose. The orientation of glucan chains is parallel in cellulose I but antiparallel in cellulose II (Atalla and Vanderhart, 1984). The thermodynamic stability of cellulose II is higher which might be attributed to the presence of one additional hydrogen bond per glucose residue. This rare allomorph only exists in some bacteria and numerous algae (Mihranyan, 2011; Park et al., 2015). Cellulose I allomorphs consist of distinct numbers of parallel glucan chains arranged to form the nanostructure known as a microfibril (Brown, 2004; Diotallevi, 2007). The enzyme complex involved in cellulose production genetically controls the arrangement and number of parallel glucan chains (Brown, 2004). There are many sizes and shapes of microfibrils. For example, Erythrocladia contains thin membrane-like microfibrils (Okuda et al., 1996) while Valonia has large square microfibrils made up of <1200 glucan chains (Brown, 1996). Moreover, a giant rectangular structure with hundreds of chains is found in Boergesenia. The tips of the microfibrils have reducing-ends of glucan chains (Kuga et al., 1988).

Type I cellulose is a combination of crystalline Iα and Iβ which differ in crystal lattice but similar in conformation (Coughlan, 1992). The two conformations of cellulose (kI and kII) have different secondary levels of the organization. Type a cellulose is less stable or resistant to chemical hydrolysis. The content of Iα and Iβ forms in native cellulose is related to the organization of the assembly of the elementary fibrils during biosynthesis of cellulose (Coughlan, 1992; Festucci-Buselli et al., 2007). Form Iβ is mostly present in organisms (e.g., algae and higher plants) that possess rosettes as the main sites of cellulose production. In primitive organisms (e.g., Laminaria japonica (brown algae), Type Iα is the

dominant form of cellulose (Ergun et al., 2016; Rongpipi et al., 2019). Due to the complex forms and types of cellulose, organisms are engaged in the synthesis and secretion of different exo- and endo-enzymes for hydrolysis of cellulose (Deng, 1994). In addition, cellulose can be found in free form (such as in seed hairs) or in combination with other organic substances (e.g., wood contains cellulose united with lignin). The highest and most pure form of cellulose is present in cottonseed hairs (99.8% cellulose) which is known as "true cellulose" (Nord and Vitucci, 1948). Generally, in leaves, wood, and plant stalks, cellulose exists in union with other compounds such as hemicellulose and lignin. For examples, hardwoods contain 18-25% lignin, 45-55% cellulose, and 24-40% hemicelluloses, while softwoods contain 25-35% lignin, 45-50% cellulose, and 5-35% hemicelluloses. Grasses normally contain 10-30% lignin, 25-40% cellulose, and 25-50% hemicelluloses (Betts et al., 1991; Howard et al., 2003; Malherbe and Cloete, 2003; Sanchez, 2009; Sasaki et al., 2009; Balat, 2011).

Cellulose is also produced in bacteria and the cellulose synthesis in Acetobacter xylinum has been widely studied (Shankaran, 2018). It is thought that the biological role of cellulose produced by bacteria is to aid in flocculation or to maintain certain environment, such as aerobic conditions or allowing attachment to plants (Sun et al., 2016). The arrangements in bacterial and plant cellulose are similar, as polysaccharide chains build microfibrils, and groups of microfibrils make ribbons (Klemm et al., 2001; Endler et al., 2010). Contrary to plant cellulose, bacterial cellulose is extremely pure and exhibits high water retention capability (which results in high crystallinity of the polymer). Retention of water in plant cellulose is 60%, while bacterial cellulose can retain water up

to 1000% of the sample weight (Klemm et al., 2005). Superior water retention of bacterial cellulose allows the polymer to possess high crystallinity (Klemm et al., 2001).

Biodegradation of cellulose

Cellulose is a major part of soil organic carbon that mainly comes from the residues of higher plants (Brown, 2004). Cellulose is also produced by several soil microorganisms such as algae, bacteria, and fungi. The bacterial and plant cellulose have different physical properties, but they have the same molecular formula ($C_6H_{12}O_6$) n and β-1,4-glucans (Yoshinaga et al., 1997). In soil, the cellulose from microorganisms and plant materials undergoes decomposition. A host of microorganisms (bacteria, actinomycetes, and microfungi) perform the degradation of the cellulose through the production of cellulolytic enzymes which are integrated and interdependent as a system (Jia et al., 2016; Jayasekara and Ratnayake, 2019). The cellulases, an enzyme system, converts the complex insoluble cellulose into simple monosaccharides or disaccharides that are soluble in water and can be transported to the cell (Figure 2.2).

It is supposed that the cellulolytic ability is commonly present in numerous fungi but limited to specific bacterial groups (Lynd et al., 2002), and the former dominate the degradation of cellulose in soil (Kjoller and Struwe, 2002; de Boer et al., 2005). The fungi present in soil play a key role in the decomposition of cellulose. Soil fungi secrete extra-cellular enzymes for the decomposition of lignocellulosic biomass (McGuire and Treseder, 2010). The mechanisms through which fungi decompose the cellulose is well-known for few species of fungi, for example, Neurospora crass, Trichoderma reesei, and Aspergillus niger Béguin and Aubert, 1994; Tomme et al., 1995; Wilson, 2011).

But more research is needed to understand how well these mechanisms represent the ecological process of cellulose degradation as it occurs in soils. Most of the microorganisms release β-glucosidases because cellobiose is the commonly accessible substrate (Lynd et al., 2002).

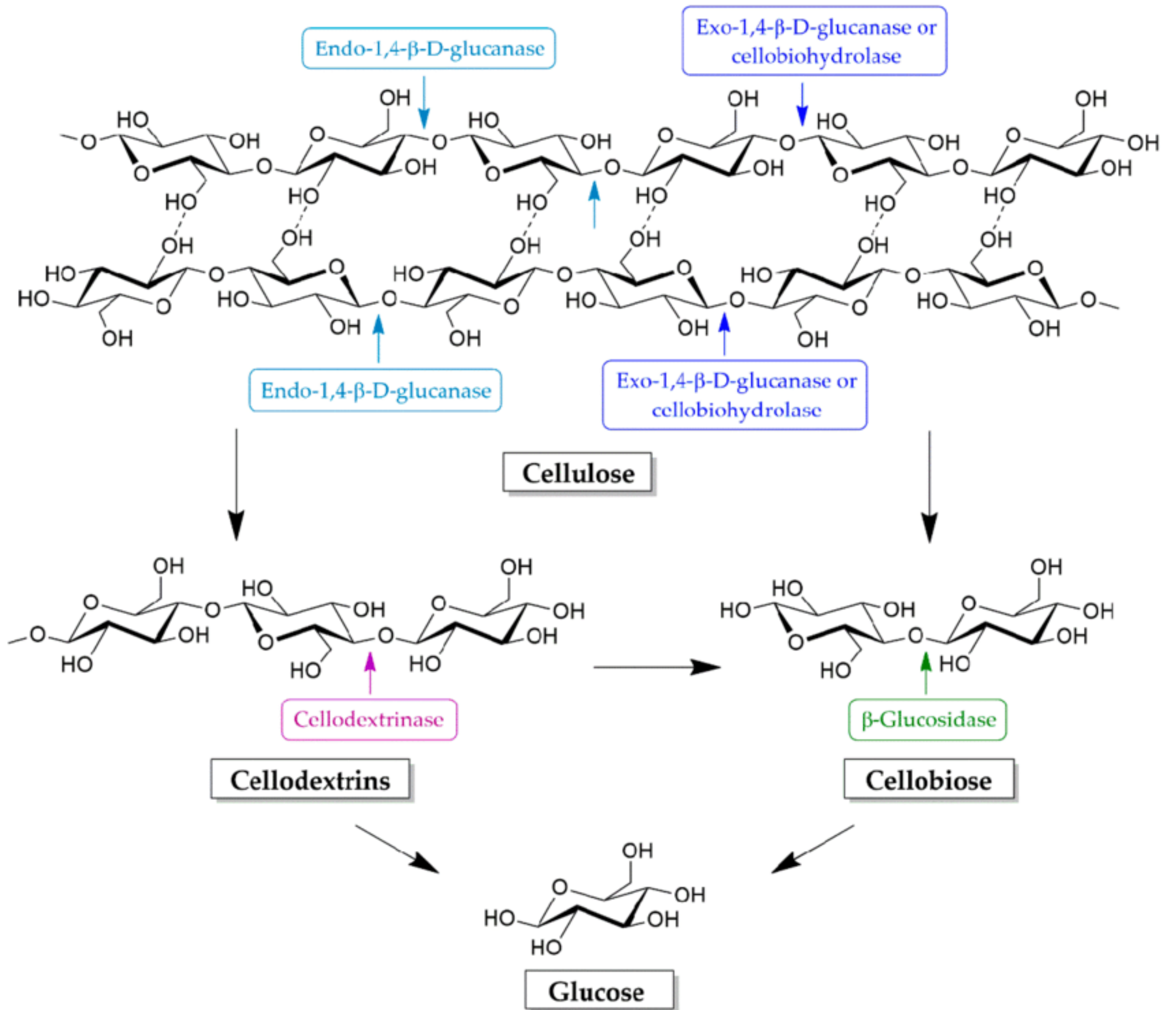
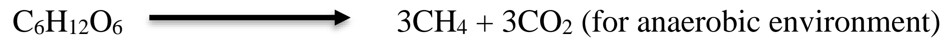


Figure 2.2. Decomposition of cellulose into glucose by the synergistic action of different enzymes.

The major portion of soil cellulose (approx. 90%) is degraded by the soil microbes aerobically while the small amount of it (10% approx.) anaerobically (Ljundahl and Eriksson, 1985). The following equation demonstrates the conversion:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O \text{ (for aerobic environment)}$$



$$C_6H_{12}O_6 \longrightarrow 3CH_4 + 3CO_2 \text{ (for anaerobic environment)}$$

The final product of cellulose decomposition by microbes is CO_2, but methane is also released in anaerobic conditions (Mountfort et al., 1982).

Biological Distribution, States, and Sources of *β*-Cellobiosidase in soils

Microbial biomass is the primary source of enzymes in the soil, but they can be derived from animal and plant leftovers (Tabatabai, 1994; Alef et al., 1995; Dick, 1997, Das and Varma, 2010). Free enzymes are intracellular, extracellular, and attached to cell components. They are the main drivers of enzymatic activities in soil. Intracellular or endo-enzymes and extracellular or exo-enzymes are released from disintegrating cells and living cells, respectively (Tabatabai, 1994). During rapid microbial growth, the enzymes are produced that may stay inside the proliferating cells or discharged into the soil. The major portion of free enzymes is in adsorbed form, not in soil solution (Tabatabai, 1994; Allison, 2006). Adsorption or complexation of free enzymes occurs on mineral and organic components or humic constituents of soil. Exo-enzymes remain in the soil for a particular period (Allison, 2006). The exo-enzymes (catalase) were reported first time at the end of the century (Skujins, 1978).

The advancement of strategies and methodologies in the research area of soil enzymes led to remarkable progress in just the previous 15 years. Now, it is well-known that the soil has a diversity of free enzymes. There are numerous sources of intra- and extracellular enzymes in soil (Dick, 1997, Acosta-Martínez et al., 2007; Das and Varma, 2010). Few enzymes may even remain active in the cell even after the cell death (Skujiņš and Burns, 1976). This might be due to the diffusion of the substrate into the cell via non-functional membranes and the final product diffuses out of the cell (Tabatabai, 1994; Spohn and Kuzyakov, 2014). It is interesting to know that some scientists consider that soil is a living tissue having enzymes comparable to the enzymes of animals. Microorganisms decompose the major portion of materials added to soil and some free enzymes can persist in the soil for a longer period Tabatabai, 1994; Renella et al., 2006).

Fungi are the major microorganism for the production of Exoglucanases (CBH). Mostly, the cellulases of fungal are glycoprotein having different forms (Coughlan and Ljungdahl, 1988). The composition of covalently bonded neutral carbohydrates was different in the CBH of T. viride (Gum and Brown, 1976). Likewise, P. funiculosum and T. reesei synthesized the CBH I and II that were immunologically different from each other (Nummi et al., 1983; Wood et al., 1980). The two CBHs of Fusarium lini have a different composition of amino acids (Mishra et al., 1983). In several fungi, the extra-cellular cellulase survives as individual bodies (Coughlan, 1985). Nevertheless, aggregation of these enzymes (containing cellulase, six proteins and xylanases, and β-glucosidase activities) has been reported in the culture filtrate of T. reesei. (Sprey and Lambert, 1983).

The most studied cellulolytic fungus, Trichoderma reesei, produces up to about 80% of the total secreted protein as CBH, and the best production strains can secrete tens

of grams per liter of these enzymes (Tangnu et al., 2010; Ilmén et al., 2011). In fungi, the two main classes of CBH are CBH1 (GH7) and CBH2 (GH6) (http:// www.cazy.org/) (Zhang et al., 2006). The structure of catalytic domains (CD) of CBH1 and CBH2 is different but both of them have tunnel-shaped active sites. The cellulose-binding module (CBM) is necessary for the crystalline cellulose hydrolysis process (Zhang and Lynd, 2006). It has been found in numerous fungal CBHs. For instance, CBHs of GH6 and GH7 have CBM at their N-terminus and C-terminus respectively. The attachment of CBM on these enzymes is due to the flexible linker. The synergistic action of CBH1 and CBH2 types for the hydrolysis of cellulose has been suggested by different studies (Zhang and Lynd, 2004, Zhang and Lynd, 2006, Nidetzky et al., 1994).

Mechanisms of Cellulose Degradation

The cellulolytic microbes decompose the cellulose via three mechanisms. The "free cellulases mechanism" is mostly followed by the aerobic microorganisms. They produce 6-10 distinct cellulases having CBM attached to their CD via a flexible peptide linker (Tomme et al., 1995; Wilson, 2004; Wilson, 2008b). The activity of the enzyme is not influenced by the CBM location (either C-terminal or N-terminal). Compared to the individual cellulase, the synergistic action of cellulases to degrade crystalline cellulose is 15 times more (Irwin et al., 1993; Wilson, 2008). The mixture of cellulases in aerobic microorganisms consists of many endocellulases and one member of each class of exocellulase (makeup <60% of total cellulase protein) (Spiridonov, N.A. and Wilson, 1998; Teeri et al., 1998; Wilson, 2009). The families of cellulases in anaerobic and aerobic microorganisms are the same, except aerobic fungi that produce GH7 cellulases and cellulosomes do not contain exo-cellulases of family 6 (Wilson, 2011). Some thermophilic

anaerobic bacterial species engaged in cellulolytic activity synthesize CBMs containing multi-domain cellulases.

Unlike other microorganisms, their efficient system for decomposition of the plant's cell wall can also hydrolyze the plant biomass which is not pre-treated (Blumer-Schuette et al., 2010). Several anaerobic microorganisms produce large multi-enzyme complexes called as Cellulosomes which are bound to the external surface of microorganisms (Ding et al., 2008). Few enzymes of cellulosomes have CBM (but the cohesin-containing scaffolding protein has CBM3) through which cellulosome binds to cellulose. The dockerin domain of cellulosomal enzymes, which is attached to CD via flexible linker peptide, makes a bond with the cohesin domain of scaffolding proteins (Doi et al., 2003; Doi et al., 2004; Wilson, 2009). Exocelluualses acting on non-reducing ends of cellulose are absent in bacterial cellulosomes, but they have numerous processive endo-cellulases (acting on non-reducing ends) and exo-cellulases (acting on reducing ends) (Barr et al., 1996). Cellulosomal enzymes also act synergistically and cellulosomes contain a large number of different types of plant cell wall-degrading enzymes. In C. thermocellum, 72 different dockerin containing cellulosomal genes are identified (Wilson, 2009). An important unanswered question about cellulosomes is: is there any defined mechanisms through which cellulosomal enzymes bind to cohesin domains of the specific scaffoldin molecule or do they bind unsystematically to cohesin domains? Different dockerins in an organism show equal affinity for all cohesion domains (Doi et al., 2003; Wilson, 2009). Even though the effectiveness of the free enzyme mechanism and the cellulosomal mechanism in the degradation of cellulose is the same, it can be predicted that cellulose in small pores cannot

be accessed by the cellulosomes due to their large size. The free cellulases attack the cellulose in small pores (Doi et al., 2003; Doi et al., 2004; Wilson, 2009).

The third approach is utilized by the anaerobic Fibrobacter succinogenes and aerobic Cytophaga hutchinsonii (Wilson, 2008; Wilson, 2009; Wilson, 2011). The genome sequence of C. hutchinsonii showed that it codes many cellulase genes and all genes do not encode a dockerin domain, but they are involved in the coding of endo-glucanases. Moreover, CBM was encoded by most the genes. Consequently, C. hutchinsonii are not engaged in the coding of processive cellulases. Based on these results, C. hutchinsonii can be distinguished from other well-known anaerobic and aerobic cellulolytic microorganisms (Iyo et al., 1996; Jun et al., 2007). The genome sequence of F. succinogenes (bacterium species found in the rumen of cattle) revealed that this bacterium does not code for any processive cellulases. It is found that one of its cloned and sequenced endo-cellulases can bind to cellulose. Moreover, dockering domains are not encoded by F. succinogenes. The mechanism for cellulose degradation is well-organized in this organism. F. succinogenes and C. hutchinsonii may use the starch-degrading mechanism of Bacteroides thetaiotaomicron for the degradation of cellulose (Wilson, 2008). This mechanism involves the transportation of starch molecules to peri-plasmic space where they undergo attack by starch-degrading enzymes. The processive cellulases are not required in this mechanism because individual molecules of cellulose rapidly degrade the endoglucanases. If this process is exploited for the degradation of cellulose, it will be very interesting to identify the mechanism by which the surface membrane proteins transport and bind individual molecules of cellulose. This information would help in designing new cellulose modifying

proteins or cellulases to increase the degradation process (Wilson, 2008; Xie et al., 2007; Wilson, 2011).

Some cellulose-degrading aerobic fungi that cannot degrade the lignin use the free-enzyme mechanism. The true brown fungi synthesize cellulases and peroxidases (Martı´nez et al., 2005). Due to oxidizing ability of peroxidases and iron, brown root fungi can easily degrade cellulose Zhou et al., 2004. The cellulases used by these fungi do not have processive cellulases and CBMs (required for degradation of unmodified crystalline cellulose). The aerobic cellulolytic microorganisms that use the free cellulase mechanism and the cellulosomes-producing anaerobic microorganisms produce the processive cellulases and CBMs. It is a fact that free cellulolytic microorganisms can produce cellulosomes and six or more cellulases while Postia placenta (brown root fungus) can only produce one endoglucanase (Doi and Tamaru, 2001; Wilson, 2004; Martinez et al., 2009).

Currently, different proteins involved in the hydrolysis of cellulose have been identified. In cellulolytic fungi, few GH family 61 genes encode for the proteins that exhibit very little cellulolytic activity (Harris et al., 2010). In the presence of magnesium ions, a protein of GH family 61 stimulated the hydrolysis of cellulose in pre-treated biomass crude T. reesei cellulases (Harris et al., 2010). When Thermobifida fusca was grown on cellulose, it synthesized the two CBM33 proteins. One of the proteins contained the family 33 domain and the other contained CBM33 attached to CBM2. Both proteins can bind to chitin and cellulose, but they give a small stimulation of cellulose hydrolysis by T. fusca cellulases (Moser et al., 2010). The swollenen protein of T. reesei (exhibiting similarity to expansin) was shown to weaken the crystalline cellulose (Saloheimo et al., 2002). The cloned

homolog of Bacillus subtilis significantly stimulated the cellulase activity of T. reesei (Kim et al., 2009).

β-cellobiosidase Activity in Soils

Decomposition of organic matter in soils is mediated by a complex set of extracellular enzymes that are produced by soil fungi and bacteria. Cellulose is quantitatively the most important organic compound in the biosphere; therefore, the products of its enzymatic hydrolysis are important as an energy source for soil microorganisms. *β*-cellobiosidase is an enzyme taking part in the decomposition of cellulose to glucose by the hydrolysis of glucosides. *β*-cellobiosidase activity plays a crucial role in the C cycle of soils and the product of its enzymatic hydrolysis is important as an energy source for soil microorganisms (Jiménez et al., 2007; Jayasekara, and Ratnayake, 2019) Since *β*-cellobiosidase are proteins which are very sensitive to different natural and anthropogenic factors (Gianfreda and Ruggiero 2006). *β*-cellobiosidase has been detected in microorganisms, plants, and animals. It is significant in the carbon cycle (Sinsabaugh et al. 1991). *β*-cellobiosidase activity has been found to be sensitive to soil management and proposed as a soil quality indicator (Kurasin and Väljamäe, 2011; Naidja et al. 2000). Only scattered information exists concerning the complexity of the relationships between soil properties and *β*-cellobiosidase activity. Therefore, determination of their activity might be helpful in monitoring soil quality, especially soil subject to differentiated organic and mineral fertilization in various crop rotations.

Microorganisms involved in biodegradation of cellulose.

The high diversity of cellulolytic microorganisms may be due to the diverse nature of plant cell walls. These wide-ranging microorganisms are abundantly present in nature. Bacteria, fungi and actinomycetes are cellulolytic microbes. They may be thermophiles or mesophiles, and anaerobe or aerobe. They can grow in extreme pH and temperature conditions (Enari and Markkanen, 1977; Bui, 2014; Saini et al., 2015).

Several bacteria and fungi decompose cellulose and release methane and CO2 (Bisaria and Ghose, 1981). Some bacteria, such as Thermomonospora sp., Streptomyces flavogriseus, Clostridium thermocellum, and Cellulomonas sp. and some fungal species, such as Schizophyllum commune, Fusarium solani, F. lini, Trichoderma reesei (previously known as T. viride), Penicillium funiculosum, Aspergillus wentii, Sclerotium rolfsii, T. lignorum, Polyporus adustus, Sporotrichum pulverulentum (previously named Chrysosporum lignorum), P. iriensis, and Eupenicillium javanicum, are capable of producing highly active cellulases (Berghem and Pettersson, 1973; Mandels et al., 1974, Ryu and Mandels, 1980; Bisaria and Ghose, 1981; Bisaria and Ghose, 1981). Under in-vitro conditions, these cellulases degrade the insoluble cellulose to produce soluble products.

The cellulase preparations secreted by several organisms can decompose the soluble derivatives of cellulose (e.g., carboxymethyl cellulose). Regardless of growth on insoluble cellulose, some organisms can synthesize very little residual cellulase (Mandels and Weber, 1969). The reason is cellulase consist of a complex enzymes or enzyme-like factors and the culture fluid after the growth of microorganisms cannot contain all

constituents of the cellulase complex. Stable cell-free enzyme preparations and sufficient vital constituents of the enzyme are requirements of practical saccharification residual cellulose (Bisaria and Ghose, 1981). The cellulose degradation, rapid growth, and adequate synthesis of enzymes for degradation of soluble cellulose derivatives are not suitable criteria for the selection of organisms as a cellulase source. Chaetomium thermophile var. dissitum, Thermomonospora sp., Humicola sp., and some other thermophilic microorganisms exhibit the native cellulose-degrading ability (Enari and Markkanen, 1977; Bisaria and Ghose, 1981). These organisms can be a good source of thermostable cellulases. Contrary to the cellulases of thermophiles, the cellulases of mesophiles exhibit little heat stability (Bisaria and Ghose, 1981; Yennamalli et al., 2013).

A highly complex and dense combination of anaerobic microorganisms in the rumen of animals digest the pre-treated biomass containing. In the cellulolytic environment of the rumen, only 10% bacteria out of 1010 bacteria per ml are cellulolytic (Russell et al., 2009). Regardless of the cellulolytic protozoa and fungi in the rumen, bacteria are considered principal decomposers of cellulose. In the rumen, the majority of cellulose-degrading microbes are attached to the feed particles (Brulc et al., 2009).

Compost is also a well-known cellulolytic environment in which fungi and bacteria start the composting process. When the temperature rises, the thermophilic bacteria carry out the decomposition process (Hansgate et al., 2005; Schloss et al., 2005; Sze´kely et al., 2009). Most of the cellulose is degraded during the thermophilic stage. Compost contains a high diversity of microorganisms while most of the cellulose-degrading bacteria are found in mature compost (Sze´kely et al., 2009).

Heterogeneity in population and the limited number of microorganisms that can be cultured are major obstacles in studying microbial ecology. Unlike the aerobic environments, the high diversity of bacteria is found in anaerobic cellulolytic environments. The synergistic action of various organisms for the decomposition of the plant cell wall is still not clear.

Different microorganisms can utilize only specific polymers. Clostridium thermocellum can degrade the xylan wall but cannot utilize the xylose for its growth (Dror et al., 2005). Similarly, F. succinogenes can grow on cellulose but hydrolyze a variety of polysaccharides (Kobayashi et al., 2008). T. fusca secretes xyloglucanase for xyloglucan hydrolysis but cannot utilize the xyloglucan for its growth (McGrath et al., 2006). The CBM-containing xyloglucanase degrade the xyloglucan of cellulose fibrils in the primary cell wall of plants. When bacterial cellulose was produced in the presence of xyloglucan, it was not hydrolyzed by a mixture of pure cellulases, unless the xyloglucanase was present (McGrath et al., 2006). T. fusca can utilize cellulose or xylan for its growth. If both cellulose and xylan are present in the biomass, T. fusca will primarily hydrolyze the cellulose. It might be attributed to the inhibition of xylanases production by cellobiose (Chen and Wilson, 2007). The substrate of most of the organisms is not known, as the particular type of plant cell wall may undergo sequential attacks of different organisms. For instance, a cellulose-degrading mixed culture has four organisms but one of them is engaged in cellulose hydrolysis and the role of the rest of the organisms is not directly associated with the hydrolysis of cellulose (Kato et al., 2008). Furthermore, the large intestine of hindgut-fermenting animals (e.g humans and horses) has cellulolytic

Table 2.1 Cellulolytic and xylanolytic enzymes producing microorganism

Organism	Enzyme	Habitat	References
Bacillus sp. SR22	Cellulase, β-cellobiosidase β-glucosidase	Cabo,Branco coral reefs	Dos Santos et al. (2018). Montenecourt and Eveleigh 1977; Nidetzky et al., 1994
Pseudoaltermonas sp. DY3	Cellulase, β-cellobiosidase β-glucosidase	Deep-sea sediment, Soil	Zeng et al. (2006). Ilmén et al., 2011
Cladosporium sphaerospermum	Cellulase β-cellobiosidase	Sediment,of Arabian sea, Soil	Trivedi et al. (2015). Devendran et al., 2016
Pseudoaltermonas haloplanktis	Cellulase	Antarctic,sea water	Garsoux et al. (2004)
Guehomyces pullulans	β -Galactosidase	Antarctic seawater	Nakagawa et al. (2006)
Shewanella sp. G5	β-glucosidase β-cellobiosidase	Beagle Channel, Tierra del Fuego coastal area, Soil	Cristobal et al. (2008)
Cladosporium sp	Xylanase	Antarctic marine sponges	Del-Cid et al. (2014)
Pseudoaltermonas haloplanktis	Xylanase	Antarctic seawater	Collins et al. (2002)
Arthrinium malaysianum	β -xylosidase	Seawater	Mukherjee et al. (2016)

Adapted from Thapa et al., 2020.

microorganisms that can provide extra energy to animals via hydrolysis plant cell walls (Flint and Bayer, 2008).

Cellulolytic Enzymes

Among the several enzymes involved in the breakage of β-1,4 bonds of cellulose, the cellulases are the most important enzymes (Garg et al. 2016). Cellulases are complex of multiple enzymes. Based on mode of action, glycoside hydrolases can be categorized into three types i.e.

i) Endo-β-1,4-glucanases (EC 3.2.1.4)

ii) Exo-glucanase (EC 3.2.1.91)

iii) β -glucosidase (EC 3.2.1.21)

The endo-β-1,4-glucanases acts on the amorphous zones of cellulose to break inner linkages, as a result, the ends of long chains are exposed. The exo-glucanase breaks the 2-4 units from the exposed ends of chains to liberate cellobiose. The products of both endo- and exo-cellulase are hydrolyzed by the β -glucosidase to generate the molecules of glucose (Wood et al. 1986; Be´guin and Aubert 1994; Shen et al. 2008; Yang et al. 2011; Chandel et al. 2012). The enzyme system of cellulose comprises either cellulosomes or free cellulases. Teeri (1997) proposed the four types of synergistic interaction between these enzymes:

1) Exo–Exo synergy (among non-reducing-end exo-glucanases and reducing-end exo-glucanases).
2) Endo-Exo synergy (among exo-glucanases and endo-glucanases).

3) Intra-molecular synergism among the carbohydrate-binding module (CBM) and catalytic modules.
4) Synergy between β-glucosidase and exo-glucanases.

The CBM assists in the cellulases binding to cellulose and disruption of cellulose fibers. In Thermobifida fusca, synergy among glycosyl hydrolases (GH) family 48 exo-glucanase and GH family 9 processive endo-glucanase is demonstrated by Kostylev and Wilson (2014). TfCel48A is a reducing-end directed exo-cellulase and exhibits low activity on low crystalline cellulose, however, TfCel9A (a processive endo-cellulase) efficiently acts on crystalline cellulose (Kostylev and Wilson 2014). Up till now, the mechanism of interaction between non-processive exo-glucanase and processive endo-glucanase throughout the hydrolysis process cannot be defined (Yarbrough et al. 2017).

Cellulases are dominant enzymes because of their excellent hydrolytic activities. They consist of functional as well as structural units called modules or domains. They have functional CBM that can form, degrade, and modify the glycosidic linkages. This is the reason that cellulases are labeled as Carbohydrate-Active enzymes (CAZy) (Lombard et al. 2014). Keeping in view the structure of catalytic modules and sequence of amino acids, cellulases are categorized into several GH families, for instance, GH1, GH5, GH8, GH12, GH48, GH61, etc. (Brumm 2013). The GH family 61of CAZY is renamed AA9 and the enzymes of class AA9 have been categorized as copper-dependent lytic polysaccharide monooxygenases (LPMOs). The enzymes responsible for carbohydrate metabolism can be expressed using two enzyme commission numbers (EC numbers): EC 3.2.1.91 (cellobiohydrolases) and EC 3.2.1.4 (endo-glucanases). Cellulases of GH family 5 and 9 are abundantly present in cellulolytic bacteria. (Sukharnikov et al. 2011). Various

cellulases of other families, such as CbhI from Fusicoccum sp. (GH7) (Kanokratana et al. 2008), endo-glucanase F from Fibrobacter succinogenes S85 (GH51) (Malburg et al. 1997), and Cel 12 A cellulase from Rhodothermus marinus (GH12) (Crennell et al. 2002), can also contribute to hydrolysis of cellulose.

Exo-cellulolytic (cellobiohydrolase/exo-cellulase) and endo-cellulolytic (endoglucanase/endo-cellulase) are two well-known actions of cellulases enzyme. Endoglucanases (EG) having cleft-like active site act on amorphous zones of cellulose and hydrolyze the interior β-glycosidic bonds. Contrary to EG, exo-acting CBH or exo-glucanases have tunnel-shaped active sites (Kurasin and Väljamäe, 2011). The processive cellobiohydrolases (CBH) cleave the chains generated by the EG to yield cellodextrins and cellobiose (Stern and Jedrzejas 2008; Beckham et al. 2011). Exo-glucanases are processive, whereas endo-cellulase can be both non-processive and processive. Processivity is a key characteristic of polysaccharides-linked active enzymes, which can be evaluated through the topology of active sites and total binding sites for monomer units (Stern and Jedrzejas 2008; Beckham et al. 2011). The preeminent producer of cellulases is Trichoderma reesei. It has Cel7A and Cel6A exo-glucanases that facilitate the cellulose hydrolysis from reducing and non-reducing ends respectively (Martinez et al. 2008).

β-glucosidases find great importance in several biological processes (Wallecha and Mishra 2003). They are also called β-D-glucoside glucohydrolase (EC 3.2.2.21). These enzymes are involved in the hydrolysis of the glycosidic link of many substances that contain oligo-glucosides, aryl glucosides, diglucosides, alkyl glucosides, and cyanogenic glucosides (Yeoman et al. 2010; Zhou et al. 2012). Consequently, glucose molecules are generated from non-reducing ends of chains. There may be the possibility of glycosyl bond

formation between glycosides and oligosaccharides. This type of activity is the result of trans-glycosylation and reverse hydrolysis. The former approach is driven by kinetics in which glycoside donor are hydrolyzed in the presence of enzymes to produce glycosyl-enzyme intermediate. The nucleophiles (excluding water) such as monoterpene alcohol, disaccharide, monosaccharide, alkyl-alcohol, or disaccharide attack the intermediate compound to produce the new product. In thermodynamically controlled reverse hydrolysis, trapping of the substrate or product concentration and a decrease in the activity of water results in shifting of reaction towards synthesis (Bhatia et al. 2002; Hays et al. 1998; Singhania et al. 2013).

Based on substrate activity, three different classes of β-glucosidases are: (1) cellobiases which hydrolyze only cellobiose to generate glucose, (2) aryl- β-D glucosidases (3) frequently observed glucosidases that are capable of hydrolyzing a variety of substrates (Hrmova et al. 2002; Langston et al. 2006; Singhania et al. 2013). According to the CAZy database (http://www.cazy.org/.), β-glucosidases belongs to GH1 and GH3 based on hydrophobic cluster analysis and sequence identity Henrissat 1991; Bohlin et al. 2013. The GH1 includes the β-glucosidases originated from plants, bacteria, and animals, while GH3 consists of β-glucosidases belong to yeasts, fungi, and ruminal bacteria. β-glucosidases of both families hydrolyze their substrate with net retention of anomeric configuration in the presence of retaining enzymes and they do so through a double-displacement mechanism (Dan et al. 2000).

The last step in the hydrolysis of cellulose is catalyzed by the β -glucosidases. Cleavage and hydrolysis of cellobiose to liberate two molecules of glucose are carried out by β -glucosidases. These enzymes are considered as a factor that can limit the rate of

cellulose decomposition. Therefore, it is important to utilize the β -glucosidase during the process to boost the saccharification activity and speed up the degradation rate (Igarashi et al. 2003; Teeri 1997; Woodward and Wiseman 1982).

Objectives and significance

Degradation of cellulose requires synergistic functions of multiple cellulolytic enzymes including cellulase (EC 3.2.1.4), β-cellobiosidase (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). Specific objectives of this study were: 1. To develop an assay method to quantify activities of β -cellobiosidase in soil; 2. To determine effect of pH, storage temperature and time, and trace elements on stability and activity of β-cellobiosidase in soil; 3. To evaluate the relationships among several cellulosic enzymes, and between their activities and microbial biomass and activities in soil; and 4. To assess the effect of crop rotation and residues management on activities of β -cellobiosidase and carbon-transforming enzymes in soils.

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CHAPTER III

ASSAY OF β-CELLOBIOSIDASE ACTIVITY IN SOILS

ABSTRACT

As the most abundant biopolymer on earth, cellulose is broken down by an array of enzymes that have varied specificities and act in synergism. An assay protocol was developed to detect and quantify activity of a cellulose degrading enzyme, cellulose 1,4-β-cellobiosidase (EC 3.2.1.91) in soil. β-Cellobiosidase is also termed exocellobiohydrolase, or β-1,4-glucan cellobiohydrolase (CBH). This enzyme hydrolyzes cellulose and cellodextrins, releasing a disaccharide, cellobiose, from the non-reducing ends of the chains. The optimum pH and temperature for β-cellobiosidase activity were approximately pH 5.5 and 60°C, respectively. The Michaelis constants (K_m) and maximum velocity (V_{max}) in the tested six soils ranged from 0.08 to 0.51 mM for Km and from 71.5 to 318.1 µmol kg soil^{-1} h^{-1} for Vmax. The temperature coefficient (Q_{10}) ranged from 1.72 to 1.99 at non-denaturing temperatures from 10 to 50°C, and the activation energy (E_a) ranged from 42.5 to 53.7 kJ mol-1. This assay procedure gave reproducible results with a coefficient of variance ≤ 4.7% and has a limit of quantification (LOQ) of 50.9 µmol p-nitrophenol release kg^{-1} soil h^{-1} for β-cellobiosidase activity in soil. The developed assay protocol offers reproducibility and precision that are comparable to bench-scale assays at reduced costs on reagents, supplies, and labor.

1. Introduction

Each year, about 5×10^{10} Mg of dry plant matter is produced by photosynthesis, which is a major precursor of soil organic carbon (SOC). Lignin, cellulose, and hemicelluloses are major components of SOC, which are decomposed by microbes. Cellulose is the most abundant biopolymer on earth, found in the cell wall of all plants. Leschine (1995) reported that 90% of cotton and 50% of wood are comprised of cellulose. The plant material can exhibit 30-60% of cellulose and the decomposition of cellulose affects the biogeochemical cycles of carbon (C) and other nutrients of plants (Paul and Clark, 1996). Cellulose degradation requires corporate action of a complex cellulose-degrading enzyme system that include three categories of cellulases (Juturu and Wu, 2014; Kuhad et al., 2011; Yang et al., 2013; Sukumaran et al., 2005). Endo-1,4-β- glucanase (EC 3.2.1.4) attacks cellulose randomly, exo-1,4-β-glucanase (EC 3.2.1.91) removes cellobiose or glucose from the non-reducing end of the cellulose chains, and β-glucosidase (EC 3.2.1.21) hydrolyzes cellobiose and other water soluble cellodextrins to glucose (Deng and Tabatabai, 1994; Percival et al., 2006; Wood, 1991). Richmond (1991) and White (1982) reported that cellulases in soil are derived mainly from plant debris with limited amounts produced by fungi, bacteria, and other soil organisms.

Cellulose 1,4-β-cellobiosidase is the accepted name of exo-1,4-β-glucanase according to the International Union of Biochemistry and Molecular Biology (NC-IUBMB, 2022). This enzyme is also termed exo-cellobiohydrolase or CBH, β-1,4-glucan cellobiohydrolase, β-1,4-glucan cellobiohydrolase,1,4-β-glucancellobiosidase, and exoglucanase (NC-IUBMB, 2019). With the development of an assay method for the detection of cellulase (endo-glucanase) in soil (Deng and Tabatabai, 1994), progress has

been made to understand soil cellulose degradation (Semenov et al., 1996; Shuangqi et al., 2011). However, in-depth understanding of the complex cellulose degradation demands understanding of all enzymes involved in the system. Limited information is available on *β*-cellobiosidase activity in soil due to the lack of a well-evaluated assay method for its detection and quantification in soil.

Nevertheless, methods are available for assaying the activity of purified *β*-cellobiosidase using p-nitrophenyl cellobiose as substrate (McCleary et al., 2012; Wu et al., 2006). While many studies assaying the activity of purified *β*-cellobiosidase using fluorometric (4-methylumbelliferone) as a substrate (Ai et al., 2012; Corrales et al., 2017; van - Tilbeurgh et al., 1982; Yue et al., 2004; Wu et al., 2006). An assay protocol employing p-nitrophenyl cellobiose and p-nitrophenyl lactoside was shown to allow selective measurement of *β*-cellobiosidase in the presence of other cellulolytic enzymes (Deshpande et al., 1984). The method commonly used for the assay of *β* - cellobiosidase activity in soil by using 4-MUB- *β* -D-cellobioside was discussed by Saiya-Cork et al. (2002). This method is not evaluated and does not give reproducible results. Because fluorescence-based assays must account for quenching, a process that decreases the fluorescence intensity and results in underestimating the actual reaction rate being measured. Further, in soil extracts, turbidity and suspended soil particles can also contribute to quenching. Quenching has been shown to reduce fluorescence by 27-61% and is highly soil-dependent (Freeman et al., 1995). Although quenching could be compensated for by using longer incubation periods, but longer periods can lead to potential microbial growth and consumption of substrates or reagents, resulting in other analytical interference (Deng et al., 2011; Dick et al., 2013). However, for a better understanding of enzymology demand

the development of accurate, simple, and reproducible assay methods. The most important and challenging aspects of soil enzyme research are the methodologies adopted for soil enzyme measurement are not universal, and this creates difficulty in comparing soil enzyme research (Burns et al.,2013).

To our knowledge, no colorimetric microplate method for the quantification of *β*-cellobiosidase activity in soil has been conducted on the evaluation of the optimal conditions for the assay of soil *β* -cellobiosidase. Because of the importance of *β* -cellobiosidase in the C cycle, we have developed a simple, precise, and sensitive method for assaying *β* -cellobiosidase activity in soils and have found out the factors affecting the observed activity. Therefore, the objectives of present study were to systematically evaluate assay conditions to develop a colorimetric microplate method for the quantification of *β*-cellobiosidase activity in soil; and to characterize its activity in soil with respect to kinetic parameters (Km and Vmax) and temperature dependence, including temperature coefficient (Q_{10}) and activation energy (E_a).

2. **Materials and methods**

2.1. **Soils**

Six air-dried soil samples (< 2mm, air-dried) were selected to represent a range of soil pH (4.7 to 7.7), organic carbon content (OC, 7 to 44 g kg-1), and texture (17.5 to 32.5% clay, 32.5 to 62.5% silt, and 7.5 to 55% sand) (Table 1). Soil pH was determined using a combination glass electrode (soil to 0.01M $CaCl_2$ ratio = 1:2.5), contents of OC by dry combustion using a Carlo-Erba NA 1500 nitrogen/carbon/sulfur analyzer (Scheperds et al., 1989), and texture by the hydrometer method (Gee and Or, 2002).

2.2. **Reagents**

Modified universal buffer (MUB): MUB stock solution (5X) was prepared by dissolving 12.1 g of tris (hydroxymethyl) amino methane (THAM), 11.6 g of maleic acid, 14.0 g of citric acid, and 6.3 g of boric acid (H_3BO_3) in about 800 mL of 0.5 M sodium hydroxide (NaOH) and then adjusting to 1 L with 0.5 M NaOH. The stock solution was stored in the dark at 4ºC.

Table 3.1. Classification, vegetation, and basic properties of soils used.

Soil		Vegetation	pH †	Organic C‡	Sand	Silt	Clay
Series	Subgroup						
					-----------	%	------------
Cordell	Thermic Lithic Haplustepts	Pasture	7.3	19	32.0	49.0	19.0
Kirkland 1	Fine, mixed, thermic Udertic Paleustolls	Native prairie (unmanaged)	6.1	44	7.5	62.5	30.0
Kirkland 2	Fine, mixed, thermic Udertic Paleustolls	Prairie CRP (managed)	4.7	28	12.5	55.0	32.5
Norge	Thermic Udic Paleustolls	Wheat (managed)	4.8	15	42.5	40.0	17.5
Richfield	mesic Aridic Argiustolls	Corn (managed)	7.7	11	30.0	42.5	27.5
Teller	Thermic Udic Argiustolls	Wheat (managed)	5.9	7	55.0	32.5	12.5

†Soil:0.1 M CaCl2 ratio = 1:2.5; ‡ C, carbon.

MUB working solution was prepared by placing 50 mL of MUB stock solution in a 125 mL beaker containing a magnetic stirring bar on a magnetic stirrer. Unless otherwise specified, the solution was titrated to pH 5.5 with hydrochloric acid (HCl) and adjusted to 250 mL with ultrapure water.

Substrate (12.5 mM): 0.145 g of p-nitrophenyl β-D-cellobioside was dissolved in about 15 mL of MUB (pH 5.5, unless specified) and diluted to 25 mL with MUB (pH 5.5). The solution can be stored at 4ºC for days or -20ºC for weeks.

Calcium chloride ($CaCl_2$, 0.5 M): 73.5 g of $CaCl_2.2H_2O$ was dissolved in about 700 mL of water and diluted to 1 L with ultrapure water.

Tris (hydroxymethyl) amino methane (THAM, 0.1 M, pH 12): This was prepared by dissolving 12.1 g of THAM in about 700 mL of DI water, adjusting to pH12 by titration with 0.1 M NaOH, and then diluting to 1 L with ultrapure water. THAM was stored at 4ºC.

Standard p-nitrophenol (pNP) stock solution (10 mM): This was prepared by dissolving 1.391 g of pNP in about 800 mL of ultrapure water and diluting to 1 L with ultrapure water. Solution was stored in the dark at 4ºC.

2.3. **Procedure**

Three replicate assays (two sample assays and one control) were prepared by weighing 0.1 g of soil (dry weight equivalent) into each 1.5-mL Eppendorf tube, then adding 0.4 mL of MUB (pH 5.5). For the two assay tubes, 0.1 mL of 12.5 mM p-nitrophenyl β-D-cellobioside was added. The tubes were vortexed for a few seconds before being placed in a water bath at 37˚C. After 1 h of incubation, 0.4 mL of THAM (pH 12)

was added to each tube to terminate the reaction and to reveal the bright yellow color of pNP. Then, 0.1 mL of 12.5 mM p-nitrophenyl β-D-cellobioside was added to the control tubes. Subsequently, 0.1 mL of 0.5 M CaCl2 was added to each tube before vortexing for a few seconds. The tubes were then centrifuged at 12,000 rpm for 10 minutes; followed by pipetting 250 µL of clear supernatant into microplate wells. The yellow intensity of the solution was measured at 405 nm with a microplate reader (Bio-Rad Benchmark Hercules, CA, USA). The control was designed to account for potential background readings from reagents and soil constituents. Additionally, another control was performed for each substrate solution using the procedure described above but without adding soil to account for non-enzymatic autohydrolysis of the substrate.

The pNP content in the solution was calculated by reference to a calibration curve developed with pNP standards. To prepare the calibration curve, a set of working standards was prepared by pipetting 0, 1, 2, 4, 6, or 10 mL of the standard stock pNP solution (10 mM) into a 100-mL volumetric flask and adjusting to 100 mL by adding ultrapure water. The working standard pNP concentrations were 0, 0.1, 0.2, 0.4, 0.6, and 1.0 mM. The calibration curve was developed by placing 0.1 mL of pNP working standards into each 1.5 mL Eppendorf tube, adding 0.4 mL MUB at pH5.5, 0.4 mL THAM (pH 12, 0.1 M), and then 0.1 mL $CaCl_2$. There were 0, 0.01, 0.02, 0.04, 0.06, or 0.1 µmol pNP in each tube. After vortexing, the yellow color intensity of the solution mixture was measured at 405 nm with a microplate reader as described above. The calibration curve was done by plotting µmol pNP versus absorbance (A) at 405 nm. The β-cellobiosidase activity was expressed as µmol pNP released kg^{-1} soil h^{-1}, which was calculated as follows:

µmol pNP = (Asample – Acontrol – Aautohylysis – interceptstandard curve) / slopestandard curve (1)

µmol pNP kg-1 soil h-1 = µmol pNP x 1000 (g/kg) / 0.1 g soil / incubation time (h) (2)

The above procedure was modified to test the effects of buffer pH, substrate concentrations, incubation time, amount of soil, and incubation temperature on *β*-cellobiosidase activity. Buffer pH tested included pH 4, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0. Substrate concentrations tested were 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2-, or 4-mM during incubation. Incubation times evaluated were 60, 120, or 240 minutes. The amount of soil tested was 0.1, 0.2, 0.3, 0.4, or 0.5 g of soil in each assay tube. The temperature effect was evaluated using incubation temperatures of 0, 10, 20, 30, 40, 50, 60, 70, or 80º C.

The coefficient of variation (CV) was calculated to determine precision of the developed method. Limits of detection (LOD) and limits of quantification (LOQ) were calculated based on standard error (SE) and slope of six p-nitrophenol calibration curves in the absence of soil using the following formulas: LOD (µmol) = 3.3 x SE/slope; and LOQ (µmol) = 10 x SE/slope as done by Deng et al. (2013). Preliminary results showed that the calibration curves were not affected by the presence of soil (data not shown). The LOD and LOQ for the detection of β-cellobiosidase using the developed protocol were then calculated and expressed as µmol pNP kg^{-1} soil h^{-1}.

Enzyme kinetic parameters, K_m and V_{max} values, were calculated using data obtained in testing the effect of substrate concentrations and three linear transformations of the Michaelis-Menten equation, including

Lineweaver-Burk transformation: $1/V = 1/V_{max} + (K_m/V_{max})(1/[S])$ (3)

Hanes-Woolf transformation: $[S]/V = K_m / V_{max} + (1/V_{max})[S]$ (4)

Eadie-Hofstee transformation: $V = V_{max} - K_m (V/[S])$ (5)

Temperature coefficient (Q_{10}) and activation energy (E_a) of the enzymatic reaction were calculated using data obtained in testing the effect of temperature, and by applying the Arrhenius equation [$k = A \exp(-E_a/RT)$], where k is the rate constant of the reaction (which is equivalent to V in Michaelis-Menten equation above), A is the Arrhenius constant, E_a is the Arrhenius activation energy, R is the gas constant (8.314 J mol-1 K-1), and T is the absolute temperature (273°C) in Kelvin (K). Linear transformation of the Arrhenius equation was done by taking the log form as shown below:

$\ln k = -(Ea/R)(1/T) + \ln A$ (6)

By plotting lnk versus 1/T, E_a and Q_{10} values were calculated. E_a values were calculated directly from the slope of the linear regressions, while Q_{10} was calculated as follows:

$Q_{10} = \exp\{10000E_a/[RT(T+10)]\}$ (7)

2.4. Data analysis

All results were expressed on an oven-dry soil weight basis and reported as averages of two replicates assays and analyses. Moisture content was determined based on weight loss after drying soil at 105℃ for 48 h. Statistical analyses and data calculation were performed in excel. All results reported are averages of duplicate assays and analyses.

3. Results and discussion

The method developed for assay of β-cellobiosidase activity in soils was based on quantitative determination of pNP in soil supernatant upon incubation of soil with p-nitrophenyl β-D-cellobioside in buffer pH that is optimal for the enzyme activity. Factors evaluated included buffer pH, substrate concentration, amount of soil, temperature during incubation, and time of incubation.

3.1 Effect of incubation pH:

The optimum pH for β-cellobiosidase activity of the six tested soil was observed as 5.5 (Figure 3.1), which is within the range for the optimum activity of β-cellobiosidase but slightly higher than optimal pH reported for this enzyme purified from various microorganisms such as the optimal pH for the activity of β-cellobiosidase purified from Trichoderma harzianum and Streptomyces coelicolor was observed around 5 (Colussi et al., 2012; Lim et al., 2016).

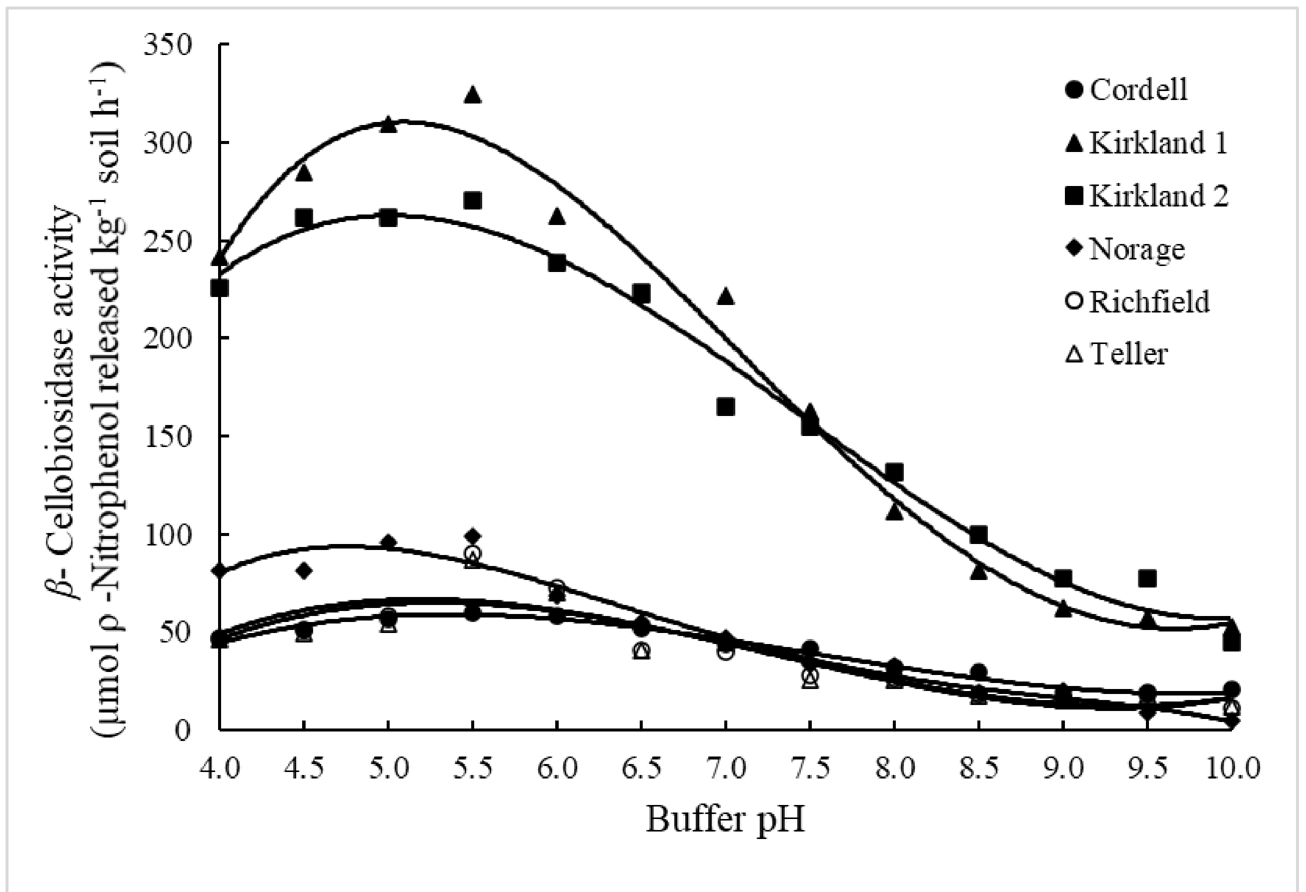

Figure. 3.1. Effect of pH on β-cellobiosidase activity in soils.

Similarly, the pH optima of several other cellulose degrading enzymes such as purified *β*-glucosidase originated from fungi Humicola brevis and Colletotrichum graminicola was recorded near 5 (Masui et al., 2012; Zimbardi et al., 2013). Likewise, pH optima for the activities of cellulases and several other carbohydralases in soils was recorded in the pH range of 5 to 6 such as cellulase at pH 5 (Deng and Tabatabai, 1994); β - glucosaminidase at pH 5.5 (Parham and Deng, 2000) and *β* -glucosidases at pH 6 (Eivazi and Tabatabai, 1988).

It has long been recognized that enzymatic reactions are pH dependent. pH influences the ionization of the functional groups of enzymes and their substrates (Pavani et al., 2017; Tabatabai, 1994; Turner, 2010). Changes in pH may lead to altered conformation of the protein that impacts protein recognition or leads to inaction of the enzyme (Robinson, 2015). Understanding the relationship between enzyme activity and reaction pH could facilitate data interpretation and link the obtained results to applications. Assaying at optimal pH of the reaction provides potential enzyme activity in the tested system and offers enhanced sensitivity of the detection method.

3.2. **Effect of substrate concentration:**

A final challenge to the reliability of soil enzyme activity measurements is the high variability in substrate concentrations among studies. In the present study, the activity of *β*-cellobiosidase increased with increasing substrate concentration and reached maximum at about 1 mM substrate concentration in most of the tested soils (Figure 3.2). So, substrate concentrations in assays should be sufficiently high as to approach or achieve Vmax, because as measured activities near Vmax they are more reliably comparable among soils within and across studies (German et al., 2011). Employing a substrate concentration 5-fold greater than the empirically determined Km in assays of enzyme activities (Brooks et al., 2012) has been suggested for soils (Burns, 1978, 1982), yet the majority of soil studies do not assess whether the substrate concentration employed achieves this. Therefore, substrate saturation was ensured at a concentration of 2.5 mM for most agricultural soils assayed using the proposed method. It is important to maintain substrate saturation during incubation when conducting an enzyme assay so that the substrate is not a limiting factor in carrying out the enzymatic reaction under the assay conditions (Frankenberger and

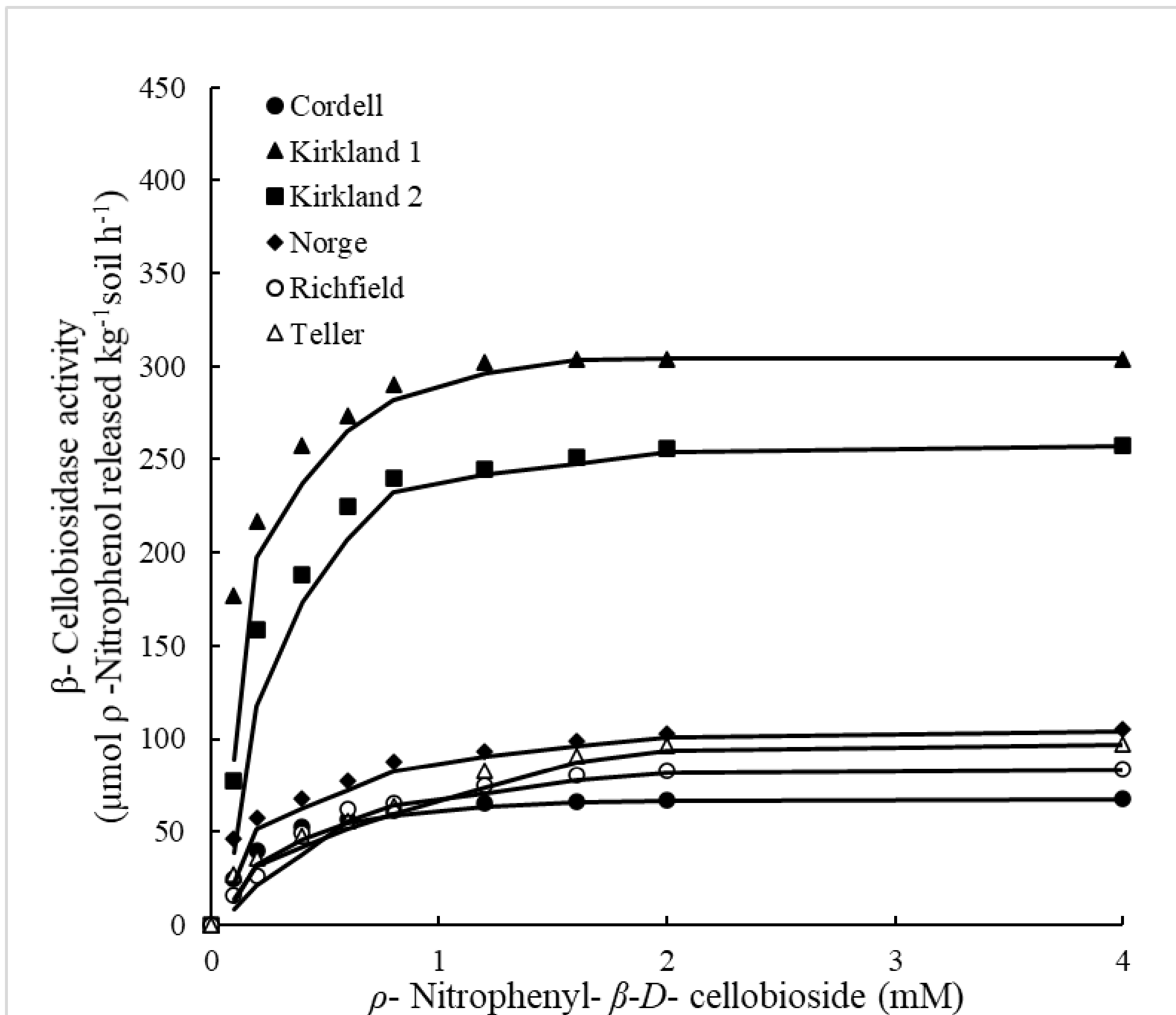

Figure 3.2. Effect of substrate concentration on *β*-cellobiosidase activity in soils.

Tabatabai, 1980). When the enzyme is saturated with the substrate, the reaction rate essentially follows zero-order kinetics. Thus, when the assay conditions are carefully controlled, the reaction rate at the saturation level will be dependent only on the enzyme concentration in the system, permitting comparison of enzyme activities between soils.

3.3. **Effect of amount of soil and effect of incubation temperature:**

Linear relationships were observed between *β*-cellobiosidase activity and the amount of soil or incubation time (Figures 3.3 and 3.4). This suggests that for soils with relatively low β-cellobiosidase activity, the measurements could be done by increasing the amount of soil or incubation time for enhanced accuracy in the quantification of *β*-cellobiosidase activity. Results are in close agreement to the observations for other hydrolytic soil enzymes that showed enhanced activity after increasing the amount of soil or incubation time (Deng and Tabatabai, 1994; Eivazi and Tabatabai, 1988; Frankenberger and Tabatabai, 1980; Parham and Deng, 2000). However, long incubation time in enzyme assays is not recommended as it can bring about challenges in data interpretation because of potentially increased microbial growth and activity during incubation (Kanazawa and Miyashita, 1986; Skujins, 1967).

Activity of *β*-cellobiosidase is highly dependent on incubation temperature with the highest activity around 60°C, with the exception of Teller soil which was about 50°C (Figure 3.5). The observed temperature optimum is slightly higher than the temperature optimum of purified *β*-cellobiosidase from Streptomyces coelicolor that showed optimal activity at 50°C (Lim et al., 2016; Lee et al., 2018). Similarly, Shin et al. (2010) observed the optimal activity of *β*-cellobiosidase purified from Fomitopsis pinicola at 50°C (Shin et al., 2010). While activity of β-cellobiosidase isolated from Aspergillus fumigatus had maximum activity at 55°C (Mahmood et al., 2013). The slightly higher optimal temperature for this enzyme in soil than purified ones suggest its greater temperature

stability in soil, which is further supported by the required higher temperature to inactivate a soil enzyme than the same enzyme in purified state (Tabatabai and Bremner, 1970).

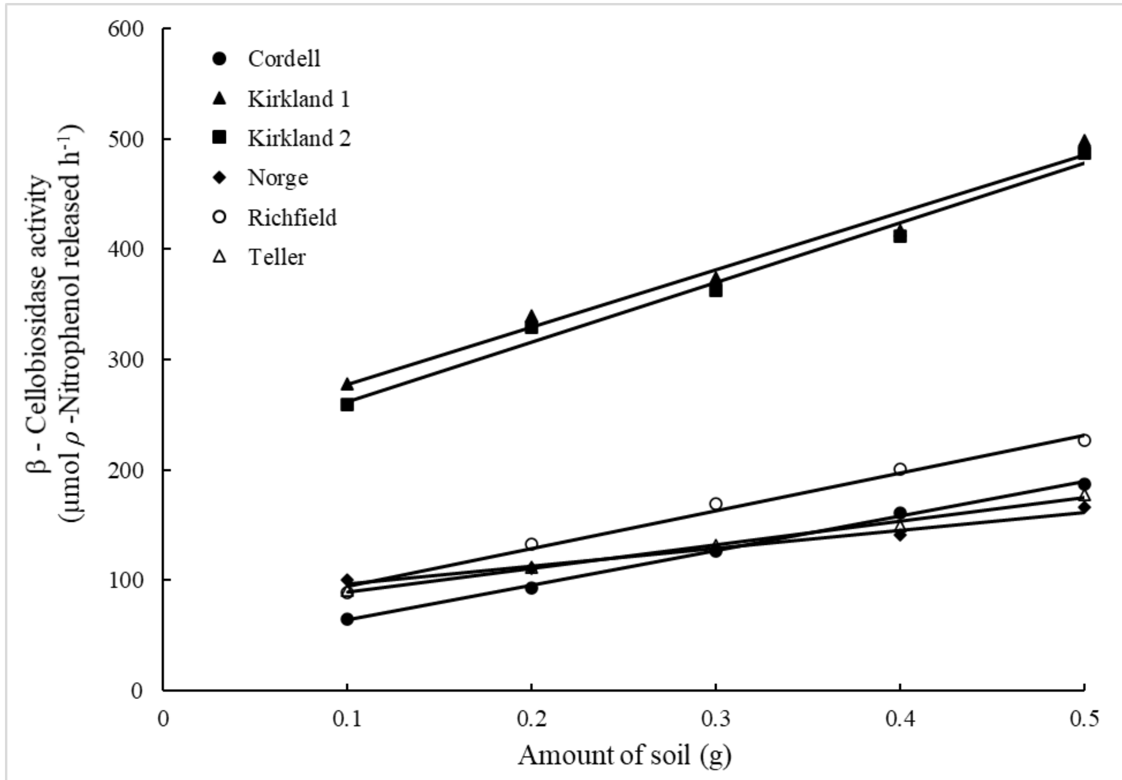

Figure 3.3. Effect of amount of soil on *β*-cellobiosidase activity in the assays.

Although most soil enzymes showed denaturation at temperatures between 60 and 70°C (Tabatabai, 1994), the assay in the proposed methods was conducted at 37°C because it is the biological temperature and the assay temperature used in many classic assay methods of soil enzymes (Tabatabai, 1994).

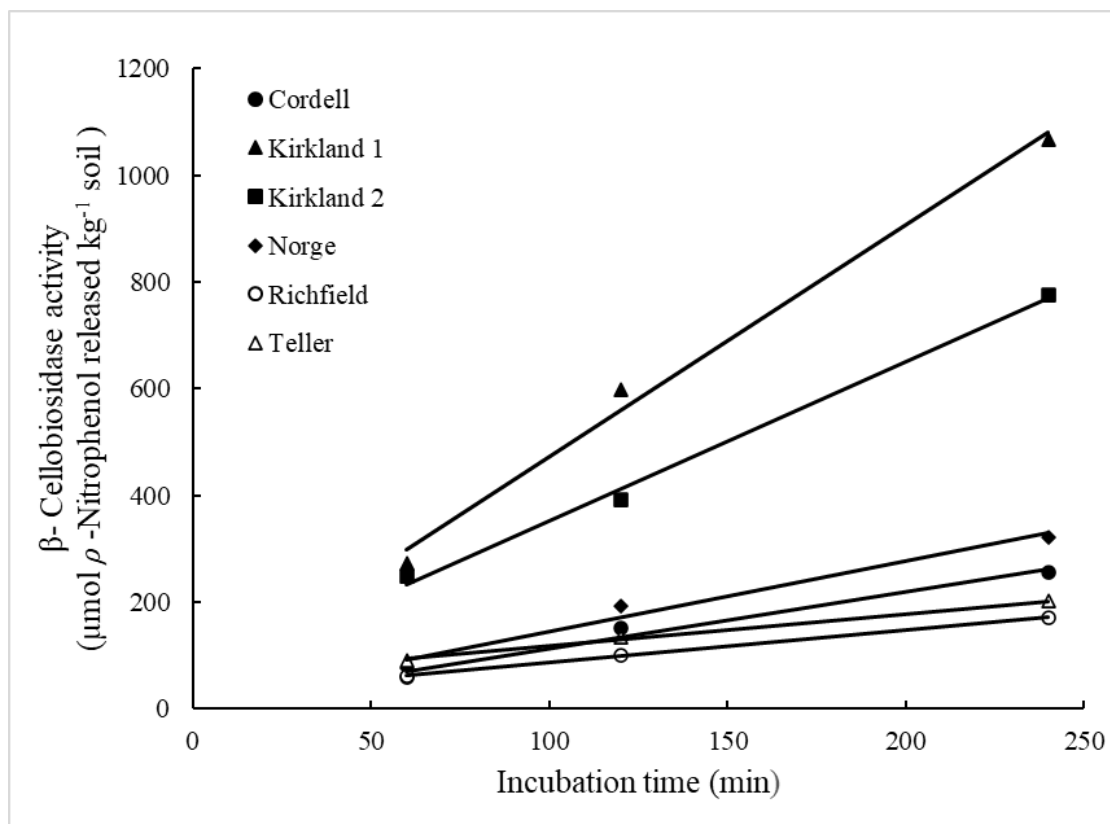

Figure 3.4. Effect of incubation time on *β*-cellobiosidase activity in the tested soils.

Using the developed protocol and under the assay conditions specified above, the developed method is reproducible, giving a coefficient of variance of $\leq$ 4.7% based on tests of six soils (Table 3.2). This method showed comparable precision to classic soil enzyme assay methods (Parham and Deng, 2000; Tabatabai, 1994), but offers markedly reduced cost and use of reagents and samples. The LOD and LOQ values calculated based on the calibration curves were 1.67 and 5.09 µmol pNP per microplate well, respectively. These are equivalent to LOD and LOQ of 16.7 µmol pNP kg^{-1} soil h^{-1} and 50.9 µmol pNP kg^{-1} soil h^{-1} for determining *β*-cellobiosidase activity using to the developed method. For soils

with β-cellobiosidase activity lower than LOQ, minor modifications of the assay protocol are needed, such as increasing incubation time or the amount of soil, to increase accuracy and precision of detection. When the detected soil β-cellobiosidase activity is > LOQ using the proposed assay protocol, quantitative results can be reported with a high degree of confidence (Thompson et al., 2002).

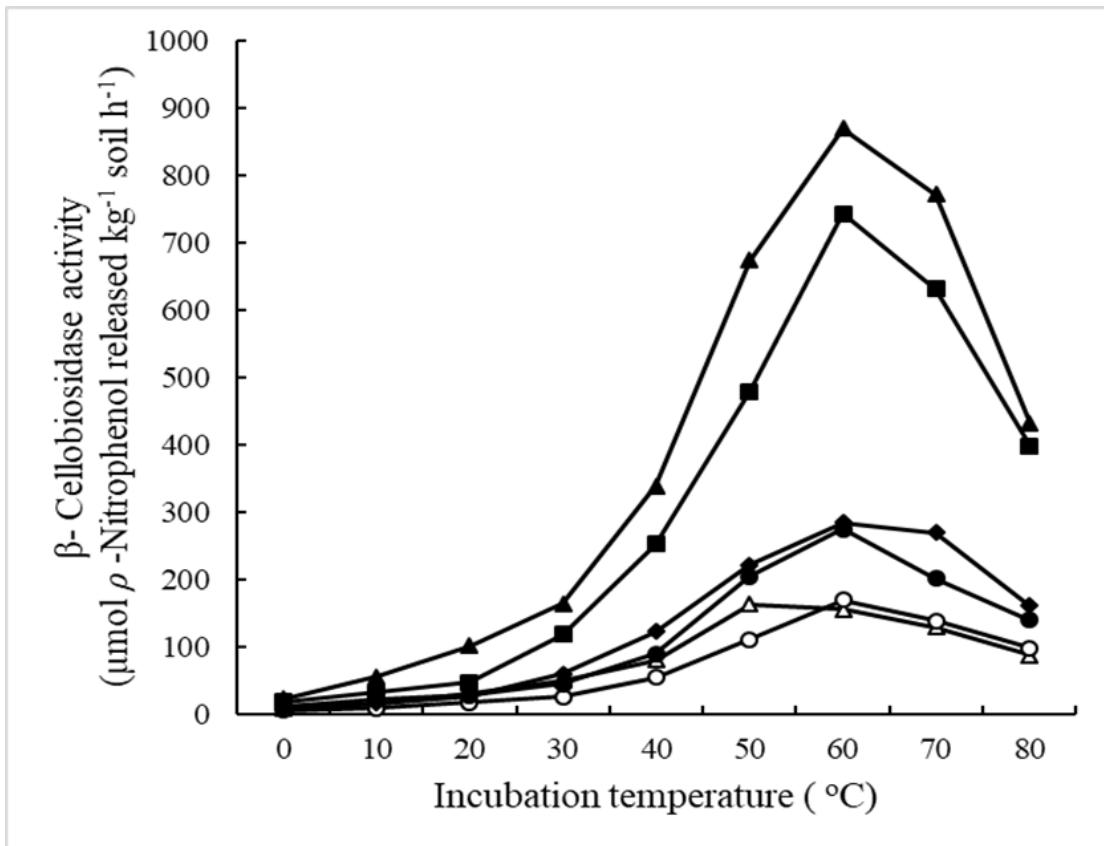

Figure 3.5. Effect of incubation temperature on β-cellobiosidase activity in soils.

Table 3.2. Precision of the method.

Soil	β-cellobiosidase activity			
	Range	Mean[†]	SD[‡]	CV%[§]
	……. µmol ρ-Nitrophenol kg^{-1} soil h^{-1}…….			
Cordell	66.9 – 75.8	73.1	3.4	4.6
Kirkland 1	302.8 – 325.1	315.5	7.2	2.3
Kirkland 2	242.9 – 257.2	250.4	5.6	2.2
Norge	82.0 – 88.3	85.8	2.2	2.6
Richfield	73.1 – 82.0	77.0	3.2	4.2
Teller	75.8 – 82.9	79.4	3.7	4.7

[†]Range of six replicated assays. [‡]SD, standard deviation. [§]CV, coefficient of variation.

3.4. **Kinetic parameters (*K_m* and *V_{max}*), temperature coefficient (*Q_{10}*), and activation energy (*E_a*)**

The relationship between substrate concentration and β-cellobiosidase activity in the tested soils obeyed the Michaelis-Menten equation. Thereby, kinetic parameters were calculated based on three linear transformations of the Michaelis-Menten equation: Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf (Figure 3.6). The K_m values in the tested soils ranged from 0.08 to 0.51 mM and the V_{max} values ranged from 71.5 to 318.1 µmol pNP released kg^{-1} soil h^{-1} (Table 3.3). In general, the three linear transformations gave similar calculated kinetic parameters. The variations among the three transformations occur because of mathematical bias; each transformation gives different weight to errors in the variables (Dowd and Riggs, 1965). The K_m value is independent of enzyme concentration and provides an indication of the affinity between the enzyme and substrate, with a low K_m indicating high affinity. V_{max} values indicate the maximum velocity of

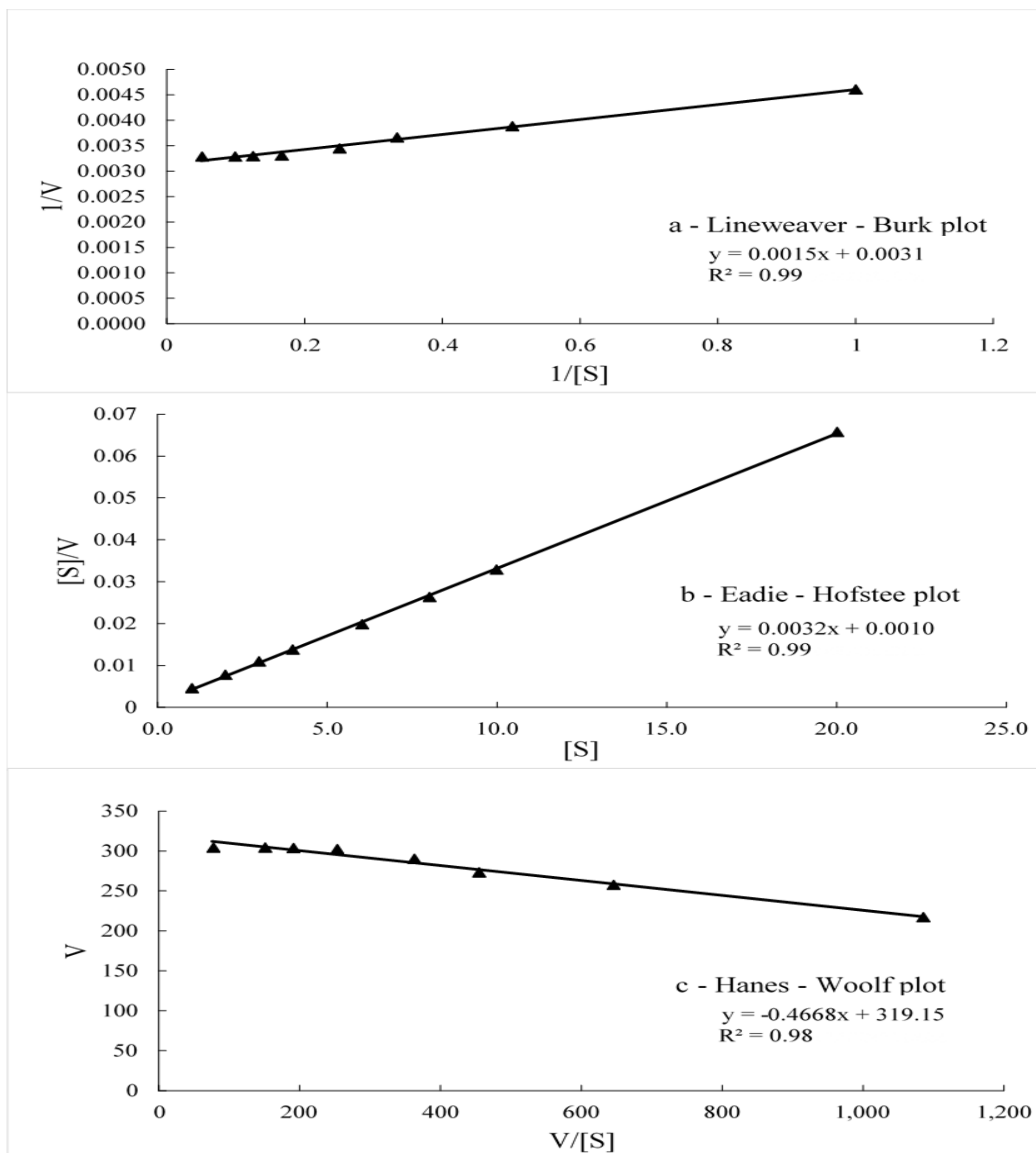

Figure 3.6. Three linear transformation plots of the Michaelis-Menten equation for *β*-cellobiosidase activity in Kirkland 1 soil as an example how Km and Vmax values were calculated. The slope and intercept of the linear regressions were used to calculate the kinetic parameters. A, Lineweaver – Burk plot, B, Eadie – Hofstee plot, and C, Hanes – Woolf plot. Velocity (V) was expressed as μmol ρ-nitrophenol released kg^{-1} soil h^{-1}. Substrate concentration (S) was in mM.

Table 3.3. Enzyme kinetic parameters, K_m and V_{max} values, of β-cellobiosidase in six soils calculated from three linear transformations of the Michaelis-Menten equation.

Soil	Liner-transformation†	K_m	Mean‡	V_{max}	Mean‡
		------------ mM ------------		---- µmol release of pNP kg^{-1} soil h^{-1} ----	
Cordell	LB	0.16	0.15	72.5	71.5
	HW	0.13		69.9	
	EH	0.16		72.1	
Kirkland 1	LB	0.10	0.08	322.6	318.1
	HW	0.06		312.5	
	EH	0.09		319.2	
Kirkland 2	LB	0.24	0.17	270.3	271.4
	HW	0.11		270.3	
	EH	0.15		273.7	
Norge	LB	0.13	0.19	101.0	107.8
	HW	0.25		112.4	
	EH	0.20		109.4	
Richfield	LB	0.63	0.47	113.6	104.1
	HW	0.33		92.6	
	EH	0.46		101.1	
Teller	LB	0.46	0.51	106.4	110.6
	HW	0.55		113.6	
	EH	0.51		111.7	

†LB, Linerweaver–Burk. HW, Hanes–Woolf. EH, Eadie–Hofstee. These transformations are shown in equations (3), (4), and (5) in the materials and methods. ‡ Mean of three linear transformations.

the enzymes under the tested conditions, which is independent of the substrate concentration but related to the amount of enzyme present in the soil. In the tested soils, the K_m values were negatively correlated with soil OC content (r=−0.80*, n=6, data not shown), but positively correlated with soil sand content (r=0.67, n=6). Organic matter and clay minerals have long been hypothesized to form a three-dimensional network that stabilizes enzymes in soil (Balota et al., 2004; Tabatabai, 1994).

Extracellular enzymes form complexes with the soil organic components and react chemically with phenols, quinones, tannins, lignin components, and humic acid (Ladd and Butler, 1975). The hypothesis is supported by observations that soil enzymes are more heat resistant than purified enzymes. The temperature required to inactivate an enzyme in soils is about 10°C higher than the temperature required to inactivate the same enzyme in the absence of soil (Tabatabai and Bremner, 1970), which is consistent with findings obtained from this study.

As discussed earlier, the optimal temperature for β-cellobiosidase activity in soil was around 60°C, while those from purified β-cellobiosidase was around 50°C (Shin et al., 2010; Lim et al., 2016; Lee et al., 2018). The negative correlation between K_m and OC suggested that the enzymes stabilized by organic substances might have lower affinity to substrates, which are thereby less efficient catalysts for the enzymatic reaction. Contrary to K_m, V_{max} values were positively correlated with OC (r=0.882**, n=6, data not shown). Because V_{max} is closely related to the amount of enzyme in the system, high OC content resulted in high content of stabilized enzyme in the system. The obtained results are

consistent with those reported by Zimmerman and Ahn (2010) in those soils with high OC contents have a significant role in the regulation of soil enzyme activity and stability.

When assaying enzyme activities, the concentration of substrate must be sufficient to maintain zero-order reaction, thus achieving a reaction rate proportional to enzyme concentration (Tabatabai, 1994). The K_m value could be used to estimate substrate concentration to be used to ensure substrate saturation and zero-order reaction during the assay duration. Often, enzyme assays are conducted using substrate concentrations that are five to 10 times of the Km (Margenot et al., 2018; Tabatabai, 1994). In the proposed assay method, 2.5 mM of p-nitrophenyl β-D-cellobioside was 4.9 - 31.3-fold of K_m values of the six soils tested. The linear relationships between enzyme activity and incubation time (Figure 3.4) further confirmed that zero-order reaction was ensured during the assay under the defined conditions. V_{max} is an indicator of enzyme concentration per volume or mass unit as well as reflecting interactions between the enzyme and soil matrix (Marx et al., 2005). Thus, V_{max} values provide comparison of enzyme activities in different soils.

Collectively, Km and Vmax values provide evaluation of the soil's capacity to cycle nutrients. A soil containing enzymes with low K_m and high V_{max} would have greater capacity to cycle nutrients than those with high K_m and low V_{max}. Data from this study indicated that soils with high OC have great capacity to cycle nutrients, which were consistent with results reported by Kujur and Kumarpatel (2014) and Masciandaro et al. (2000). All reactions are temperature dependent. Temperature dependence of enzymatic reactions is often evaluated by temperature coefficient (Q10) and activation energy (Ea) (Calsavara et al., 2001; McClaugherty and Linkins, 1990). In the non-denaturing temperature range of 10 - 50oC, the Q10 values ranged from 1.72 to 1.99 and decreased with increasing incubation temperature for

the tested soils (Table 3.4). Low Q10 values are indicative of the low kinetic energy required for the reaction catalyzed by this enzyme. As incubation temperature increased, enzyme catalytic cellobiohydrolase originated from Aspergillus oryzae ranged from 2.1 to 2.6 (Sorensen et al., 2015). It is interesting that Q_{10} values of this enzyme in soils were lower than those purified from A. oryzae. This high catalytic efficiency of β-cellobiosidase in soil implies that A. oryzae is not likely a major contributor of this enzyme activity in the tested soils.

Table 3.4. Temperature coefficient (Q_{10}) and activation energy (E_a) of β-cellobiosidase activity in soils tested in the non-denaturing temperatures ranging from 10 to 50°C.

Soil	Q_{10} for temperatures specified (°C)						E_a[†] (kJ mol^{-1})
	10	20	30	40	50	Average	
Cordell	1.87	1.80	1.73	1.67	1.62	1.74	43.2
Kirkland 1	1.97	1.89	1.81	1.75	1.69	1.82	46.9
Kirkland 2	2.18	2.07	1.98	1.89	1.82	1.99	53.7
Norge	2.10	2.00	1.91	1.84	1.77	1.93	51.2
Richfield	2.02	1.93	1.85	1.78	1.72	1.86	48.5
Teller	1.85	1.78	1.71	1.66	1.61	1.72	42.5

[†]Calculated based on data shown in Figure 7 using equations (6) and (7) in the materials and methods.

The Q_{10} values also indicate the importance of controlling temperatures during incubation because a 1oC difference in incubation temperature would lead to about 10% variation in data obtained when the Q_{10} equals 2. In biological systems, enzyme reaction rates increase with increasing temperature until reaching enzyme denaturation temperature. Generally speaking, enzymatic reactions are less sensitive to temperature changes than chemical reactions. Chemical reaction rate might be doubled with every 10°C elevation of temperature, while most biological reaction rates would increase by a Q_{10} of < 2 (Frankenberger and Tabatabai, 1980).

The β-cellobiosidase reaction in the tested soils obeyed the Arrhenius equation from 10 to 50°C as indicated by the linear relationships (Figure 3.7). The Ea values of the reaction catalyzed by β-cellobiosidase in these soils ranged from 42.5 to 53.7 kJ mol-1

(Table 3.4). An activation energy value of 35.5 kJ $mo1^{-1}$ was reported for arable loamy haplic Luvisol (Razavi et al., 2015). The range of activation energies for *β*-cellobiosidase in soils were somewhat lower than purified β-cellobiosidase originated from Aspergillus oryzae (60 to 70 kJ mol^{-1}, Sorensen et al., 2015), a trend consistent with that observed for Q_{10} values.

Low Q_{10} and Ea values are closely related. According to Segel (1975), a Q_{10} value of 2 is equivalent to an E_a of about 12,600 cal mol^{-1} (53 kJ mol^{-1}). Data from this study indicated a correlation coefficient (r) between Q_{10} and E_a value of 0.9996*** (n=6; data not shown). The calculated E_a value at Q_{10}=2 was 52.9 kJ mol^{-1}. This confirms that these two values are highly correlated for enzymes in the soil system and can be used to predict each other. Low Q_{10} and E_a values are not only indicative of low required kinetic energy but also indicative of efficiency in catalyzing the enzymatic reaction. The observed variations in temperature dependence of *β*-cellobiosidase among soils tested indicated variations in the source and status of this enzyme in different soils.

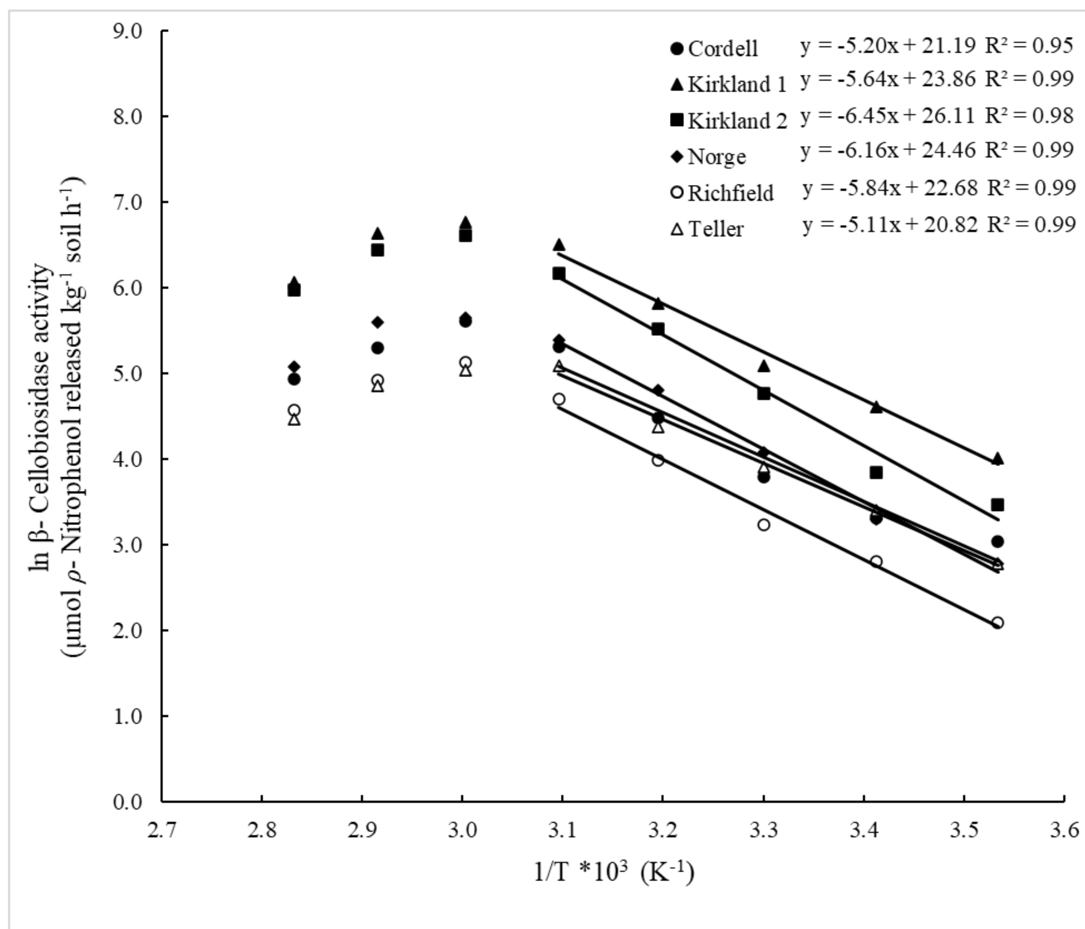

Figure 3.7. Linear transformation plots of the Arrhenius equation for β-cellobiosidase activity in the selected six soils at temperatures ranging from 10 to 50ºC.

4. **Conclusions**

A simple, sensitive, and precise method for assaying β-cellobiosidase activity in soils was developed. This method employed determination of the pNP content when a soil was incubated at 37˚C for one hour in the presence of 2.5 mM p-nitrophenyl β-D-cellobioside. Based on assays of this enzyme activity in six soils, the standard deviation

ranged from 2.2 to 7.2 (µmol pNP kg^{-1} soil h^{-1}) and the coefficients of variation (CV) were ≤ 4.7%. LOD and LOQ were 16.7 and 50.9 µmol pNP kg^{-1} soil h^{-1}, respectively. The optimal activity of β-cellobiosidase in soil was around pH 5.5 at the incubation temperature of 60°C. The Michaelis constant (K_m) and maximum velocity (V_{max}) for its activity in the tested soils ranged from 0.08 to 0.51 mM and 71.5 to 318.1 µmol pNP kg soil^{-1} h^{-1}, respectively. The K_m values were negatively, while the V_{max} values were positively correlated with soil OC content, suggesting that organic substances not only promoted affinity between the enzyme and substrates, but also enhanced stability of this enzyme in soil. Activities of this enzyme in soil were highly temperature dependent, with Q_{10} values ranged from 1.72 to 1.99 and decreased with increasing temperature from 10 to 50°C. The activation energy (E_a) ranged from 42.5 to 53.7 kJ mol^{-1} for soils tested. As temperature increases, the enzymatic reaction became more efficient and required less kinetic energy for the catalyzed reactions in soil.

Acknowledgements

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CHAPTER IV

TYP THE RELATIONSHIPS AMONG COIL ENZYMES, AND BETWEEN THEIR ACTIVITIES (RESPIRATION) AND MICROBIAL BIOMASSE TITLE HERE

Abstract

Variability in soil enzyme activity may have important implications for the knowledge of underground ecosystem functions driven by soil extracellular enzymes. To illustrate the variation in soil enzyme activity after land use and management, we collected soil samples from three long-term (9 years) switchgrass experiments located in central Oklahoma. Soil samples were taken from 0-10, 10-20 and 20-30 cm. These samples were analyzed to determine cellulase, *β*-cellobiosidase, *β*-glucosidase, urease, alkaline phosphatase, arylsulfatase, acid phosphatase, microbial biomass carbon (MBC) and microbial respiration. The results revealed that the soil enzyme activities significantly decreased after land management and varied with depth. Except urease, all the tested enzyme showed a net reduction (up to 61%) in their activities in switchgrass fields as compared to natural undisturbed soils. Moreover, lower MBC (up to 54%) in switchgrass plots and lower microbial respiration (up to 96%) was observed in native undisturbed soils. In addition, the Pearson's correlation coefficients results revealed an overall significantly positive correlation ($r=$ ranged from 0.68 to 0.98 at $p< 0.05$; $p<0.01$; $p<0.001$) between soil enzyme activities and MBC except urease ($r= -0.77$). Overall, our results showed that land

management by planting switchgrass significantly resulted in the reduction of MBC and their activities as compared to native soils.

Introduction

The removal of natural vegetation followed by cultivation can cause severe changes to physical, chemical, and biological soil properties. These changes are often associated with reduction of soil organic matter, deterioration of soil structure, and decreases in microbial biomass and activity (Waldrop et al., 2000; Islam and Weil RR, 2000; Solomon et al., 2002; Dinesh et al., 2004; Bossio et al., 2005; Celik, 2005; Dawson and Smith, 2007; Nourbakhsh, 2007). Previous studies have reported that land use/cover would affect the soil physical, chemical, biological properties, and soil organic matter dynamics, which subsequently alters the soil quality and fertility (Li et al., 2013). Further, the variation in land use/cover influences the soil microbial functions by affecting the soil carbon and nitrogen cycle (Sousa et al., 2011).

Soil microbes despite comprising a small fraction of the total mass of soil organic matter play a critical role in soil processes, soil organic matter decomposition, nutrient cycling, etc. They are significantly influenced by human interventions involving land conversions (Smith and Paul, 2017; Kabiri et al., 2016). Additionally, the management practices, variation in quality and availability of substrate, fine roots activity, litter quality, vegetation composition, plant biomass, and belowground processes also change the soil organic matter content, hence, affect the soil microbial community structure and functions. Soil enzyme activities related to soil microbial biomass are often used for comparison of different land use with varying soil organic matter content (Waldrop et al., 2000; Bastida

et al., 2008). Measurement of soil enzyme activities in key nutrient cycling (C, N, and P) and oxidation–reduction processes have been used widely as a potential indicator for determining the effect of land use conversions and management practices on soil health (Acosta-Martínez et al., 2008; de Medeiros et al., 2015). Therefore, an investigation of soil microbial characters and enzyme activities is critical in studying the land conversions and focusing the soil management. Further, enzymatic activities, soil microbial biomass and respiration (Anderson and Domsch, 1978) are good indicator of soil nutrient dynamics (Raiesi and Beheshti, 2015; Goenster et al., 2017) and would provide an early warning of soil quality deterioration (Cookson et al., 2007; Huang and Song, 2010).

Microbial biomass and activity are generally closely related because it is through the biomass that the transformations of the important organic elements (C, N, P, and S) occur. The microbial biomass also acts as a small but labile reservoir for these elements. Therefore, the ideal parameter for assessing the role of microorganisms in various soil processes would correlate soil enzymatic activities not only with microbial respiration but also with microbial biomass.

Therefore, in present study, soil enzyme activities (cellulase, β-cellobiosidase β -glucosidase, arylsulfatase, acid phosphatase, alkaline phosphatase, urease) microbial biomass carbon and respiration were determined in three long-term (9 years) switchgrass field experiments and for comparison from the nearby fields having mixed vegetation.

The objectives of the study were to evaluate the relationships among different soil enzymes and their activities with microbial biomass in soils having in managed and unmanaged soils.

Materials and Methods

A. Site description

In current experiment, the soils were taken from three long-term (9 years) switchgrass experiments located in central Oklahoma. The first site is Efaw (EF) (Easper loam and Pulaski fine sandy loam mix, 36°7’52.64” N, 97°6’16.75” W). The second site is Cow Creek (CC) (Easper loam, 36°7’2.77” N, 97°5’52.06” W). The third one is 40 North (40N) (Huska Silt Loam, 36°8’21.48” N, 97°4’44.4” W). These sites have received nitrogen at 75 kg N per year.

B. Sampling and analysis

Soil samples were taken from different depths i.e., 0-10, 10-20 and 20-30 cm. To study the effect of land management, soil samples were taken from the nearby field with natural vegetation as controls. The pH of the soils ranged 5.7-6.6. The samples were air-dried, crushed, and passed through 2 mm sieve prior to analyze the physicochemical characteristics of the soils (Table 4.1) by following: soil texture was determined using composite samples by the hydrometer method (Gee and Or, 2002). Total carbon was found through dry combustion analysis by placing soil samples wrapped in foil into a Leco TruSpec combustion analyzer (Nelson and Sommers, 1996). Soil pH was determined using a standard glass electrode (1:2) Soil/CaCL2 solution (McLean, 1982).

Table 4.1. Classification, vegetation, and basic properties of soils used.

Soil							
Location	Series Subgroup	Vegetation	pH†	Organic C‡	Sand	Silt	Clay
				---- g kg^{-1} ---	-------------- % ------------		
Cow Creek	Easpur/Fluventic Halustolls	Bermuda grass	6.4	14.9	47.5	38.7	13.8
Cow Creek		Switchgrass	6.2	10.4			
Efaw	Easpur/Fluventic Halustolls	Bermuda grass	6.0	33.1	26.3	52.4	21.3
Efaw		Switchgrass	6.0	22.0			
40 North	Huska/Mollic Notrustalfs	Indian grass	5.2	26.9	36.3	41.2	22.5
40 North		Switchgrass	6.3	18.3			

†Soil: Water ratio = 1:2; ‡ C, carbon.

For the measurement of cellulase activity, 5 g of field moist soil was incubated with 20 ml buffered (Acetate buffer (pH 5.5, 50 mM) 2% Carboxymethyl cellulose (CMC) as substrate and 0.5 ml toluene for 24 h at 30°C in a sealed Erlenmeyer flask. Then, the suspension was thoroughly mixed and centrifuged three times for 10 min at 17,390g. Aliquot of the supernatant (10 ml) was treated with 2 g of K-saturated cation-exchange resin followed by the shaking of the obtained mixture for 30 min. reducing sugars in the supernatant were analyzed by the Somogyi-Nelson method (Deng and Tabatabai, 1994).

The method of Tabatabai, (1994) was followed to measure the urease activity. For this, field moist soil (5 g) was incubated in 9 ml THAM buffer and 0.2 ml of toluene in volumetric flask. The contents in flask were agitated for a few seconds and 1 ml urea solution (0.2 M) was added and agitated again. The flask was stoppered and placed in an incubator for 2 h at 30°C. Then, 35 ml of KCl-Ag_2SO_4 solution was added to flask and mixed. The flask's contents were allowed to cool down to room temperature followed by further addition of KCl-Ag_2SO_4 solution to make 50 ml volume. After mixing the contents thoroughly, NH^{+4}- N was analyzed in obtained suspension by distilling the 20 ml of suspension with 0.2 g of MgO for 4 min.

Arylsulfatase activity was measured by incubating soil (1g) in 4 mL of acetate buffer, 0.25 mL of toluene, and 1 mL of p-Nitrophenyl sulfate (PNS) solution in a sealed Erlenmeyer flask at 37°C for 1 h. Then 4 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$ were added in the flask and mixed. The resulting suspension was filtered, and filtrate was analyzed by using a spectrophotometer at 410 nm. Similar procedure was adopted for controls but 1 mL of PNS solution was added after the addition of 4 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$ (Tabatabai, 1994).

The method of Tabatabai, (1994) was followed to determine the β-glucosidase activity. For this, three replicate samples (two assays and one control) were prepared by weighing 1.0 g of soil (based on the oven dry weight) into each 20 ml test tube containing 4 mL of modified universal buffer (MUB, pH 6) and 0.25 ml of toluene. Then, 1 mL of 0.05 M p-Nitrophenyl-β-D- glucopyranoside to two assay tubes and vortexed each tube for a few second. The tubes were then placed in a water bath at 37˚C. After 1 h incubation, 4

mL of 0.1 M tris (hydroxymethyl) amino methane (THAM; pH 12) and 1 mL of 0.5 M $CaCl_2$ was added to terminate the reaction.

Activity of Acid and Alkaline Phosphatases

For this purpose, 1g of field moist soil was incubated with 4 mL of MUB (pH 11 for assay of alkaline phosphatase and pH 6.5 for assay of acid phosphatase), 0.2 mL of toluene, and 1 mL of p-nitrophenyl phosphate solution in a sealed Erlenmeyer flask 37 °C. Then, 4 mL of NaOH (0.5 M) and 1 mL of $CaC1_2$ (0.5 M) was added to flask after 1 h. The suspension was filtered after mixing the contents of flask and filtrate was analyzed for yellow intensity (Tabatabai, 1994).

Microbial Biomass Carbon and Respiration

Following the chloroform fumigation-incubation method (Vance et al., 1987), composite soil sample from each plot (30 g fresh weight) was used to prepare triplicate subsamples in 50 ml beaker. Fumigation and control were used as treatments. For fumigation, samples were kept in vacuum desiccator having lining of moist paper towels. A 50 ml beaker containing acid washed chloroform was placed in the center of the desiccator. The sealed desiccator was kept in laboratory hood and then evacuated to allow the boiling of chloroform for 30 seconds. The boiling was repeated 4 times. The samples were incubated in chloroform vapor-saturated atmosphere for one day. After incubation, chloroform was removed through vacuum air flushing of the desiccator (repeated 8 times). The soil was then transferred to plastic bottles, and 2 ml of NaOH was put into a glass bottle open at the top. The bottles were tightly closed and left to sit in the dark for ten days. After the ten-day incubation period, the NaOH was transferred from the glass bottles to 125 ml flasks, 2 ml of $BaCl_2$, and one drop of phenolphthalein indicator was added to the

mixture, then swirled together. Samples were then titrated with HCl until the solution turned from pink to clear.

Microbial respiration was calculated from desiccators controls and the soil was then transferred to plastic bottles and 2 ml of NaOH was put into a glass bottle open at the top. Bottles were tightly closed and left to sit in the dark for 10 days. After the 10-day incubation period, the NaOH was transferred from the glass bottles to 125 ml flasks, 2 ml of $BaCl_2$, and one drop of phenolphthalein indicator was added to the mixture, then swirled together. Samples were then titrated with HCl until the solution turned from pink to clear.

C. Statistical Analysis

Data were analyzed by three-way analysis of variance techniques (ANOVA). Tukey’s test was performed using Statistix 8.1 (Analytical Software, USA) for mean comparisons while XLSTAT software (version 2021.3.1) was used for principal component analysis (PCA). Pearson’s correlation coefficients (Person’s R) were used to reveal the correlation between the soil enzyme activities and soil microbial biomass and respiration.

Results

It was observed that soil management caused a significant effect on the soil MBC and their activities. Activities of all the tested enzymes except urease decreased due to the cultivation of switchgrass as compared to the native non-cultivated soils with mixed vegetation. The soil management by cultivating switchgrass caused reduction in soil microbial biomass carbon as compared to the native non-cultivated soils while microbial respiration increased due to the cultivation of switchgrass.

Activities of β-cellobiosidase, arylsulfatase, and acid phosphatase showed the similar trend in response to switchgrass cultivation. Higher enzyme activities were observed under native non-cultivated sites as compared to switchgrass managed fields. In case of arylsulfatase, 53% higher enzyme activities were recorded in native Cow Creek soil with mixed vegetation as compared to the respective soil with switchgrass cultivation. Efaw and 40 North soils showed 10 and 16% higher enzyme activities, respectively, under natural mixed vegetation management as compared to respective switchgrass managed soils at 0-10 cm depth. Similarly, 41 and 42% significantly higher acid phosphatase activities were observed in native non-cultivated 40 North and Efaw soils compared to soils with switchgrass cultivation at 0-10 cm depth. While 42, 48 and 52% significantly higher β-cellobiosidase activities were observed in non-cultivated Efaw, Cow Creek and 40 North fields when compared with respective switchgrass managed fields, respectively, at 0-10 cm depth (Figure.4.1). Urease was the only enzyme whose activity was increased under switchgrass managed soils. The maximum urease activity (51.74 µg NH^{4}-N g^{-1} soil h^{-1}) was observed in switchgrass cultivated Efaw soil which was 103% higher than respective native non-cultivated Efaw soil at 0-10 cm depth following by Cow Creek fields under switchgrass experiment that resulted in 71% higher enzymatic activity as compared to native non-cultivated cow creek soil at 0-10 cm depth. While 35% higher urease activity was observed in switchgrass cultivated 40 North soils as compared to respective non-cultivated soil with mixed vegetation. Similar trend in the urease activity under switchgrass managed and non-cultivated soils was observed under the depth of 10-20 and 20-30 cm. Soil management resulted in 19 to 43% reduction in the activity of alkaline phosphates at

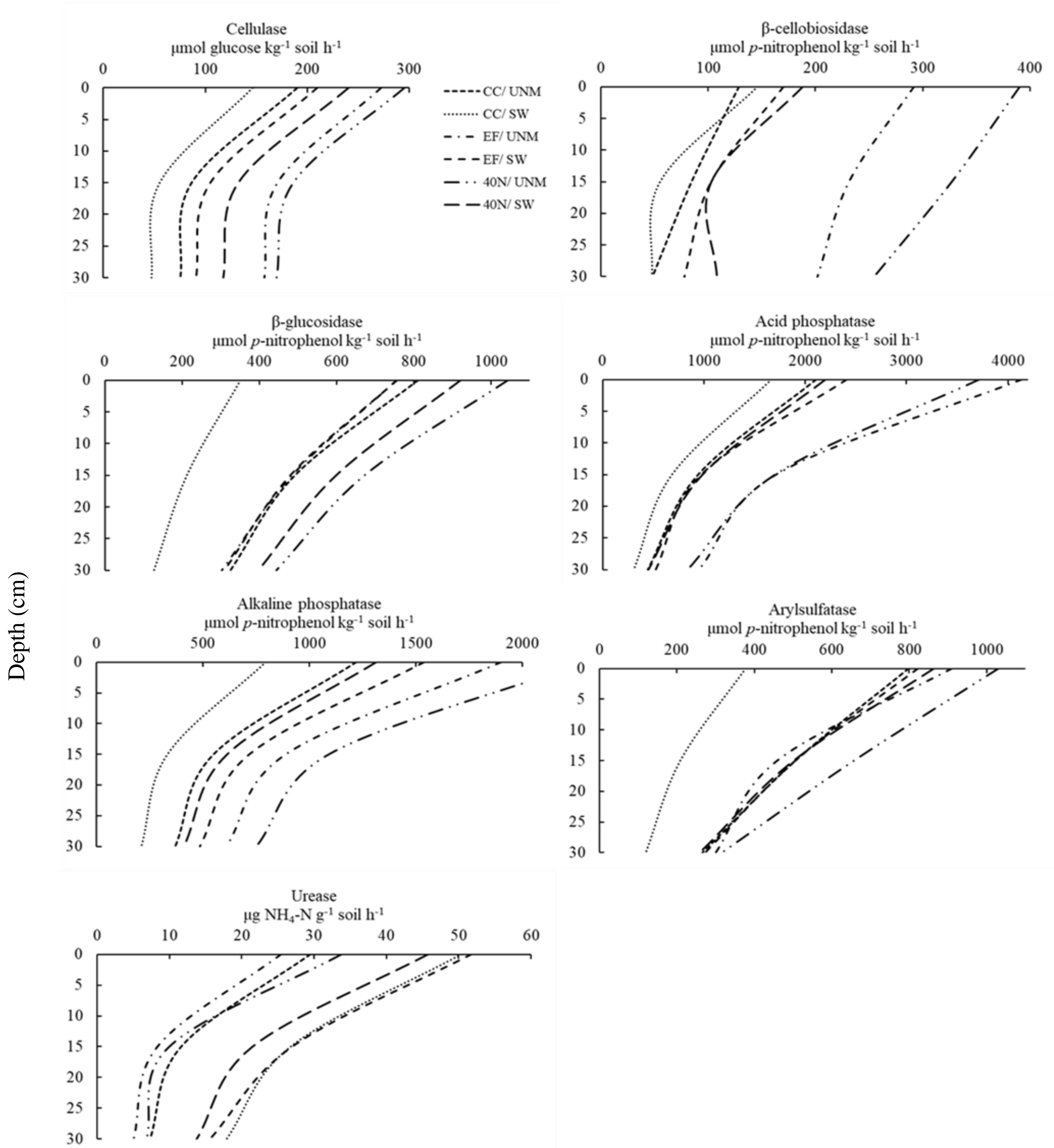

Figure 4.1. Distribution of C-transforming enzymes with 0-30 cm soil depth.

0-10 cm depth. Similar trend was observed in the subsurface soil. Maximum significant reduction 43% as compared to respective non-cultivated soil) in alkaline phosphatase due the switchgrass cultivation was observed 40 North soils followed by cow creek soil where 35% reduction in enzyme activity was observed due to cultivation of switchgrass. In Efaw soil, soils with switchgrass cultivation caused 19% decrease in the alkaline phosphatase activity. Similarly, 18 to 23% reduction in cellulase was observed in switchgrass managed soil as compared to the soil with natural native vegetation. No effect of soil management using switchgrass on the enzyme activity of *β*-glucosidase in Efaw soil was observed. However, 12 and 57% reduction in β-glucosidase activity in switchgrass managed 40 North and Cow Creek soils were recorded, respectively, compared to respective unmanaged soils.

In case of microbial biomass carbon, cultivation of switchgrass resulted in 29, 34 and 48% reduction in 40 North, Efaw and Cow Creek soils, respectively, as compared to respective native non-cultivated soils with mixed vegetation. However, in case of microbial respiration, different soils responded differently to soil management under all the three soil depths (Figure.4.2).

Switchgrass cultivation resulted in 67 and 96% significantly higher microbial respiration in Efaw and Cow Creek soils, respectively, as compared to respective non-cultivated soils at 0-10 cm depth. While 43% reduction in microbial respiration due to switchgrass cultivation in 40 North soil was observed as compared to native non-cultivated mixed vegetation soil. Microbial biomass carbon and microbial respiration at three depths (0-10 cm, 10-20 cm, and 20-30 cm) are shown in Figure 4.1. Microbial biomass carbon (MBC) was significantly higher in the unmanaged mixed grassland sites which ranged from 17.14 to 23.22 mg biomass-C 30 g-1, compared to the managed switchgrass sites

which ranged from 8.95 to 15.21 mg biomass-C 30 g-1 soil at 0-10 cm soil depth. Similarly, microbial respiration in switchgrass cultivated soils ranged from 0.18 to 0.37 mg CO2-C 30 g^{-1} soil day^{-1}, while native mixed vegetation grasslands ranged from 0.18 to 0.32 mg CO_2-C 30 g^{-1} soil day^{-1}.

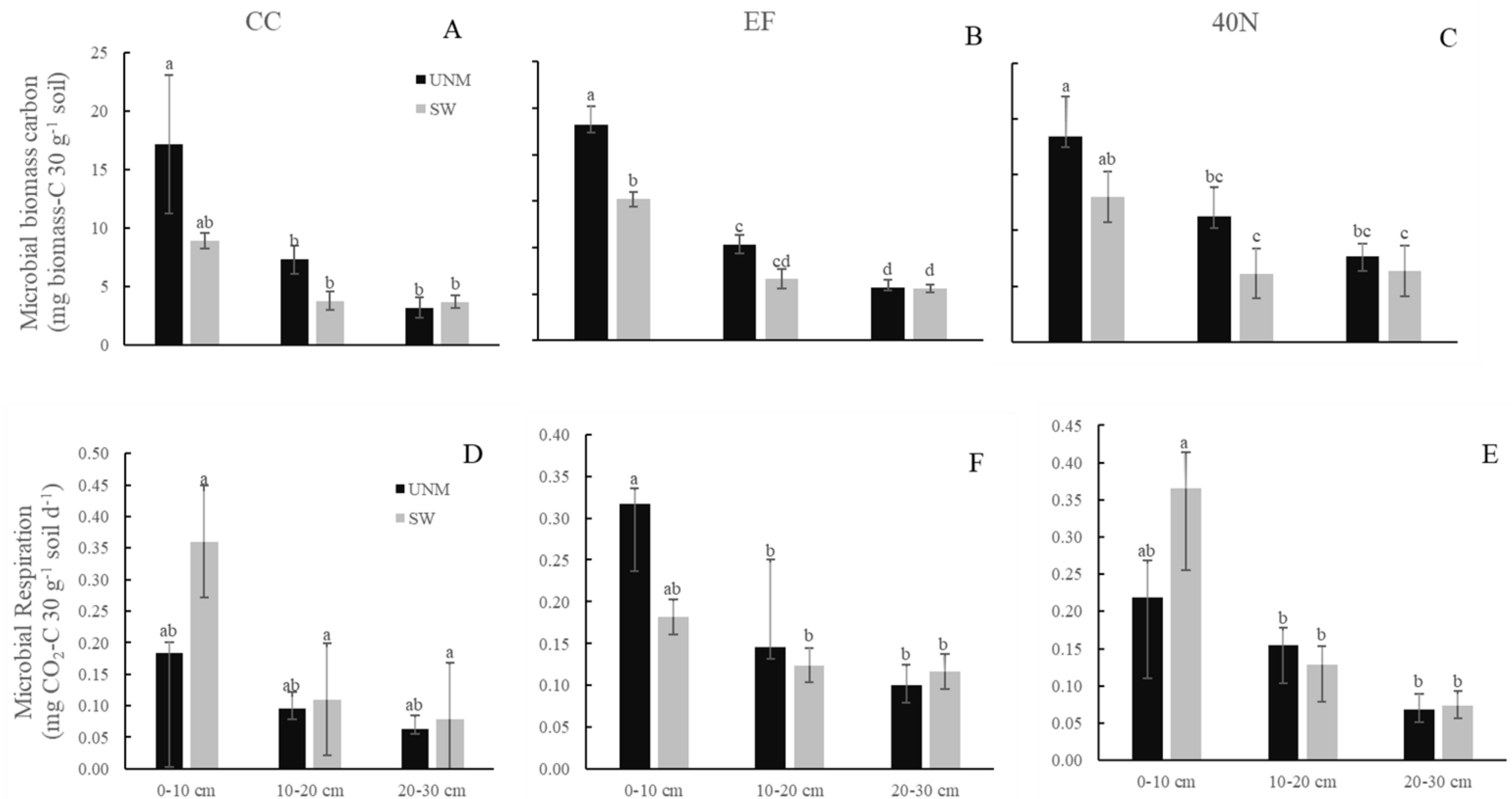

Figure 4.2. Microbial Biomass carbon and microbial respiration in soils of three depths at the studied locations (CC, EF, and 4N are locations as described above), separated by unmanaged mixed grass (UNM). and switchgrass (SW). Different letters on bars indicate significantly different means according to the least significant difference test as P< 0.05. Error bars indicate standard deviation of averages samples.

Relationship between soil microbial biomass carbon, respiration and soil enzymes was evaluated by using Pearson correlation coefficient (r) under 5% ($p < 0.05$), 1% ($p < 0.01$) and 0.1% ($p < 0.001$) significance levels. Very strong significant correlation coefficient between enzyme activities and microbial biomass carbon ranged from 0.68 to 0.98*** (Table 4.3). Acid phosphatase (r=-0.98***), alkaline phosphatase (r=-0.96**), cellulase (r=-0.94**) and arylsulfatase (r=-0.82*) showed significantly very strong correlation with microbial biomass carbon. While β-glucosidase (r=-0.68) and β-cellobiosidase (r=-0.80) showed strong positive correlation with microbial biomass carbon. However, urease showed a strong negative correlation with microbial biomass carbon (r=-0.77).

Most of the enzymes showed week to negative correlation with microbial respiration that ranged from 0.01 to 0.45 (Table 4.2). Maximum correlation coefficient (r=45) was observed between urease and microbial respiration followed by correlation between microbial respiration and alkaline phosphatase with r=29. Very weak correlation between microbial respiration and β-cellobiosidase (r=0.01), cellulase (r=0.13) and acid phosphatase (r=16) was observed. However, arylsulfatase and β-glucosidase showed a negative correlation with microbial respiration with r=-0.08 and r=-0.12, respectively. Negative to very strong correlation between different enzymes was observed that ranged from 0.48 to 98*** at three different significance levels.

Table 4.2. Correlation matrix (r) of soil microbial biomass, respiration, and microbial enzyme activities across the locations, management, and the depths in the 54 soils.

Variables	Urease*	Alkaline phosphatase	Cellulase	β-glucosidase	Arylsulfatase	Acid phosphatase	β-cellobiosidase	Microbial Biomass Carbon
Alkaline phosphatase§	-0.61							
Cellulase+	-0.63	0.94**						
β-glucosidase§	-0.49	0.76	0.79					
Arylsulfatase§	-0.60	0.87*	0.84*	0.96*				
Acid phosphatase§	-0.74	0.91*	0.92*	0.54	0.70			
β-cellobiosidase§	-0.55	0.87*	0.90*	0.98***	0.96**	0.69		
Microbial Biomass Carbon†	-0.77	0.96**	0.93**	0.68	0.82*	0.98***	0.80	
Microbial respiration‡	0.45	0.29	0.13	-0.13	-0.08	0.16	0.01	0.11

Significance level of correlations: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, +glucose produced µg kg^{-1}soil h^{-1}. §µmol ρ - Nitrophenol released kg^{-1}soil h^{-1}, †mg biomass-C 30 g^{-1} soil, ‡mg CO_2-C 30 g^{-1} soil d^{-1}, *µg NH^4-N g^{-1} soil h^{-1} .

The interrelationship among soil enzyme, soil microbial biomass carbon and microbial respiration were further evaluated by Principal Component Analysis. The obtained results were expressed by PC1 and PC2, which explained 88.44% of total variability (Table 4.3).

Table 4.3. Principal Component loadings of soil enzyme activities, microbial biomass carbon and respiration under native mixed vegetation and switch grass cultivated system of three different soils.

Parameter	PC1	PC2
Urease	-0.728	0.496
Alkaline phosphatase	0.962	0.254
Cellulase	0.964	0.125
β-glucosidase	0.857	-0.150
Arylsulfatase	0.932	-0.102
Acid phosphatase	0.896	0.145
β-cellobiosidase	0.935	-0.020
Microbial Biomass Carbon	0.960	0.086
Microbial Respiration	0.026	0.993
Eigenvalue	6.585	1.375
Variability (%)	73.164	15.275
Cumulative %	73.164	88.439

The first principal component analysis (PC1) explained 73.16% of the total variance, was positively correlated with most of the variables tested and contributed mostly by activities of cellulase, *β*-cellobiosidase; alkaline phosphatase, *β*-glucosidase; arylsulfatase; acid phosphatase; microbial biomass carbon and microbial respiration while PC2 explained 15.28% and was contributed by urease only (Figure. 4.2).

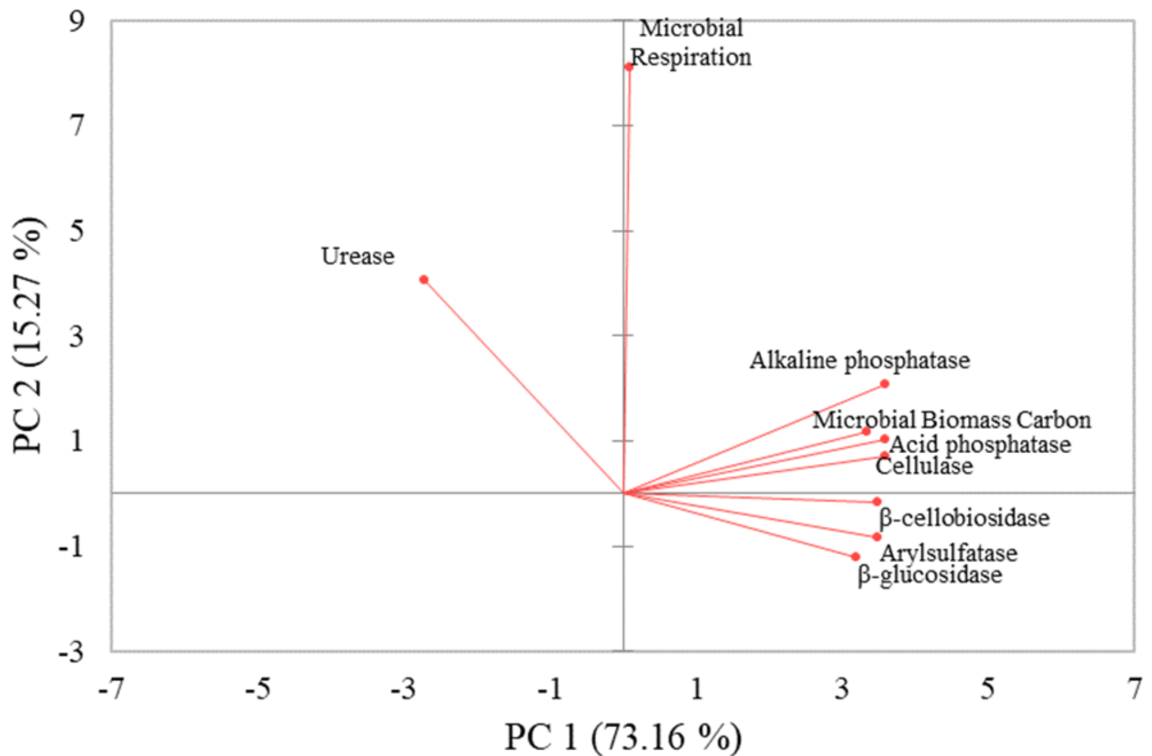

Figure 4.3. Factor scores of soil enzyme activities, microbial biomass carbon and respiration under native mixed vegetation and switch grass cultivated system of three different soils.

Discussion

Relationship of microbial biomass and enzyme activities

The combination of soil microbial biomass and enzyme activity measurements have been widely used over the last 10 years to study microbiological responses to agriculture management (Wick et al., 2000; Clegg, 2006; Udawatta et al., 2009). In the present study, we detected a significant strong correlation between soil enzyme activities (except urease) and soil microbial biomass carbon (MBC) following land management by growing switch grass. Our results agreed with many studies that had reported the positive correlation between soil enzymes and MBC (Wick et al., 2000; Clegg, 2006; Van Der Heijden et al., 2008; Udawatta et al., 2009). Similar to our results, Frankenberger and Dick (1983), reported that for the 10 soils tested, the various enzyme activities, with the exception of urease had positive correlation with MBC. They observed that enzyme activities were influenced to a greater degree by that portion of the microbial population which was active in soils than by any other factors that may affect enzyme activity. Similarly, Hojati and Nourbakhsh (2006) found positive correlations between soil enzymes and MBC and concluded that soil microbial activity and biomass were an important source of enzymes involved in C, N and P cycling.

We found negative correlation between urease activity and MBC which were in line with the results of Garcıa et al. (2000) who observed negative correlation between urease activity and MBC. However, contrary to our results, a strong correlation between urease activity and microbial biomass was observed by Nannipieri et al. (1978). In our study, alkaline and acid phosphatases showed a very strong significant positive correlation

with MBC even at significance level of $p<0.01$ and $p<0.001$, respectively. Many studies have reported strong positive correlations among phosphatase activity and microbial biomass (Kandeler and Eder, 1993; Nannipieri et al., 1983). A highly significant positive correlation between cellulase activity and MBC was observed in our study. However, Wang et al. (2013) observed negative correlation between soil microbial biomass and cellulase activity. After phosphatases and cellulase, highest correlation coefficient was observed in the case of arylsulfatase and MBC. Similarly, a very high correlation coefficients between arylsulfatase activities and microbial biomass was reported by Li and Sarah (2003) in all the studied sites. This indicates that microbial biomass directly influences enzyme activity. Many other authors also observed very high positive correlation coefficients between arylsulfatase activities and microbial biomass (Klose et al., 1999; Boyrahmadi and Raiesi, 2018). The activity of β-cellobiosidase and β-glucosidase showed positive but non-significant correlation between MBC which is corroborated by many other researchers (Ajwa et al., 1999; Garcıa et al., 2000; Turner et al., 2002; Tripathi et al., 2007).

Relationship of enzyme activities and respiration

In the current study, normal to weak positive correlation was observed between microbial respiration and enzyme activities (except β-glucosidase and arylsulfatase). Highest correlation was observed between urease activity and microbial respiration when compared with respect to other tested enzymes. While the correlation coefficient showed the very weak negative correlation between microbial respiration and β -glucosidase and arylsulfatase. Similarly, several studies have reported strong to weak correlation between microbial respiration and soil enzymatic activities. For example, respiration and

phosphatase activity were found to be correlated in reclaimed strip mine spoils by Hersman and Temple (1979) and Carpenter-Boggs et al. (2003) found positive non-significant correlation between microbial respiration and alkaline and acid phosphatase. Also, weak non-significant correlation was observed between microbial respiration and alkaline phosphatase by Kuperman and Carreiro (1997). The weak correlation between microbial respiration and enzymatic activities indicated the low intensity of metabolic processes. Contrary to our results, Li and Sarah, (2003) reported a very strong significant positive correlation between soil microbial respiration arylsulfatase activity and Fereidooni et al. (2013) found a significant correlation between soil microbial respiration and urease activity. Similarly, Oliveira and Ferreira, (2014) evaluated the correlation between substrate induced respiration and different enzyme activities and found positive significant correlation between respiration induced by substrate and different tested enzymes (i.e., cellulase β-glucosidase alkaline and acid phosphatase).

Microbial biomass and respiration

Different researchers have reported variability between the relationship of microbial biomass and respiration. In our study, a weak non-significant positive correlation was observed between the microbial biomass carbon and respiration corroborated by Carpenter-Boggs et al. (2003) who observed non-significant positive correlation between microbial biomass and respiration. Similarly, Santr and Sirasicraba, (1991) reported that soil respiration was not related to microbial biomass, since it remained on the average nearly constant. However, results obtained by Li and Sarah, (2003) contradicted our findings as they observed very strong correlation between soil microbial respiration and microbial biomass carbon. Fereidooni et al. (2013) also observed significant positive

correlation between microbial biomass and microbial respiration. While Frankenberger and Dick, (1983) observed that microbial biomass and respiration were significantly correlated (for both O_2 uptake and CO_2 evolution for amended soils). But no significant correlation was observed obtained between biomass and CO_2 evolution in non-amended soils.

Positive correlations between enzyme activities and microbial biomass carbon in unmanaged and switch grass fields (except for urease) reflect the microbial origin of the enzymes (Nayak et al., 2007; Wallenius et al., 2010). Positive correlations between microbial biomass carbon and enzymatic activities confirm the extreme importance of soil organic carbon for soil enzymatic activity maintenance because soil organic carbon is crucial for microbial growth (Bowles et al., 2014; Raiesi and Beheshti, 2015). Raiesi and Beheshti (2015) suggest that specific enzymatic activity may influence soil organic matter losses, due to the negative correlations found between specific enzymatic activity and soil organic carbon and microbial biomass carbon contents. We found the negative correlation between the urease activity and microbial biomass carbon strengthen the possibility that this enzyme is produced through microbial growth stimulation by carbon supply and consequent nitrogen limitation, as suggested by Bowles et al. (2014). The greater correlations between enzyme activity and organic carbon were consistent with previously published results (Myers et al., 2001; Kremer and Li, 2003; Mungai et al., 2005; Udawatta et al., 2008). Increased enzyme activity was because of increased organic matter and litter quality and quantity and improvement of the physical properties of the soil. Increased enzyme activity is proportionally linked to microbial function, leading to improved nutrient cycling and availability, which favors root growth, promotes beneficial plant-microbial interactions, and eventually increases the total carbon pool of soil (Udawatta et al., 2009).

Conclusion

In this study, effect of land management on soil enzymes and their relationship with microbial biomass carbon and respiration was evaluated in three different soils. It was observed that land utilization by planting switchgrass resulted in the significant reduction of soil enzymes except the urease when compared with natural unmanaged soils. A strong correlation between the tested soil enzymes and microbial biomass carbon was observed under managed and unmanaged soils. However, a negative correlation between urease activity and microbial biomass carbon was observed. A weak to negative correlation between tested enzymes and microbial respiration was observed. Further, higher enzymes activities were observed in surface soil layer (0-10 cm) as compared to deeper layers (10-20 cm, 20-30 cm).

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CHAPTER V

Factors affecting β-cellobiosidase activity in soils

V.1. Effect of Soil pH on Stability of Cellulose 1,4 - β- cellobiosidase (β-cellobiosidase)

(Short communication)

Factors such as temperature, availability of nutrients, pH, moisture, etc. can pose direct effects on extracellular enzyme activities (Petersen and Anderson 2005; Sinsabaugh et al., 2008; Baldrian et al., 2012). Soil pH plays a key role in affecting nutrients availability, controlling the variety and structure of microbial communities, affecting plant responses, and modifying solid-phase equilibrium. Furthermore, soil pH affects enzyme activity because amino acids, acting as functional groups, are sensitive to pH. It can bring chemical and structural modifications in amino acids indispensable for binding as well as catalysis. The pH can influence the concentration of activators and inhibitors in soil solution and optimum substrate concentration that ultimately can affect the activity of an enzyme (Tabatabai, 1994). Previously, Renella et al. (2006) found that soil pH controlled the activity of soil enzymes exhibiting various optimum pH. A large-scale analysis at the global level using data of >40 ecosystems revealed that all measured activities of soil enzymes are associated with the pH of the soil (Sinsabaugh et al., 2008).

Instead of focusing on the use of chemical approaches to assess fertility and other qualities of soil, biological approaches are being requested to determine the various soil processes and sustainability (Dick, 1994). Maintenance of productivity in cropping systems depends on natural biological processes. To assess key functions of soil, preference may be given to biological approaches over chemical approaches. It should be possible to determine the beneficial soil pH depending on soil enzymes sensitivity (Wittmann et al. 2004; Nannipieri et al. 2012). The research on soil enzymes mainly focused on the development of methodologies for their measurement and provision of knowledge about the origin of soil enzymes and factors affecting their activities in soil (Dick and Tabatabai, 1992; Ruggiero et al., 1996; Gianfreda and Bollag, 1996).

It is crucial to determine the activity of enzyme as well as its regulating factors to describe the soil quality and fertility, metabolic potential and to assess the soil fungi and bacteria which have further applications in studying biochemical processes and evaluation of soil quality (Dick 1992, 1994, 1997; Trasar-Cepeda et al. 1998; Acosta-Martinez et al. 1999; Olszowska 2009, 2016). Comprehension of enzyme activities along with information on other properties of soil help in choosing the suitable soil use methods (Shaw and Burns, 2003). As mentioned by Koper and Piotrowska (1999), variation in the activity of enzymes depends on the system of soil used.

In the past, efforts have been made to assess the impact of pH on the stability of different soil enzymes (Frankenberger and Johanson, 1982). However, the impact of pH on the stability of β-1,4-cellobiosidase in different soils has not been studied so far. Therefore, the present study was designed to determine the relationships between β-1,4-cellobiosidase activities, and pH in different soils.

The six soils were used (Table 5.1) in the present study. These surface soils (0-to 10-cm) were collected from different fields having Native prairie, Prairie und, Wheat, Corn, Pasture, and Wheat vegetation at different locations of Oklahoma. The pH and organic carbon of the soils ranged 4.7 to 7.7 and 0.7 to 4.4%, respectively. The three replicates of samples (two assays and one control) for each soil were taken by weighing 0.1 g of oven-dried soil into each 2 mL Eppendorf tube. The soil samples were mixed with 0.1 mL of 2.5X modified universal buffer (MUB) at the desired pH (1-13) and the tubes were placed for 24 hours at laboratory temperature. After 24 hours, the buffer solutions were kept allowing their evaporation until the soil moisture contents were about 60% of their water-holding capacity. The soil samples were then treated with 0.4 mL of MUB equivalent to the pH optimum of β-cellobiosidase (pH 5.5)

The pH at each step was recorded before investigations to ensure that soil solutions were kept at the desired pH. Then, 0.1 mL of 12.5 mM 4-Nitrophenyl-β-D-cellobioside was added to two assay tubes. Each tube was vortexed few seconds. The tubes were then inserted in a water bath (37˚C) in a trackable order to stop the reaction after equal time interval in all the tubes. After one hour of incubation, 0.4 mL of tris (hydroxymethyl) aminomethane (THAM; pH 12) was added to terminate the reaction and tubes were vortexed immediately to mix. Then, 250 µl of clear supernatant was taken into microplate wells and the yellow intensity of the solution at 405 nm with a microplate reader was measured.

Table 5.1. Characteristics of soils used

Soil							
Series	Subgroup	Vegetation	pH[†]	Organic C[‡]	Sand	Silt	Clay
					----------- % ------------		
Cordell	Thermic Lithic Haplustepts	Pasture	7.3	19	32.0	49.0	19.0
Kirkland 1	Fine, mixed, thermic Udertic Paleustolls	Native prairie (unmanaged)	6.1	44	7.5	62.5	30.0
Kirkland 2	Fine, mixed, thermic Udertic Paleustolls	Prairie CRP (managed)	4.7	28	12.5	55.0	32.5
Norge	Thermic Udic Paleustolls	Wheat (managed)	4.8	15	42.5	40.0	17.5
Richfield	mesic Aridic Argiustolls	Corn (managed)	7.7	11	30.0	42.5	27.5
Teller	Thermic Udic Argiustolls	Wheat (managed)	5.9	7	55.0	32.5	12.5

[†]Soil: Water ratio = 1:2; [‡] C, carbon.

Data regarding the impact of pH on β-cellobiosidase stability in six different soils (Kirkland-1, Kirkland-2, Teller, Richfield, Cordell, and Norge) is depicted in figure 5.1. The pH stability study reveals that β-cellobiosidase remained active over a wide range of pH (2-11). Results revealed the maximum activity of the enzyme in all the soil at pH 5. However, a progressive decrease in the enzyme activity was observed above and below this pH. The pH stability of soil β-cellobiosidase changed with the soil being examined. The soils Kirkland-1 and Kirkland-2 showed the highest enzyme activity at all pH levels when compared with enzyme activity in other soils at respective pH. The enzyme activity in Kirkland-1 at pH 5 was 198 µmol kg^{-1} h^{-1} in terms of p-nitrophenol released which was 1, 106, 110, 117, and 178% more when compared with the enzyme activity of Kirkland-2, Norge, Richfield, Teller, and Cordell soils, respectively, followed by Kirland-2 whose enzyme activity was 196.6 in terms of p-nitrophenol released which was 104, 108, 115 and 175% more as compared to the enzyme activity of Norge, Richfield, Teller, and Cordell, respectively.

In Kirkland-1 and Kirkland-2 soils, decrease in the enzyme activity was less between pH values 4-9 and the impacts of pH on β-cellobiosidase stability indicated gradual irreversible denaturation at pH values <3 and >10. β-cellobiosidase was active during pH range of 4-6 in teller soil, 3-9 in Richfield, 2-9 in Cordell, and 3-10 in Norge soil.

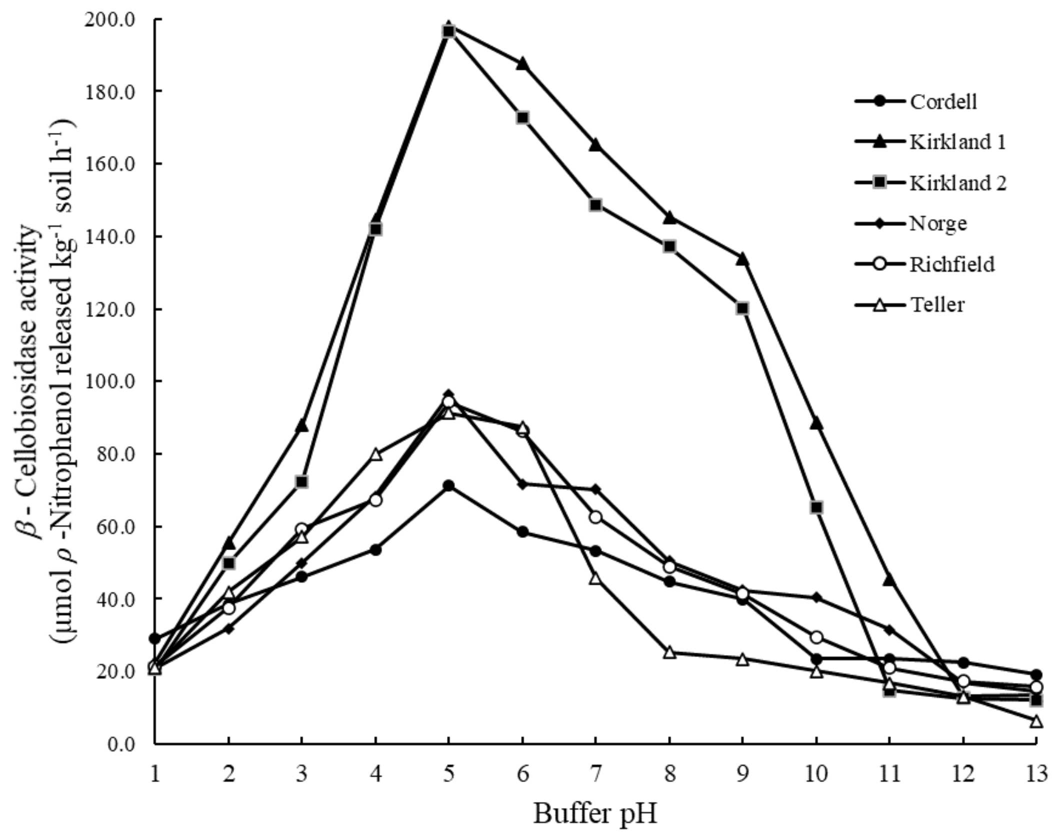

Figure 5.1. pH stability of soil *β*-cellobiosidase

In the study, maximum enzymatic activity was observed at the optimum pH of cellobiosidase in six different soils while the high acidic and basic conditions caused a considerable reduction in pH stability of the enzyme. Similar results were presented in several studies that show the reduction in pH stability of different soil enzymes with an increase in acidic and basic pH of the soils (Singh and Nye, 1984; Frankenberger and Johanson, 1982; Kishore et al., 2012). Bisswanger (2014) described that the pH governs the enzymes activity in the assay mixture. In present study, the curve for the activity of many enzymes bell-shaped, showing a rise from zero to maximum in the region of strong acid and again decline to zero in the strong alkaline region. Two major factors controlling this behavior are (I) the three-dimensional structure of an enzyme, and (II) the protonation state of functional groups of amino acids and cofactors engaged in catalysis. Structural damage of protein is irreversible process while protonation is reversible. When one functional group undergoes protonation to promote the catalytic reaction, the protonation of another important group breaks it down. Consequently, bell-shaped curve is formed by two titration curves (a decreasing and an increasing one).

The observed optimum pH for cellobiosidase is 5-6. Since the optimum pH is a value at the maxima of the pH-activity curve, it is generally selected as a standard pH for enzyme's assay. Moreover, the activity of enzymes is maximum (V_{max}) at this value of pH. Bisswanger (2014) described that the optimal pH of numerous enzymes fell within the physiological range (about pH 7.5) not exactly at this pH, but often fall within pH 7-8. On contrary, the optimal pH of few enzymes deviates from the common physiological range. For example, pH optima for pepsin, acid phosphatase, alkaline phosphatase is 2, 5.7, and 10.5, respectively. Naturally, an enzyme is relatively stable at its optimum pH, therefore,

optimum pH is suggested for testing, storage performance of enzyme assays. It is noticeable that adding an aliquot from a stock solution of the enzyme to the assay mixture will not alter the pH of the assay. Though enzymes exhibit stability within their optimum pH range, very high pH (either acidic or basic) results in irreversible damage to the tertiary structure of the enzyme. It is a time- and effective pH-dependent process, the more the deviation from pH optima the higher the inactivation rate. At strong basic (>11) and acidic (<3) pH, inactivation occurs nearly at once.

Various factors such as temperature, chemical properties of buffer, ionic strength, contamination of metallic ions, the concentration of cofactors, enzymes, and substrates, influence the pH stability of enzymes (Bisswanger, 2014; Papaneophytou et al., 2014; Segel, 1975). In the present study, an assessment of soil enzymes was performed under standard conditions to keep these factors constant. The activity of enzymes was changed with alterations in the concentration of hydrogen ion due to the irreversible denaturation of the enzyme and reversible reaction of deionizing and ionizing prototropic groups occurring at the active center of enzyme-protein. When the medium was buffered back to pHopt of the enzyme, restoration of biological activity and native structure was observed. As a result, stability in activity was recorded near cellobiosidase's pH_{opt}. However, extremely acidic, and alkaline environments tend to decrease the catalytic potential of soil enzymes due to the effects posed by pH on the globular structure of the protein. Disorder in the conformation of peptide chains of three-dimensional proteins results in denaturation. The secondary arrangement in proteins including ionic, hydrogen, and hydrophobic bonding is an important factor that affects the structure of the native protein. High concentration of H-ions (pH 1-2) and OH-ions (pH 12-14) disturb the hydrogen and ionic bonds required to

ensure the effective configuration of enzyme-protein. Some significant alterations in denatured enzyme-proteins are loss of biological activity, high levo-rotation, increased vulnerability to the action of proteolytic enzymes, decrease in solubility at isoelectric point (PI) of the enzyme, increased reactivity of chemical groups (e.g., S-S, -SH, phenolic hydroxyl groups of tyrosine), and increased asymmetry (White et al., 1968; Bhagavan, 1978).

In soils, the changes in the stability of enzymes to pH can be attributed to various other factors that promote the activity of enzymes and the adsorption properties of soils. Among soils, variation in pH stability of enzyme activities might be due to diverse vegetation, soil fauna, and microorganisms acting as suppliers of different enzymes. The humus and clay contain a negative charge and adsorb the positive charge bearing functional groups below the isoelectric point (PI) of protein (Zaborsky. 1973). At a point where pH is higher than PI of protein, ionization of carboxyl and amino groups would increase and decrease, respectively. Below the PI of protein, the amino group would have a positive charge thus showing more attraction to negatively charged micelles. The cations can also affect the adsorption of enzymes by neutralizing repulsive electrostatic potential of negative charge containing proteins and soil colloids which could lead to the formation of a humus-or clay-cation-protein bridge. In this bridge, cation act as a linker between two negatively charged particles. The reason for electrostatic attraction between adsorbed cations trapped by micelles in the soil sites for cation exchange and proteins is reactive groups (such as sulfhydryl, phenolic OH- and carboxyl groups) of enzyme-protein.

The key characteristics of soil that significantly influence the enzyme adsorption to soil colloids are CEC, pH, contents of organic matter, and surface area. These

characteristics of soil used in the present study vary greatly that may lead to a change in pH stability of immobilized enzymes. Above the PI of the enzyme, decreased enzymatic activity might be due to proteins desorbed from soil colloids (McLaren and Estermann, 1957).

Clay mineralogy and the occurrence of organic matter are two factors that controlling the availability of negative charges on micelles needed for the adsorption of enzyme-proteins. Comparatively, organic matter and clay have a high surface area and more active sites than sand and silt, thus by increasing the proportion of organic matter and clay, adsorption can be increased. Burns (1978) conducted a study to review the possible immobilization mechanisms for enzymes of soil including physical entrapment within organo-colloids, chemical incorporation into humic polymers, and lipophilic association with organic matter. Adhesion between enzyme-protein and clay-humic fractions may cause resistance to pH denaturation while most of the ionizable groups would not undergo hydroxylation and protonation. As described by McLaren (1960), if entrapping of enzyme occurs in soil particles, the possibility of ionization of reactive groups at the active center will depend on the position of the -COOH to -NH: groups. Furthermore, -NH; groups would repel the H + ions which subsequently would change the pK, value of -COOH and affect the pH stability of the entrapped enzyme.

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V.2. Effect of storage temperature and time on β-cellobiosidase (EC 3.2.1.91) activities in soils

Abstract

Analysis of freshly taken field soil samples is the most reliable method of determining soil enzyme activity. However, because of the long handling time involved, so this method becomes nearly impossible especially for many soil samples. Soil samples are, therefore, often stored prior to analysis, either frozen or air-dried. Unfortunately, both drying and freezing can substantially change enzyme activity depending on soil type and enzyme. In this study, the effect of storage temperature and duration of soil samples of β-cellobiosidase activity was investigated for soils of three long-term (9 years) switchgrass experiments located in central Oklahoma. Soil samples were taken from moist and air-dry field conditions and were stored at three different temperatures: at room temperature ~23ºC, 0ºC, and -18ºC. The activity of β-cellobiosidase was measured on the 0, 30, 60, 90,120, 150 and 180 days after storage at the defined temperatures. Results showed that the storage of the soil samples resulted in significant alteration in the β-cellobiosidase activity which can be attributed to the soil type, period, and storage conditions of the soil samples. Moist soils showed higher enzymatic activities at all the storage temperature and duration when compared with enzymatic activities of the dry soils. However, most of the soil showed nonlinear changes in enzyme activities under different temperature and duration. Maximum changes were observed over the initial duration of 90 days. Then, a very slow decline in β-cellobiosidase activity was detected. The reduction of enzyme activity by air-drying process ranged from -4.04% to -22.42%, averaging 13.23%. Enzyme activity increased by average 4.6% when soils were stored at room temperature for 30 days; but decreased by average 3.26% and 8.03% when soils were stored 30 days at 0 and -18ºC, respectively. While enzyme activity increased by average 11.79% when soils were stored at room temperature for 60 days; but decreased by average 12.24% and 14.73% when soils were stored 60 days at 0 and -18ºC, respectively. After long-term (>90 days) storage at varying

temperatures, the variation in soil β-cellobiosidase activity was less prominent. Preservation of air-dried samples at room temperature may be recommended for mass investigations.

Introduction

Enzymes act as catalyst in all types of biochemical reactions, and they play a central role in nutrients cycle in soil. Soil enzymes not only originate from microbes (Ladd, 1978) but also derive from animals and plants. In addition to their association with actively growing cells, they can be released by living and dead cells (Tabatabai, 1994). The freely available soil enzymes make complex with the humic colloids and may stabilize on organic matter or surface of clay (Boyd and Mortland, 1990). Thus, enzymes play an integral role in several phenomenon of ecosystem such as cycling of nutrients, decomposition of organic matter, carbon sequestration, etc. (Sinsabaugh et al., 2008). Insights in activities of soil enzymes has helped to understand the impacts of different management practices on the functions of natural ecosystem (Saiya-Cork and Sinsabaugh, 2002; Sinsabaugh et al., 2008; Wallenstein et al., 2009; Weedon et al., 2011) and agricultural system's productivity (Bolton et al., 1985; Dick et al., 1988; Bandick and Dick, 1999).

Mostly, in-situ assessment of soil enzymes activity is advantageous. However, metabolic, proteomic, and genomic technologies are not same as accessible to ecologists and agronomists (Wallenstein and Weintraub, 2008). Freshly collected samples can be used for assessment of enzyme activity when in-situ measurements are unfeasible (DeForest, 2009; Wallenius et al., 2010). Analyses of enzymes require considerable efforts, collection of soil samples and their further processing as well as measurement of enzyme activities at specific time depending upon formation of fluorescent or colored products followed by substrate addition (Eivazi and Tabatabai, 1977; Saiya-Cork and Sinsabaugh, 2002). It is

challenging to analyze the bulk of fresh samples in precise time which may limit the number of processed samples and subsequently possible comparisons. As a result, there are different proposed methods for storage of samples before their analysis (Lee et al., 2007; DeForest, 2009). Generally, storage of soil samples using conventional methods results in considerable changes in the activity of enzyme. Based on soil as well as enzyme, drying at room temperature and freezing at -20ºC can bring significant changes in activity (Wallenius et al., 2010; Abellan et al., 2011). In recent years, sensitivity in various ecosystems has been widely studied (Koch et al., 2007; Wallenstein et al., 2012; Stone et al., 2012). Stone et al. (2012) found that the increase in temperature resulted in decreased sensitivity of soil enzyme activity to temperature.

Various projects are primarily more focused on understanding the associations between types of vegetation, time of sampling, experimental treatments than on determination of absolute activity of enzyme. Alterations in enzyme activities due to storage of soil samples should be under consideration. Usually, laboratory experiments of very fresh collected samples cannot be performed instantly, so the questions such as "under what conditions should soil samples be stored and how long?" arise when we are planning the experiments. This problem often occurs when many samples required for mass investigations are kept at different places. Repetition of analyses to confirm the initial data may also require. Choosing a storage condition is mainly associated with climatic conditions at different places. Therefore, storage of soi samples at -20°C for one year is observed in some countries (Lorenz et al., 2006). Soil samples can be stored at 12°C in Netherlands as this value is close to mean annual temperature of Netherlands' soils (Lorenz et al., 2006).

The storage temperature for soil samples in Denmark, Switzerland, Great Britain, Germany is 4°C whereas storage of airdried soil samples is practiced in Italy (Lorenz et al., 2006). It is generally recommended to store the samples at 2-4°C particularly when determination of biological parameters is main concern (Ross, 1970; Ross et al., 1980; Dick, 1997; Dick et al., 1997; Bandick and Dick, 1999; Khaziev, 2005; Lorenz et al., 2006, Bloem et al., 2006; Pancholy and Rice, 2006) .It is evident that some biological characteristics of soil such as respiration, enzyme activities and microbial biomass, do not change throughout initial period of storage. for determination of biological parameters, various researchers reported different suitable storage intervals for soil samples: 7 days (Trabue et al., 2006), 28 days (Dick et al., 1996), 3 months (Stenberg et al., 1996), and several years (Galstyan, 1978; Galstyan, 1982). However, temperature and storage duration are highly unpredictable and fluctuate from few days to many weeks and between 4°C to 37°C (Galstyan, 1978; Galstyan, 1982; Joergensen et al., 1990). Conditions for storage are often not well-defined and do not consider the properties of soil samples.

The storage temperature (e.g., freezing vs refrigeration) and duration between soil collection and assays for enzymes can differ significantly among studies, so different results can be described. Many publications did not describe the exact procedures, thus making the comparisons of results more difficult (Marx et al., 2001; Saiya-Cork et al., 2002). According to past studies, refrigeration is best option when storing the samples for very shorter period (Ross, 1965; Kandeler and Gerber, 1988; Lee et al., 2007). But these studies employed colorimetric methods for determination of enzymes and performed occasional measurement of freezing storage period at short intervals.

The main objective of the present study was to investigate the effects of temperature and storage duration on β-cellobiosidase activity in different soils. Additionally, air drying not only helps in processing of soil samples but may also urge the idea to adopt activities of soil enzymes as important component of soil quality index. Hence, interest lies in determination of consistency in enzyme activity between field-moist and air-dried soil samples.

Materials and Methods

The soils used in the experiment were taken from three long-term (9 years) switchgrass experiments located in central Oklahoma. Soils from an adjacent area with mixed native vegetation were used to serve as controls. The first site is Efaw (Easper loam and Pulaski fine sandy loam mix, 36°7’52.64” N, 97°6’16.75” W). The second site is Cow Creek (Easper loam, 36°7’2.77” N, 97°5’52.06” W). The third one is 40 North (Huska Silt Loam, 36°8’21.48” N, 97°4’44.4” W). These sites have received nitrogen at 75 kg N per year.

Soil samples were taken from the surface soil 0-10 cm. Soil texture was determined using composite samples by the hydrometer method (Gee and Or, 2002). In dry combustion analysis, total carbon was found by placing soil samples wrapped in foil into a Leco TruSpec combustion analyzer (Nelson and Sommers, 1996). The pH, and organic carbon of the soils ranged 5.2-6.4, 10.4-33.1 g kg^{-1} respectively. Air-drying, crushing sieving (using 2 mm sieve) of soil samples was performed. The physicochemical characteristics of the soils (Table 5.2.).

A total of 36 samples under field-moist (18 samples) and air-dry (18 samples) conditions were evaluated for the *β*-cellobiosidase activity. To investigate the effects of storage temperature and time on *β*-cellobiosidase activity, these experimental variants were tested: (1) airdried samples stored at room temperature~23ºC, (2) airdried samples stored at 0ºC, (3) airdried samples stored at low negative (in a freezer, -18ºC) temperature, (4) field-moist samples stored at room temperature~23ºC, (5) field-moist samples stored at 0ºC, (6) field-moist samples stored at low negative (in a freezer, -18ºC) temperature. *β*-cellobiosidase activities were performed on the 0, 30, 60, 90,120, 150 and 180 days after storage at the defined temperatures by following the procedure described below.

Three replicate assays (two sample assays and one control) were prepared by weighing 0.1 g of soil (dry weight equivalent) into each 1.5-mL Eppendorf tube. Then, the soil samples were treated with 0.4 mL of MUB equivalent to the optimum pH of *β*-cellobiosidase (5.5) for assay the activity. The pH at each step was measured before investigations to ensure soil solutions were kept at the desired pH in the soil solution. Then, 0.1 mL of 12.5 mM 4-Nitrophenyl–*β*–D-cellobioside was added to two assay tubes and vortexed each tube for a few second before placing the tubes in a water bath at 37˚C in a trackable order to stop the reaction after equal time interval in all the tubes. After one hour of incubation, 0.4 mL of tris (hydroxymethyl) amino methane (THAM; pH 12) was used to terminate the reaction and tubes were vortexed immediately to mix.

For control tubes, 0.1 mL of 12.5 mM 4- Nitrophenyl-*β*-D-cellobioside was added and vortexed for few seconds. Then, 0.1 mL of 0.5 M CaCl2 was added to each tube and vortexed a few seconds. Each tube was centrifuged at 12,000 rpm for 10 minutes and 250

μl of clear supernatant was taken into microplate wells and the yellow intensity of the solution at 405 nm with a microplate reader was measured. The contents of p-nitrophenol in the solution were calculated by reference to a calibration curve developed with p-nitrophenol standards at concentrations of 0, 0.1, 0.2, 0.4, 0.6, 1, 1.5, and 2 mM. The reported values were averages of duplicate determinations and articulated on a moisture-free basis. Moisture was calculated from weight lost after drying for 48 h at 105°C.

Table 5.2. Physicochemical characteristics of sol used in the experiment.

Soil							
Location	Series Subgroup	Vegetation	pH†	Organic C‡	Sand	Silt	Clay
				---- g kg^{-1} ---	-------------- % ------------		
Cow Creek	Easpur/Fluventic Halustolls	Bermuda grass	6.4	14.9	47.5	38.7	13.8
Cow Creek		Switchgrass	6.2	10.4			
Efaw	Easpur/Fluventic Halustolls	Bermuda grass	6.0	33.1	26.3	52.4	21.3
Efaw		Switchgrass	6.0	22.0			
40 North	Huska/Mollic Notrustalfs	Indian grass	5.2	26.9	36.3	41.2	22.5
40 North		Switchgrass	6.3	18.3			

†Soil: Water ratio = 1:2; ‡ C, carbon.

Results

Before storage, all the field-moist and air-dried fresh samples were assayed for enzymatic activity which was different for different soils. However, higher enzymatic activity was observed in moist-field soil samples as compared to the air-dried soil figure (5.2). Moreover, under a moist state, soil samples taken from switch grass field showed lower enzymatic activity as compared to the control soil samples while the air-dried soil samples taken from switch grass field showed lower enzymatic activity as compared to the control soil samples. The changes in the β-cellobiosidase activity during storing of the samples had differin patterns for the different soils. It was observed that the β-cellobiosidase activity in field-moist and air-dried samples of all analyzed soils were higher at ~23ºC than lower temperatures figure (5.2.1).

The data showed the variability and unpredictable pattern of the changes in the β-cellobiosidase activity upon storing the moist samples of Cowcreek soil figure (5.2.1, a-d). The activity of enzyme was gradually decreasing (but still lower than respective control) during the storing of the samples for 30-90 days at different temperatures. The decrease in the β-cellobiosidase activity as compared to respective control was observed when moist samples were stored for 90-180 days at low negative (in a freezer, -18ºC) temperature. In Cowcreek sample, a significant increase of 1.98 % compared to initial value was recorded in β-cellobiosidase activity after 30 days at room temperature~23ºC. After 90-180 days of sample storage at low negative temperature, β-cellobiosidase activity was decreased to 39.83-43.68 % of the value recorded after 30 days of sample storage at the same temperature. However, moist Cowcreek soil exhibited lowest activity of enzyme as compared to moist samples of other soils.

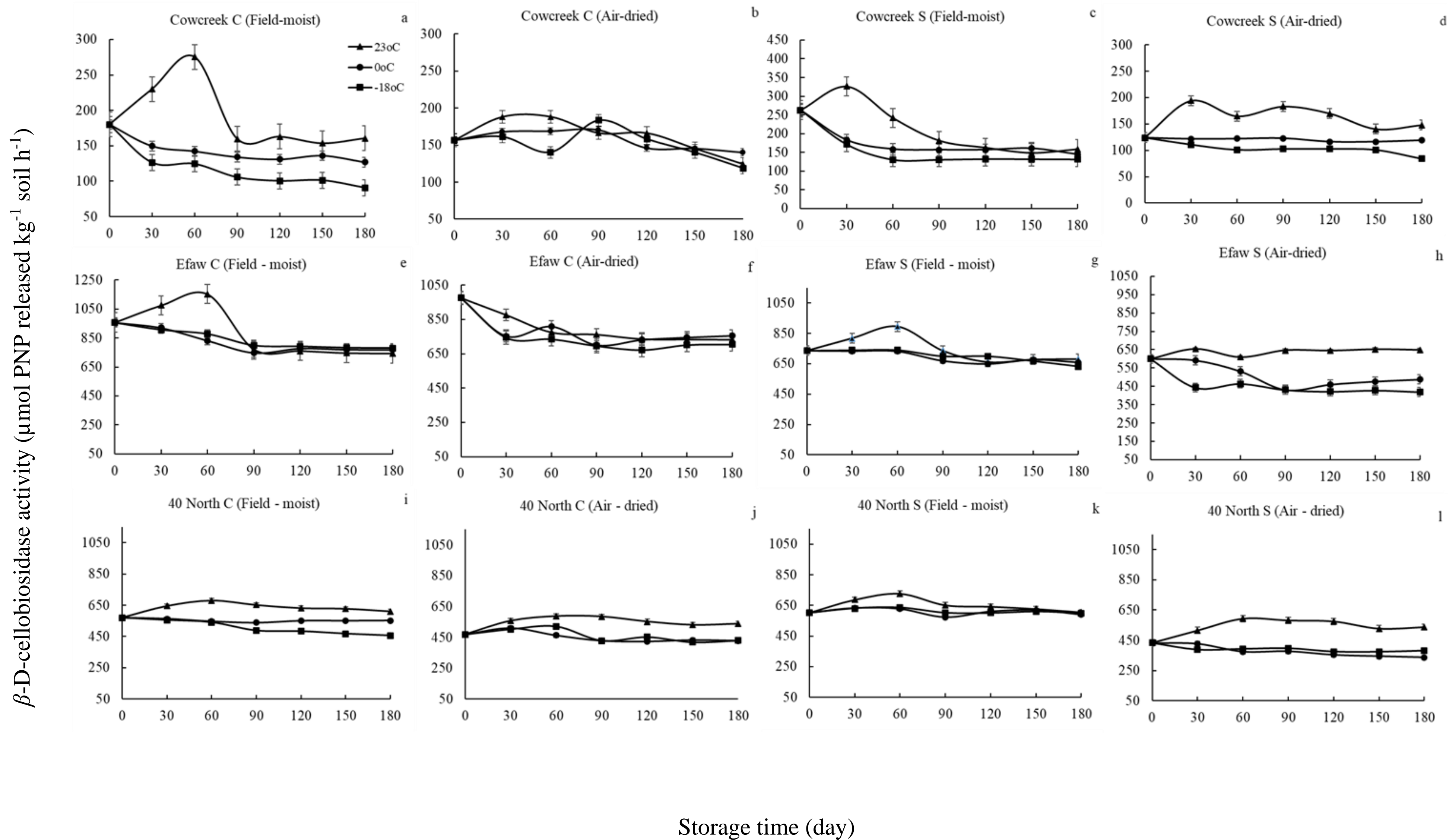

Figure 5.2. Effect of storage temperature and time on β-cellobiosidase (EC 3.2.1.91) activities in soils

In moist samples of Efaw soil, the β-cellobiosidase activity was lower as compared to respective control at all temperatures figure (5.2.1, e-h). After 30 days, maximum activity of β--cellobiosidase in moist control and Efaw sample (0.83 and 6.3% higher than their initial period of storing, respectively) was recorded at room temperature. After 60 days of storing, the activity was increased to 21.23 % of the value recorded after 30 days when temperature was ~23ºC but decreased by 12.34-14.73 % at 0ºC and -18ºC as compared to the value recorded at ~23ºC. After 90 days, the β--cellobiosidase activity was decreased to 6.48 - 21.26 % of the values recorded after 60 days of storage.

In field-moist 40 North soil, the β--cellobiosidase activity was higher than Cowcreek soil as compared to respective control at different temperature and storing time figure (5.2.1, i-l). During 30 days of storage, the activity of enzyme gradually decreased as the temperature decreased but still higher than the initial values recorded. Maximum activity of moist soil samples was observed upon storing them for up to 60 days at room temperature~23ºC which was 19.47% higher than the initial value recorded at same temperature.

The maximum β-cellobiosidase activity was observed in air-dried control upon storing it for 90 days at low negative (in a freezer, -18ºC) temperature and it was 50.36 % lower than the air-dried sample stored at same temperature. In Cowcreek samples, activity was increased to 32.28% of the initial value at ~23ºC after 60 days of storage. After 120 days, a reduction of 33% was recorded in β-cellobiosidase activity when temperature was

low negative (in a freezer, -18ºC) temperature, after 180 days, it reduced to 39 % of the initial value.

Like moist samples of Efaw, its air-dried samples also exhibited low β-cellobiosidase activity as compared to respective control upon storing for different time periods. It was observed that samples showed high β-cellobiosidase activity at room temperature during different storage period as compared to other temperatures and maximum activity (5% higher than the initial value) was recorded when sample was stored for 150 days. After 30 days, 4% increase in β-cellobiosidase activity when sample was stored at ~23ºC as compared to initial value at same temperature. But the activity then decreased at 0 and -18ºC as compared to room temperature. Similar trend was observed upon storing the sample for 60 days.

In air-dried 40 North, unlike the moist samples, overall β-cellobiosidase activity was low as compared to respective control at different temperature and storing time. Air-dried 40 North showed lower β-cellobiosidase activity at room temperature during different storage period as compared to other temperatures as observed in air-dried Efaw soil. At however, maximum activity in soil samples was observed at ~23ºC when sample was stored for 60 days, and it was 32.78 % higher than the initial value at the same temperature. After 60 days, β-cellobiosidase activity was decreased by 12.34 % at 0ºC, as compared to the value recorded after 30 days at the same temperature.

Discussion

Even with the advancements in the methodology in recent years, (Lee et al., 2007; DeForest, 2009), clear standard methods have not been developed for handling of soils before they undergo analysis of enzymes. Most of the studies require storage of sampled soils because they involve collection of a large number of samples that cannot be handled in one day, thus impeding the measurement of enzymatic activities in freshly samples soils. Though freezing and air-drying are commonly used storage methods before the sampled soils undergo enzyme assay. However, researchers reported that these storage methods can alter the entire activity of enzymes (Lee et al., 2007; DeForest, 2009; Wallenius et al., 2010; Abellan et al., 2011). In current study, it was found that storing the soil samples either in moist or dry form significantly changed the absolute activities of enzymes as compared to freshly sampled soils.

Any change in absolute enzymatic activity because of storage is negligible in other studies in which comparisons of treatment are main concern if storage pose similar effects on the treatments. Sometimes, researchers hypothesize that storage effects all treatment in the same way. Previously, only few studies investigated the changes in the treatments in relation to storage. keeping in view the β-cellobiosidase activity, our study observed that storage caused significant changes in association among two soils when storing the samples in both air-dry and field-moist state. Similarly, DeForest (2009) and Lee et al. (2007) reported that storage act in a different way on different soils.

The enzymes are adhered to organo-mineral complexes present in soil (Busto and Perez-Mateos, 2000), resulting in highly stabilized form of enzymes (Perez-Mateos et al., 1991). Therefore, response variation in soils to drying and freezing vary depending on organo-mineral complexes and their concentration. In addition, different practices to manage the soil, for example tillage (Monreal and Bergstrom, 2000), also control the number of organo-mineral complexes and stability of enzymes in stored soil. In our study, soil sampled from the switch grass experiments showed different enzymatic activity as compared to the soil with mixed vegetations. Moreover, soils with different organic matter content have different enzymatic activity both at air-dried and field-moist state upon storage.

In our work, it was observed that air-dried soil samples showed slight or non-significant alteration in β-cellobiosidase activity even after six months of storage. This has also been reported in previous studied that activity of enzymes has barely influenced by air-drying indicating that some soil enzymes show resistance to air-drying (Fenn et al., 1992; Bandick and Dick, 1999; Li and Sarah, 2003).

Some fractions of soil enzymes are linked with living and nonliving microorganisms as well as soil matrix. The rest of the soil enzymes are found in soil solution as free enzymes (Burns, 1982). The enzymes associated with soil colloids are immobilized and they are prone to denaturation to a very small extent (Garcia et al., 1993). These immobilized enzymes significantly affect the soil quality and play a key role in decomposition. It has been previously found that air-drying and then wetting the soil

followed by incubation resulted in reduced microbial biomass and carbon in sampled soils. It was also concluded that enzymatic activities in the tested soils were not highly relying on microbial biomass in a short period and enzymatic activities remained. As in our study, incubating the sampled soils for six month had little effects on β-cellobiosidase activity under air-dried state. It suggests that the enzymes have to be extracellular in nature released during cell lysis and held on humus and clay complexes (Burns, 1982; Nannipieri et al., 1983; Dick, 1994).

Moreover, Garcia et al. (2000) reported the prolonged half-life and protection of enzymes by the soil colloids in calcareous soils having well-stabilized organic matter. It is likely to diminish the activities of free enzymes utilizing airdried samples along with similar experimental conditions as provided to fresh samples. Free enzymes are more prone to the denaturation (Nannipieri et al., 1990). In present study, air-drying of the samples had insignificant effects on the activity of β-cellobiosidase, which supports the hypothesis which states that most of the activities of this specific enzyme observed in stabilized enzymes. Knight and Dick (2004) found that high enzymatic activity of β-glucosidase in Oregon soils is associated with extracellular stabilized enzymes. They also concluded that this extracellular enzyme is main cause of shifting in overall activity owing to soil management practices. Therefore, using airdried samples helps in assays due to their long-term stability. However, more investigation is required to confirm that these results are reliable and consistent.

In present study, storage and temperature significantly affected the activity of soil enzymes and these findings are in line with other studies (Mandal et al., 2007; Lin, 2010). In current study, the changes in ratio of enzyme activity in one soil to other were usually larger for moist samples and smaller for air-dried samples. So, air-drying seems to be a superior method of storage than freezing. But some authors also have shown freezing as better option than air drying (Peoples et al., 2010). Though, our results do not provide one best storage method that is suitable for all conditions. For accurate interpretation of effects of treatments on activity of soil enzymes, investigators should clearly understand the impacts of storage. Establishment of correction factors for treatments and their application before comparison can be useful in various cases.

Conclusions

The importance of sample storage for enzyme measurements is often underestimated and procedures are often not well documented. The best storage method depends strongly on the enzyme and the sampling procedure. The procedures of measuring enzymes can significantly influence the data we generate and the way we interpret them. Results from this study support the following conclusions: (1) Air-dried soils can be stored either at room temperature ~23ºC, 0ºC, and -18ºC without significant influence on enzyme activity, but the influence of temperature on enzyme activity was inconsistent. However, soil samples gave higher enzymes activities at room temperature as compared to the lower temperatures. (2) Although the enzyme activity was lower in air dried soil samples as

compared to field moist samples, however, air-dried soil samples were least affected in alteration in enzyme activity with time as compared to the moist-filed samples.

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V.3. Effect of trace elements on cellulose 1,4-β-cellobiosidase activity in soil

(Short communication)

Cellulose β-1,4-Cellobiosidase (EC 3.2.1.91) plays a significant role in cellulose degradation, which hydrolyzes cellulose by releasing cellobioses from the nonreducing end of the cellulose chains. Recently an assay method was developed to quantify its activity in soil (Alserae and Deng, unpublished). Understanding factors that affect the activity of this enzyme in the soil is important in efforts undertaken to strengthen the functional dynamics of its activity in maintaining soil health and productivity.

Trace elements could activate or inhibit the enzyme. Many trace elements are essential to the growth and health of biological systems at low levels but are toxic at high concentrations. In soil, concentrations of trace elements could be elevated resulting from natural deposition or anthrophonic activities (Huang and Shindob, 2000; Yang et al. 2006), such as those that originate from impurities in fertilizers, industrial wastes, and/or sewage sludge (Berrow and Webbe, 1972; Charter et al. 1993). Some trace elements such as Ag(I), Hg (II), and As (III) cause serious effects on soil productivity and function and negatively impact soil biochemical processes (de Mora et al. 2005; Effron et al. 2004; Lorenz et al. 2006; Malley et al. 2006). Studies have shown that the activities of many extracellular enzymes in soil could be inhibited by trace elements, such as β-cellobiosidase (Sinegani and Emtiazi, 2006), β -glucosidase (Effron et al. 2004; Geiger et al. 1998; Trasar-Cepeda et al. 2000; Wyszkowska et al. 2010), phosphatases (Effron et al. 2004), β -glucosaminidase (Ekenler and Tabatabai, 2002), and cellulase (Deng and Tabatabai, 1995;

Geiger et al., 1998; Sinegani and Emtiazi, 2006). Therefore, the objective of this study was to evaluate the effect of trace elements on the activity of β-cellobiosidase enzymes in soils.

Air-dried soils with particle size < 2 mm were selected with a range of pH (4.7 to 7.7), organic carbon content (7 to 44 g kg^{-1}), and texture classes (12.5 to 32.5% clay, 32.5 to 62.5% silt, and 7.5 to 55% sand). Additional description of these soils is shown in Alserae and Deng (unpublished). The trace elements used were Fisher-certified reagent-grade chemicals (Fisher Scientific Co., Chicago). Of these, Ag(I), Co(II), Cu(II), Fe(II), Zn(II), Al (III), and Mn(II) were added as the sulfate; Cu(I), Ba(II), Hg(II), Ni(II), Cr(III), Co (II) and Fe(III) as the chloride; Pb as the acetate; and As(III), As(V), B(III), Se(IV), and Mo(VI) as $NaAsO_2$, Na_2HAsO_4, $Na_2B_4O_7$, H_2SeO_3, and Na_2MoO_4, respectively.

To determine the effect of a trace element on β-cellobiosidase activity, 0.1 g of soil in a 2-mL Eppendorf tube was treated with 0.2 mL of a solution containing 2.5 µmol of trace element, which resulted in the trace element concentration of 25 µmol g^{-1} soil. Additional, trace element concentrations of 0, 2.5, 5, 15, and 25 µmol g-1 soil were performed to evaluate the effect of Mn, As, Se, and Mo on *β*-cellobiosidase activity in soils. Trace element solutions were added dropwise to moisten the soil samples. After 10 min of equilibration, the moist soil was used to determine the activity of *β*-cellobiosidase by using the method developed by Alserae and Deng (unpublished). The results of *β*-cellobiosidase activity from the soils treated with trace elements were compared with those obtained with 0.1 g of soil treated with 0.2 mL of water in place of the trace element solutions. The equation ((1-B/A) x 100) was used to calculate percentage inhibition of *β*-cellobiosidase

activity due to trace elements, where A and B is the β-cellobiosidase activity in untreated and trace element treated soils, respectively.

All 20 tested trace elements added at 25 μmol g-1 soil inhibited the activity of β-cellobiosidase in the six soils tested (Table 5.3). The degree of inhibition ranged from 4.2% to 42.1% and varied considerably depending on the Soil and trace element tested. Of the metals tested, Se (IV), As (V), Mn (II) were the least effective inhibitors of β-cellobiosidase activity, demonstrated < 16 % inhibition by following average addition of 25 μmol g^{-1} soil to soils. While the rest of the 17 trace elements showed average inhibition of 17.4-34.5% at 25 μmol g^{-1} soil. Between elements of two different oxidation states, Cu (II) was a more potent inhibitor than Cu(I); Fe (II) was stronger than Fe (III); and As (III) was a stronger inhibitor than As (V). The degree of inhibition to soil β-cellobiosidase activity by many of the trace elements studied are considerably lower than that those reported for inhibition of acid or alkaline phosphatases but higher than that reported inhibition of β-glucosidase at the same treatment concentration of 25 μmol g^{-1} soil (Eivazi and Tabatabai, 1990; Juma, and Tabatabai, 1977).

Trace elements inhibit the enzymatic reactions by complexing the substrate, reacting with the enzyme active sites, or reacting with the enzyme-substrate complex. It has been reported that some of the trace elements added to soils could react with soil organic matter and form mineral-organic complexes (D'Ascoli et al. 2006; Tejada et al. 2008). Consequently, the complexed trace elements lose the capacity to react with the enzyme active sites.

Therefore, the inhibitory capacity of trace elements varied among the tested soils. Purified β-cellobiosidase activity was severely inhibited by several divalent cations, including Co (II), Cu (II), Ni (II), and Mn (II) at 10 mM (Lim et al., 2016). The reductions were 40, 41, and 51% when purified β-cellobiosidase was incubated with 1 mM of Hg (II), Pb (II), and Fe (II), respectively (Haq et al., 2018). These findings are consistent with finding in this study in that most of the divalent metal ions were potent inhibitors of β-cellobiosidase activity. Therefore, trace elements in soil could impact the soil's capacity to cycle nutrients (Koper et al., 2005; Wyszkowska and Kucharski, 2003).

Table 5.3. Inhibition of *β*-cellobiosidase activity in soils by 25 μmol g^{-1}soil of trace elements†

Trace element	Oxidation state	Inhibition of β-cellobiosidase activity in soils specified (%)						
		Kirkland 1	Kirkland 2	Teller	Richfield	Cordell	Norge	Average
Ag	I	27.3	32.2	26.4	31.3	34.0	39.1	31.7
Cu		5.3	27.2	18.6	23.7	11.1	21.7	17.9
Ba	II	21.3	24.0	21.4	13.0	15.8	18.6	19.0
Cd		17.3	25.1	19.7	35.5	12.4	21.7	21.9
Co		14.0	18.3	39.6	30.7	20.6	35.5	26.5
Cu		14.1	27.0	33.3	28.0	26.9	22.1	25.2
Fe		33.2	20.8	35.4	39.4	13.6	31.9	29.1
Hg		36.5	28.4	36.5	42.1	18.7	30.1	32.0
Mn		9.1	18.5	10.7	18.2	12.3	26.1	15.8
Ni		27.9	28.9	13.9	19.9	16.2	22.1	21.5
Pb		16.8	13.2	15.6	19.5	18.4	24.0	17.9
Zn		30.0	20.4	14.5	11.9	24.8	21.7	20.6
Al	III	22.1	13.2	27.5	18.2	22.5	35.0	23.1
As		29.9	36.8	33.2	36.1	31.3	39.5	34.5
B		11.3	21.6	29.8	37.0	22.2	33.9	26.0
Cr		13.2	20.6	22.2	22.0	30.7	40.0	24.8
Fe		27.8	29.5	20.1	28.5	31.5	31.9	28.2
Se	IV	11.1	12.9	10.2	4.2	6.0	6.5	8.5
As	V	6.0	22.8	9.7	27.5	5.9	7.2	13.2
Mo	VI	18.7	21.6	25.6	13.0	6.2	19.5	17.4
L.S.D. ($P < 0.05$)		6.5	7.6	10.8	11.2	12.8	9.2	

†Activity of *β*-cellobiosidase at untreated soils (μmol pNP release kg^{-1} h^{-1}) were 319.2, 239.1, 86.2, 83.7, 71.3, and 101.1 in Kirkland 1, Kirkland 2, Teller, Richfield, Cordell, and Norge, respectively.

However, trace elements in agricultural soils did not always exhibit inhibitory effects on enzyme activities (Stuczynski et al., 2003). Dar (1996) reported that the addition of Cd at 10 μg g^{-1} soil did not result in significant changes in alkaline phosphatase activity; but the addition of Cd at 50 μg g^{-1} soil decreased its activity significantly. Concentration of the trace element is a key factor influencing the observed effect. In this study, a stimulatory effect of B(III), Mn (II), Se IV), and As (V) on *β*- cellobiosidase activity was observed following additions of low concentrations (2.5 and 5 μmol g^{-1}soil) of these metals. Of the four trace elements studied, Mn (II), and As (V) led to activation of *β*-cellobiosidase activity in soils by 46 and 31%, respectively, when their concentrations were added at 2.5 μmol g^{-1} soil (Figure 5.3).

While the activations resulting from the addition of Se (IV) and As (V) at 5 μmol g^{-1} soil were 4.8 and 34%, respectively. However, the As (V) activation was from 0.39 to 2.49 % when it was added at 15 μmol g^{-1} soil. The Mo ions did not show any activation to *β*-cellobiosidase under any of the four concentrations (Figure 5.3). These findings could be attributed to, that few sulfhydryl groups (S-H) of *β*-cellobiosidase reacted with the trace element ions when 2.5 and 5 μmol g^{-1} soil was used.

There was a marked decrease in the effectiveness of trace-element ions in inhibition of *β*-cellobiosidase activity when the concentrations of 0.5 and 5 μmol g^{-1} soil were compared with 15 or 25 μmol g^{-1} soil. These reductions were expected because smaller proportions of the S-H groups of *β*-cellobiosidase reacted with the trace element ions when

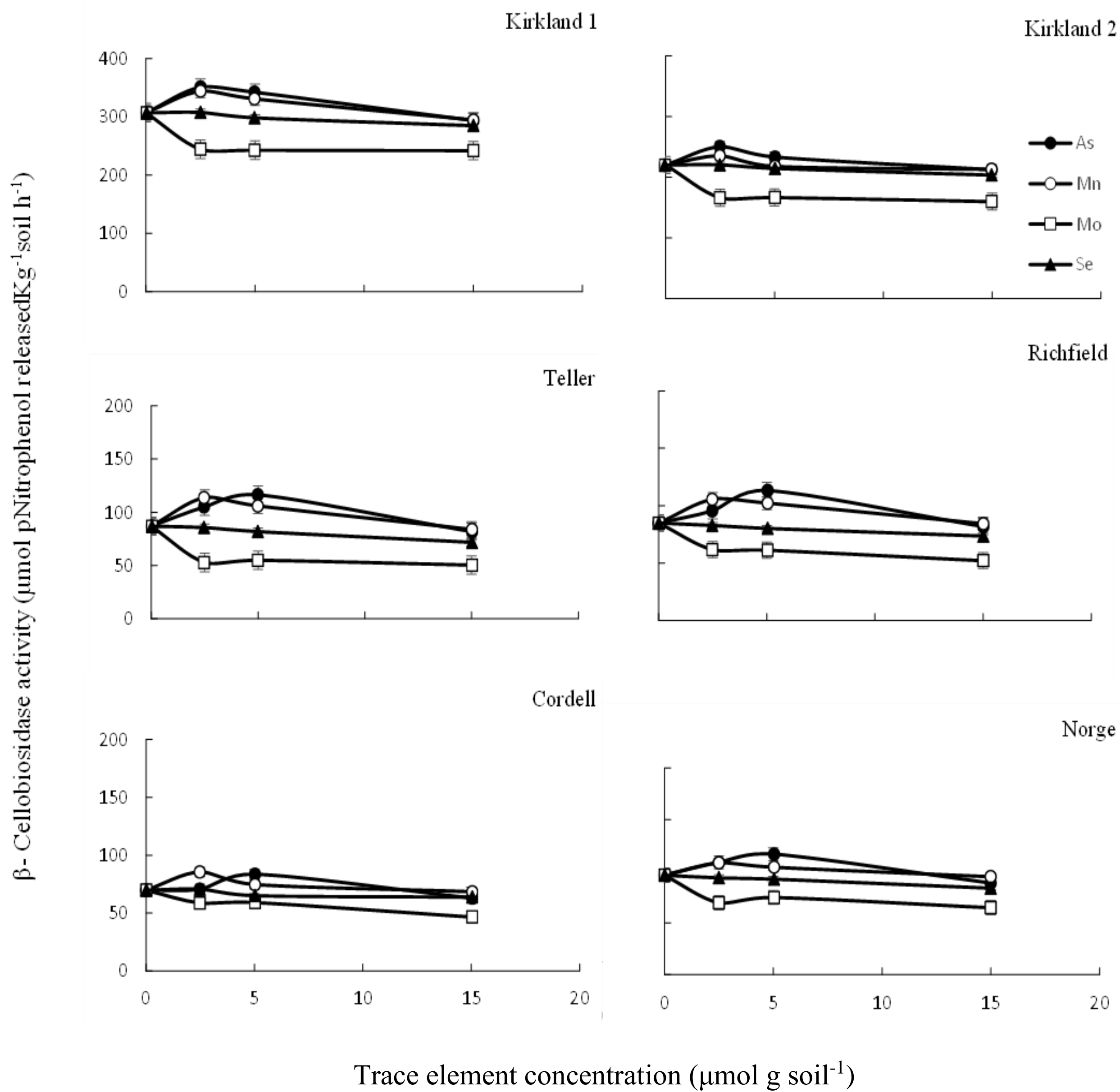

Figure 5.3. Effect of trace element concentrations on β-cellobiosidase activity in six soils.

0.5 or 5 μmol were used. This might be attributed to the concentration of the trace elements input or pollution, which is in line with the findings of Ekenler and Tabatabai (2002).

They observed that enzymes could be stimulated when the soil trace elements only slightly exceed natural values but can be inhibited by excessive heavy metal concentrations. Further, it has been found that the cellulase and *β*-glucosidase activities were slightly reduced at 1 mM concentration of copper compared to 600 μM (Geiger et al.1998).

Biological activities of the soils are a fundamental parameter of its ecological status. The potential toxic trace elements present in the soil due to anthropogenic activities could disturb soil biology. Further, chemical, and physical properties of the soil might be influenced by trace metals. The primary sources of these pollutants in the agricultural soils are impure chemical fertilizers and organic amendments such as animal manure and sewage sludge. However, such agricultural practices influence physical, chemical, and biochemical properties of arable soils. It is well documented that by increasing the concentration of the trace elements in the soil, the activity of most soil enzymes was significantly reduced, and it might be because of the interaction among the enzymes and the trace elements that did not reduce microbe's population. Potentially toxic elements influence the enzymatic activity by complexing the substrate, either reacting with the enzyme active sites or with the enzyme-substrate complex. Further, the potency of the trace elements depends on the kind and concentration added because the part of the S-H groups of enzyme-active sites interacts with the metal ions.

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CHAPTER VI

EFFECT OF CROP ROTATION AND RESIDUES MANAGEMENT ON CELLULASE, *β* -CELLOBIOSIDASE, *β*-GLUCOSIDASE, *α*- and *β*-GALACTOSIDASE, *β* -D-FUCOSIDASE, AND INVERTASE ACTIVITIES IN SOIL.

Abstract

Agricultural practices that cause minimum soil damage and promote sustainability are desired for future food security. Activities of soil enzymes can offer evidence on potential effects of soil management on soil and its processes such as nutrient cycling and decomposition. Crop rotation and residues management have significant effect on soil physicochemical and biological properties. In the present study, carbon transforming activity of soil enzymes (*β*-cellobiosidase, *β*-glucosidase, cellulase, *α*- and *β*-galactosidase, invertase and *β*-D-fucosidase) were assessed in a long-term cropping system experiment established in the 70's in central Oklahoma with five different crop rotation systems (Double crop wheat graze; Modified double-crop, early season; Wheat/Soybean double-crop; Monocrop soybean; and Modified double-crop, full-season). The wheat/soyabean double cropping system resulted in significantly higher enzyme activities (*β*-cellobiosidase (331.80 µmole kg^{-1} soil h^{-1}), *β*-glucosidase (3835.26 µmole kg^{-1} soil h^{-1}), cellulase (782.96

μmole kg^{-1} soil h^{-1}), α- and β-galactosidase (1940.06 and 3536.36 μmole kg^{-1} soil h^{-1}, respectively), invertase (70.01 μmole kg^{-1} soil h^{-1}) and β-D-fucosidase (1404.44 μmole kg^{-1} soil h^{-1})) as compared to the rest of the cropping systems followed by the monocropping soyabean system in which most of the enzyme were in higher in their activities compared to the other cropping systems. The minimum enzymatic activity of most of the enzymes was recorded in double crop wheat graze system.

Introduction

Extensive research work has showed that one of the major challenges faced by the humans is loss of surface biodiversity and related services and functions of the ecosystem (Tilman et al., 1997; Cardinale et al., 2012; Hooper et al., 2012). In case of linking the plant biodiversity to ecosystem processes and subsurface populations, we cannot accurately predict that whether variations in plant communities will affect the important subsurface process including decomposition. Soil helps water storage, carbon (C) sequestration, support plant growth and offer various services to humans, thus comprehensive understanding of associations between soil processes and plant diversity is mandatory. It is known that plant species richness can affect the accumulation of soil C, nutrient cycles, microbial biomass, however specific relations differ across systems and sites Zak et al., 2003; Eisenhauer et al., 2010; Mueller et al., 2013). For instance, Zak et al. (2003) reported that an increase in species richness (from 1 to 16 species) in Minnesota grassland resulted in improved microbial biomass in soil, mineralization rate of nitrogen (N) and respiration after seven years.

However, species richness did not affect the bulk soil N and C. Impacts of belowground plant diversity with varying degree have been reported by different studies. Moreover, agricultural landscapes show higher spatial variations along with inconsistent agroecosystem management and physicochemical characteristic of soil Drinkwater et al., 1995; Vasseur et al., 2013), which ultimately influence the structure and activities of soil biota (Acosta-Martínez et al., 2008; Schipanski and Drinkwater, 2012). Soil microorganisms also facilitate the biochemical conversions of organic matter thus supporting the important ecosystem functions. Transformation of complex forms of natural communities to simplest agricultural systems is diminishing the global biodiversity (Heywood, 1995; Sala et al., 2000; Tilman et al., 1997).

Sequential crop rotation produces temporal biodiversity which, in developed countries, involves rotation of two crops in 2 years (for example soybean-corn), while long term crop rotation and monoculture are common practices. Crop rotation and residues management can transform chemical, biological and biochemical soil characteristics as well as structure, activity, and distribution of enzymes and microbial populations (Doran 1980a, b; Dick 1984). Rotating the crops results in improved soil fertility and structure owing to successive cultivation of different crops (Balota et al., 2003; Govaerts et al., 2008).

Crop rotation also lessen the prevalence of phytopathogens and pests. Adopting long-term cropping systems can influence various soil characteristics, however only few

studies have demonstrated the effect of crop rotation on soil enzyme activities (Dick 1984; Dick et al. 1988).

Further research is needed on *β*-cellobiosidase, *β*-glucosidase, cellulase, *α*- and *β*-galactosidase, *β*-D-fucosidase, and invertase because such information is important in understanding the effect of crop rotation on C transformation, thereby improving soil fertility and productivity.

β-cellobiosidase hydrolyzes cellulose and cellodextrins, releasing cellobiose, a disaccharide, from the non-reducing ends of the chains. *β* -glucosidase enzyme act as rate-limiting factor and accounts for the conversion of cellobiose and cellodextrins into glucose (Singhania et al., 2013; Yao et al., 2016).

Cellulase enzyme complex has *β*-glucosidases as its integral component and is important for transformation of cellulose to glucose through hydrolysis (Bai et al., 2013). *β* -glucosidases produce gentiobiose and sophorose to stimulate cellulase enzyme system (Ramani et al., 2012; Bai et al., 2013) and normally present in several organisms including bacteria and fungi (Bai et al., 2013). As a catalyst, cellulase mediates the cellulose decomposition by cleaving the *β*-1,4-glycosidic bonds.

Galactosidases are the enzymes that are responsible for hydrolyzing galactosidic linkages. They are categorized as α-galactosidases and *β*-galactosidases based on anomeric C atom arrangement in galactose of substrate (Dey and Pridham 1972; Katrolia et al. 2012). *α*-galactosidases catalyze hydrolysis of the terminal α-galactosyl moieties of polysaccharides and oligosaccharides (both are commonly found in plants). *β*-

galactosidase, an exoglycosidase, catalyzes the hydrolysis of β-glycosidic bond between galactose and its organic moiety. β-D-fucosidase catalyzes the hydrolysis of β-D-fucose (6-deoxy-D-galactose) residues from the terminal non-reducing end of β-D-fucosides. Because of the structural similarity between β-D-fucose and β-D-galactose, this enzyme also hydrolyzes β-D-galactosides (Ma and Deng, 2020). Invertase (β-D-fructofuranoside fructohydrolase) hydrolyze the sucrose and generate fructose and glucose (Frankeberger and Johanson, 1983).

Nevertheless, mechanism underlaying the effects of crop rotations on soil biogeochemical processes are not well-studied despite the evident benefits of crop rotations, their long-term application in agriculture (Bullock, 1992) and recent prevalence in cropping systems of U.S (Padgitt et al., 2000). Looking into the above-mentioned facts, this studied was aimed to assess the effect of long-term crop rotation and residues management on the C transforming enzymes i.e., β-cellobiosidase, cellulase, β-glucosidase, α- and β-galactosidase, invertase and β-D-fucosidase, in the soil.

Materials and Methods

Soil samples used in this study were taken from a long-term cropping system experiment established in the 70's in central Oklahoma on a Wynona silt loam with 0 to 1% slope. The five cropping systems under conventional-till; include Double crop wheat graze, Modified double-crop, early season, Wheat/Soybean double-crop, Monocrop soybean, and Modified double-crop, full-season. The modified double-crop systems consisted of early-season soybean [Glycine max (L.) Merr.] planted in April and harvested

in September of year 1, followed by winter wheat [Triticum aestivum L] planted in September and harvested in June of year 2, then followed by another planting of soybean in year 2 which was harvested in October or November (Farno et al., 2002; Keim et al., 2003). The plots were 36.6 m × 19.8 m. In this region of United States, planting double-crop soybean after harvesting winter wheat is an important cropping system (Farno et al., 2002). There were four replicated plots for each cropping system tested. Crop production practices used for tillage, fertilization, and pesticides were considered as standard for the region. Wheat, and early-season and monocrop soybean were planted using conventional tillage. Conventional tillage operations consisted of disking, chiseling, and field cultivating. Composite samples were taken from each treatment plot at first depth (0-10 cm) to determine basic soil properties and carbon-transforming enzyme activities (Table. 6.1). Soil texture was determined as described in Gee and Or (2002). Total carbon and nitrogen were found through dry combustion analysis by placing soil samples wrapped in foil into a Leco TruSpec combustion analyzer (Bremner, 1996; Nelson and Sommers, 1996).

Activities of carbon transforming soil enzymes were determined as the following: β-cellobiosidase as described in chapter three, β-glucosidase, α-galactosidase, β-galactosidase as described in Eivazi and Tabatabai (1988), cellulase, β-D-fucosidase, and invertase as described in Deng and Tabatabai (1994), Ma and Deng, (2020), and Frankenberger and Johanson (1983).

Statistical Analysis

Data were analyzed by one-way analysis of variance techniques (ANOVA) (Steel et al., 1997) and means were compared using Tukey's test using Statistix 8.1 (Analytical Software, USA) while XLSTAT software (version 2021.3.1) was used for principal component analysis (PCA).

Table 6.1. Characteristics of soils used

Crop rotation	pH†	Organic C‡	Total N§	Sand	Silt	Clay
		---------- kg^{-1} ----------		---------- % ---------		
Double crop wheat graze	5.6	7.1	0.75	45	46.5	8.5
Modified double crop early season	5.7	7.3	0.77			
Wheat/soyabean double cropping	6.2	9.1	0.91			
Monocrop soybean	6.4	8.1	0.84			
Modified double crop full season	6.0	10.6	1.05			

†Soil:0.1 M $CaCl_2$ ratio = 1:2.5; C‡, carbon, N§ nitrogen.

Results

Effects of the different crop rotation systems on the determination of enzymatic activities are presented in the figures (6.1-6.7). Different cropping systems affected the soil enzyme activities significantly. The effect of different cropping system on β-cellobiosidase activity is shown in the Figure 6.1. The maximum β-cellobiosidase activity (331.80 µmol kg^{-1} soil h-1) was observed in case of wheat/soyabean double cropping system (WSDC) followed by modified double crop system till early season (MDCES) with 299.55 µmol kg^{-}

^{1} soil h^{-1} enzyme activity. The next treatment that showed higher enzyme activity (268.43 μmol kg^{-1} soil h^{-1}) was monocrop soyabean (MCS) while the treatments modified double crop till full-season (MDCFS) and double crop wheat graze gave lower enzyme activity as compared to other treatments (Figure 6.1).

The *β*-glucosidase activity was significantly different in different crop rotation systems (Figure 6.2). For example, the maximum *β*-glucosidase activity (3835.93 μmol kg^{-1} soil h^{-1}) was observed in case of wheat/soyabean double cropping system (WSDC) that was 144% higher than the enzyme activity observed in double crop wheat graze followed by monocrop soybean (MCS) (3553.93 μmol kg^{-1} soil h^{-1}) with 126% higher enzyme activity as compared to DCWG. The minimum enzymatic activity was recorded in DCWG system that was 1574.19 μmol kg^{-1} soil h^{-1}. The order of *β*-glucosidase activity in different cropping systems was as follows WSDC>MCS>MDCES>MDCFS>DCWG.

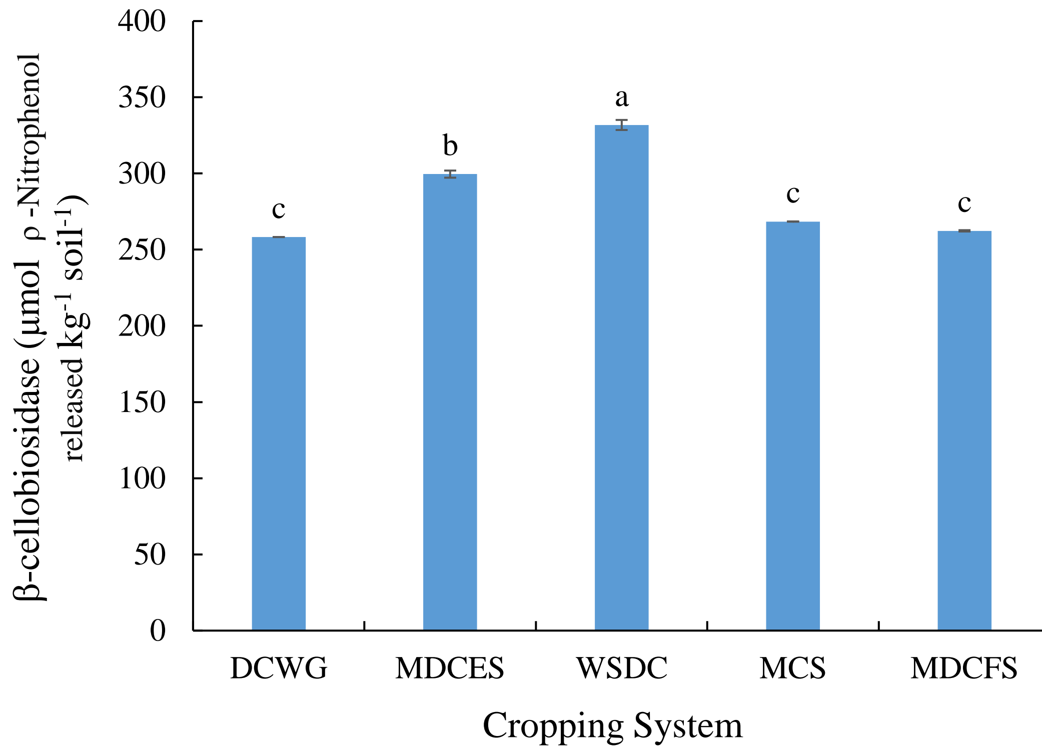

Figure 6.1. Effect of cropping system on the activity of *β*-cellobiosidase. Bars sharing same letters are statistically at par at 5% level of probability. WSDC: wheat/soyabean double cropping system; MDCES: modified double crop system early season; DCWG: double crop wheat graze; MCS: monocrop soybean; MDCFS: modified double crop full season.

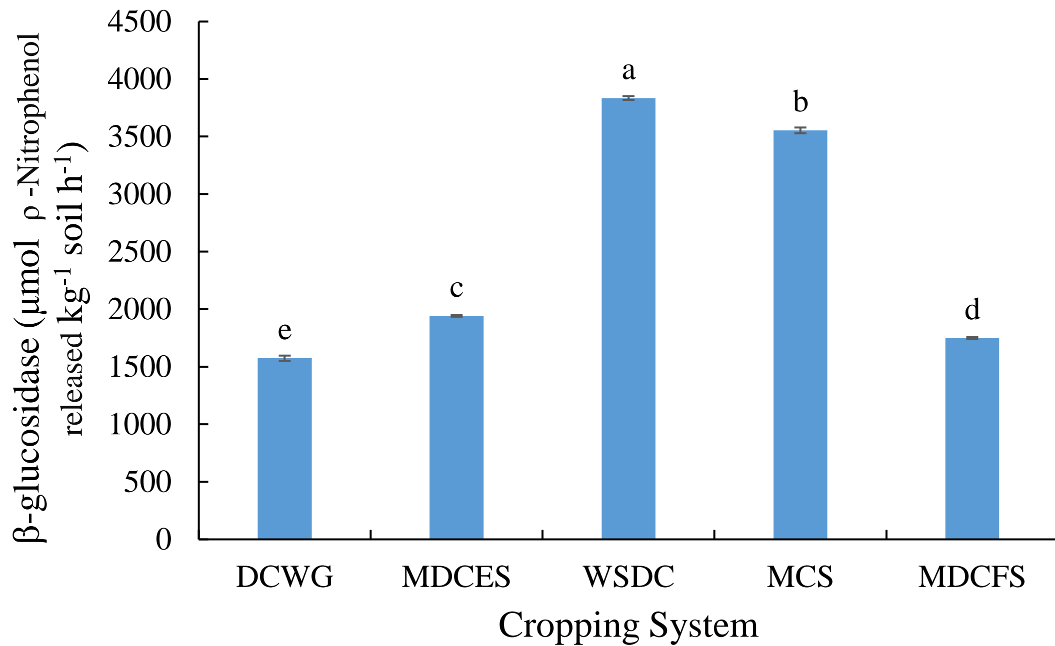

Figure 6.2. Effect of cropping system on the activity of *β*-glucosidase. Bars sharing same letters are statistically at par at 5% level of probability. WSDC: wheat/soyabean double cropping system; MDCES: modified double crop system early season; DCWG: double crop wheat graze; MCS: monocrop soybean; MDCFS: modified double crop full season.

Similarly, *β*-galactosidase activity was maximum in WSDC system that was 177% higher than the enzyme activity recorded in DCWG and significantly different from rest of the treatments followed by monocrop soyabean (MCS) that showed 155% higher *β*-galactosidase activity than DCWG system (Figure 6.3). The next treatment with higher enzyme activity was MDCES that gave 29% more activity than DCWG. The MDCFS and DCWG were observed to have lower enzyme activity that were 1450.30 and 1275.99 µmol kg^{-1} soil h^{-1}, respectively.

The activity of α-galactosidase in soils under different crop rotation systems is depicted in the Figure 6.4. It was observed that WSDC gave highest α-galactosidase activity (1940.06 µmol kg^{-1} soil h^{-1}) that was 3-fold higher than enzyme activity observed in DWG and significantly different from rest of the treatments followed by α-galactosidase activity in MCS rotation system with 1557.09 µmol kg^{-1} soil h^{-1}. The lowest enzyme activity was observed DCWG system (477.94 µmol kg^{-1} soil h^{-1}). All the treatments were statistically significant from each other and the order of α-galactosidase activity in different cropping systems was as follows WSDC>MCS>MDCES>MDCFS>DCWG.

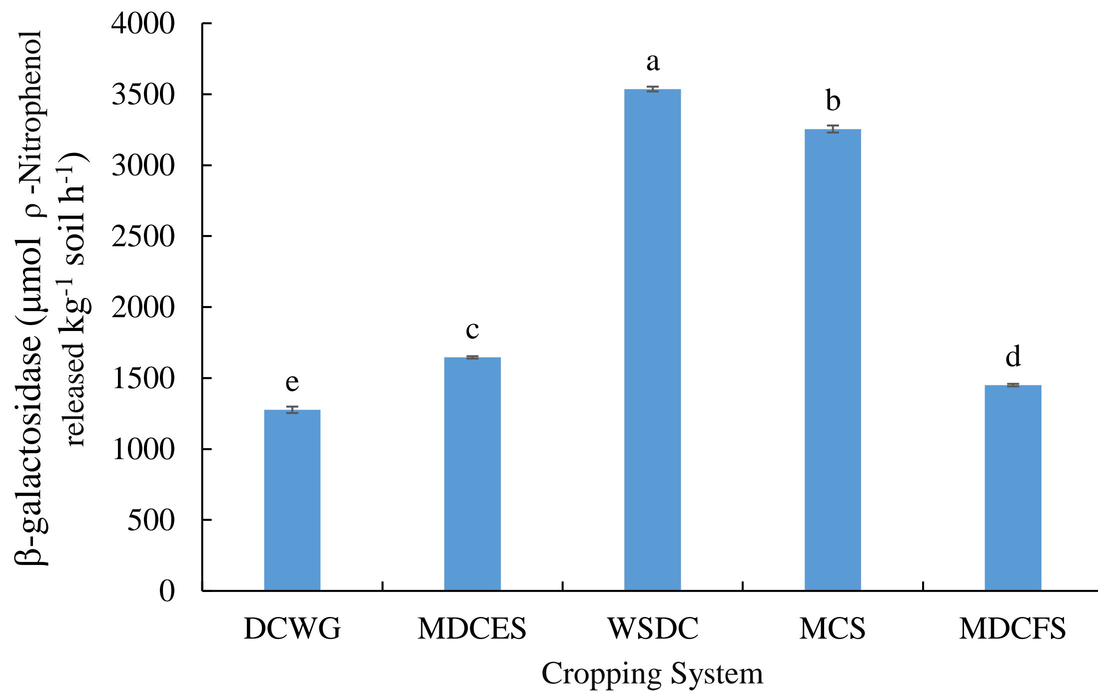

Figure 6.3. Effect of cropping system on the activity of β-galactosidase. Bars sharing same letters are statistically at par at 5% level of probability. WSDC: wheat/soyabean double cropping system; MDCES: modified double crop system early season; DCWG: double crop wheat graze; MCS: monocrop soybean; MDCFS: modified double crop full season.

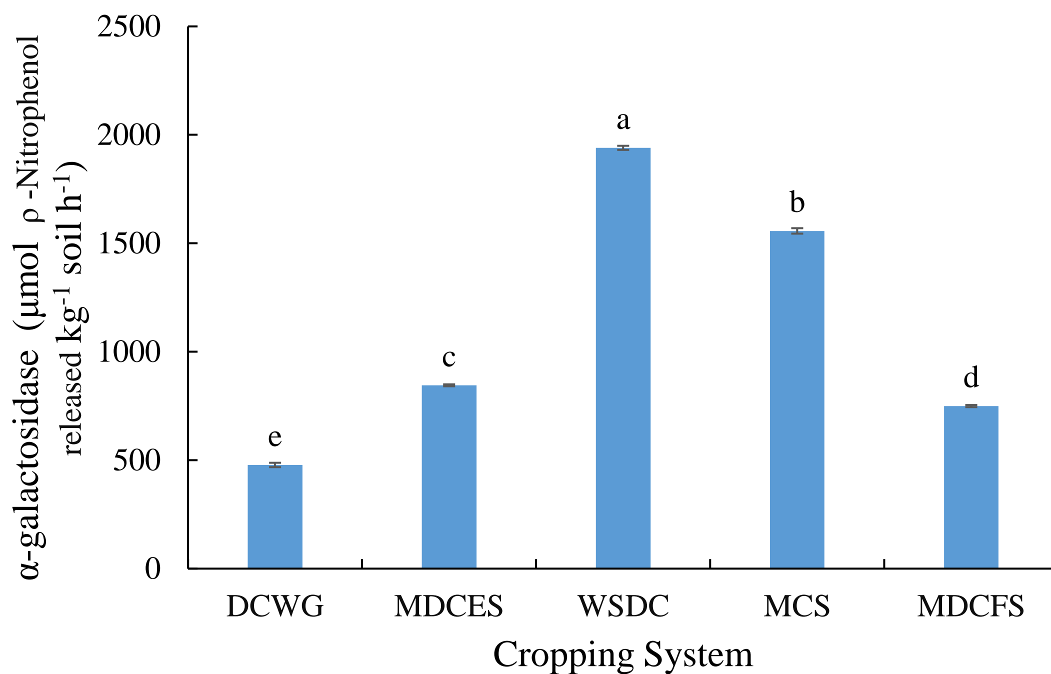

Figure 6.4. Effect of cropping system on the activity of α-galactosidase. Bars sharing same letters are statistically at par at 5% level of probability. WSDC: wheat/soyabean double cropping system; MDCES: modified double crop system early season; DCWG: double crop wheat graze; MCS: monocrop soybean; MDCFS: modified double crop full season.

The activity of cellulase (782.96 μmol kg^{-1} soil h^{-1}) was maximum in WSDC system and statistically significant than rest of the treatments (Figure 6.5). While the minimum enzyme activity was observed in MDCFS that was statistically similar to DCWG and DCWG and MDCFS are statistically non-significant to each other. The order of α-galactosidase in different cropping systems was as follows WSDC>MDCES>MCS>DCWG>MDCFS. Similarly, higher invertase activity was observed in WSDC that was 82% higher than MDCFS soil followed by MDCES that was statistically similar to MCS (Figure 6.6). The invertase activity was lower in DCWG and MDCFS compared to the rest of the treatment. The order of invertase activity in different cropping systems both was as follows WSDC>MDCES>MCS>DCWG>MDCFS.

Figure 6.7 shows the data regarding the β-D- Fucosidase activity of soil under different crop rotation systems. The maximum β-D- Fucosidase activity was recorded in WSDC that was 2.89-fold higher than enzymatic activity observed in DCWG and significantly different from rest of the treatments. The next treatment with higher enzyme activity was MDCES with 501.15 μmol kg^{-1} soil h^{-1} β-D- Fucosidase activity and significant over rest of the treatment. The MCS and MDCFS were statistically similar to each other and gave 9 and 6% higher enzyme activity as compared to DCWG.

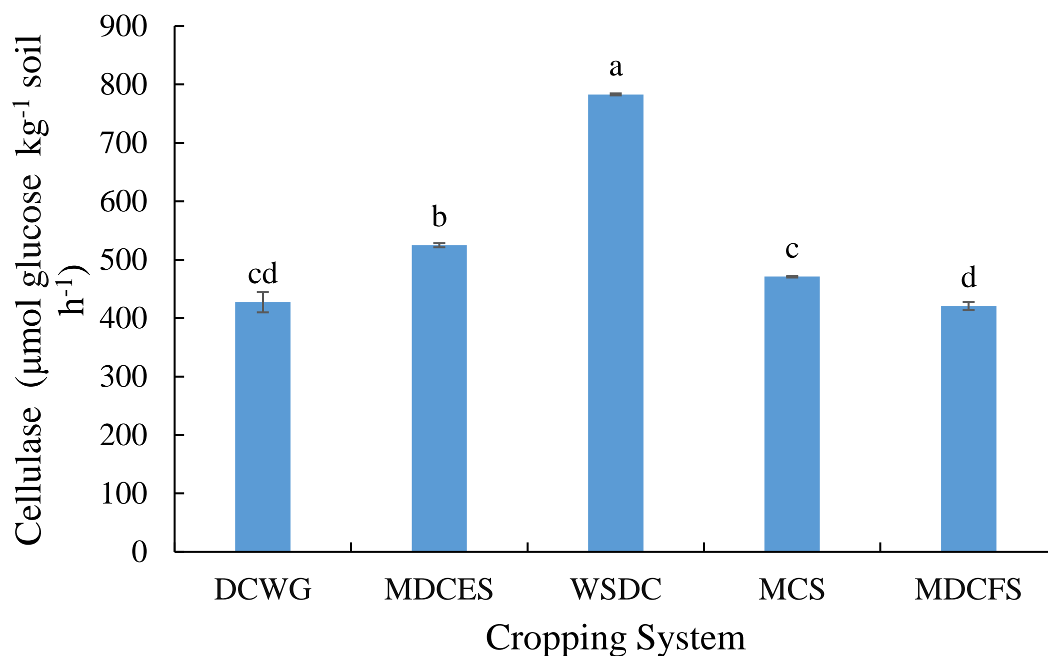

Figure 6.5. Effect of cropping system on the activity of cellulase. Bars sharing same letters are statistically at par at 5% level of probability. WSDC: wheat/soyabean double cropping system; MDCES: modified double crop system early season; DCWG: double crop wheat graze; MCS: monocrop soybean; MDCFS: modified double crop full season.

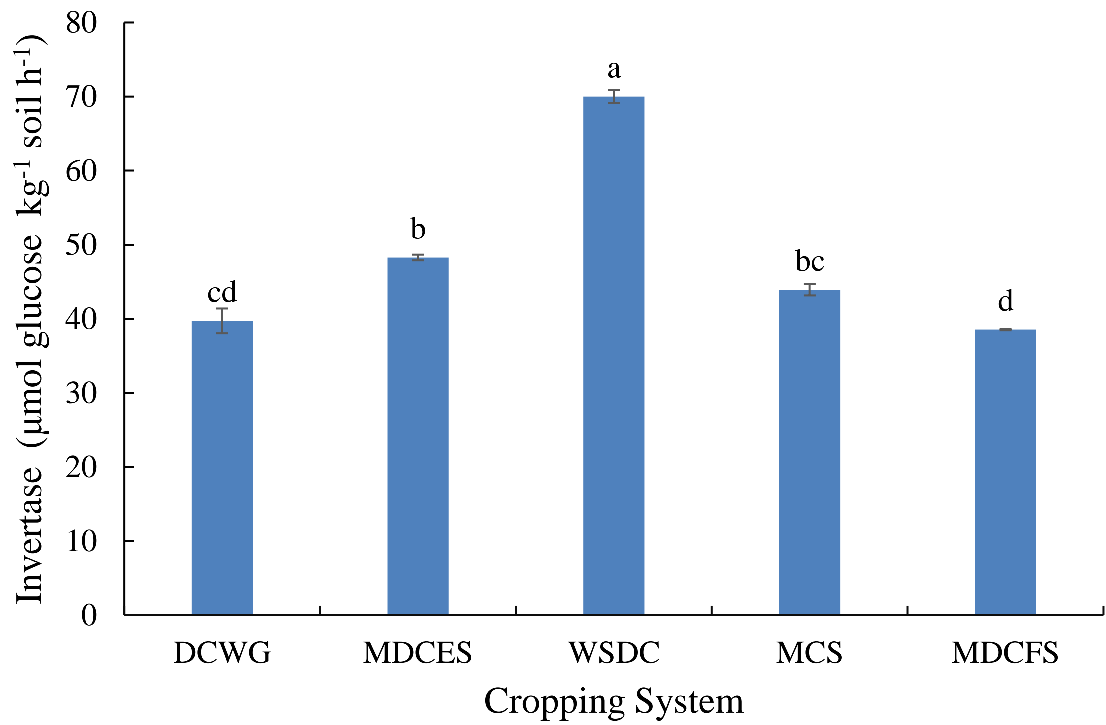

Figure 6.6. Effect of cropping system on the activity of invertase. Bars sharing same letters are statistically at par at 5% level of probability. WSDC: wheat/soyabean double cropping system; MDCES: modified double crop system early season; DCWG: double crop wheat graze; MCS: monocrop soybean; MDCFS: modified double crop full season.

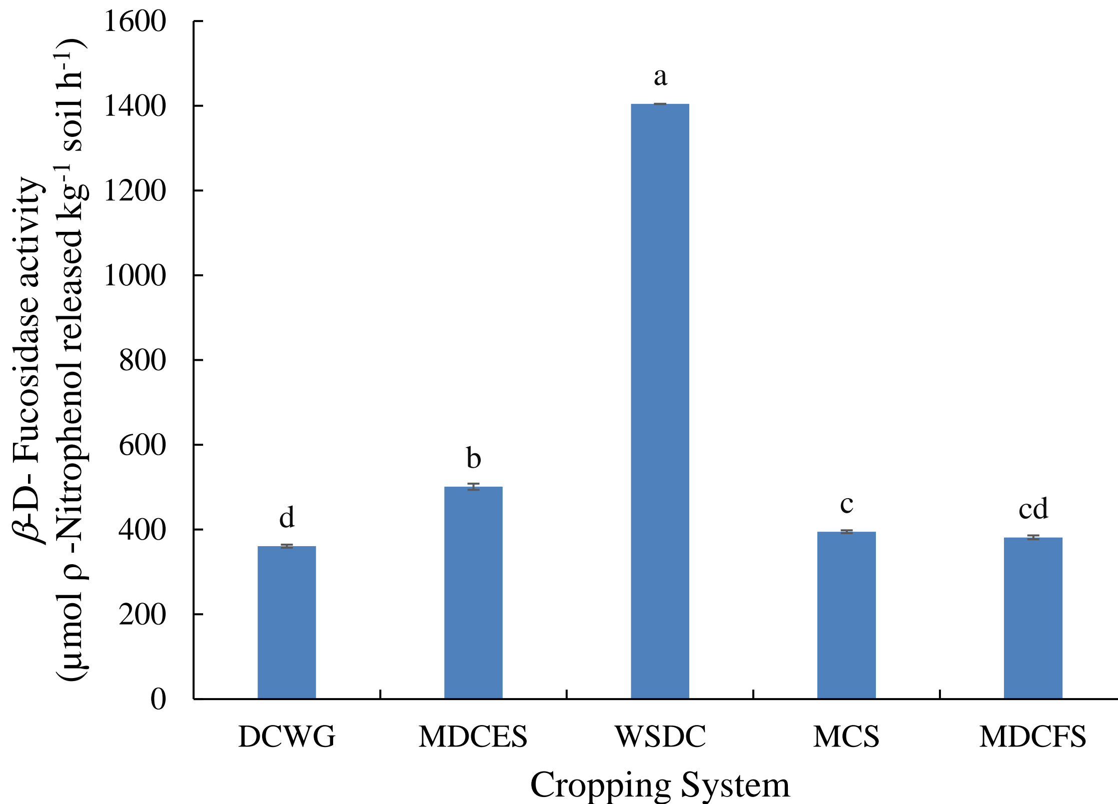

Figure 6.7. Effect of cropping system on the activity of β-D- Fucosidase. Bars sharing same letters are statistically at par at 5% level of probability. WSDC: wheat/soyabean double cropping system; MDCES: modified double crop system early season; DCWG: double crop wheat graze; MCS: monocrop soybean; MDCFS: modified double crop full season.

Principal Component Analysis was used to evaluate the interrelationship among soil enzymes and different crop rotation systems. The obtained results were expressed by PC1 and PC2, which explained 98.466% of total variability (Table 6.2). The first principal component analysis (PC1) explained 84.351% of the total variance and contributed mostly by activities of β-galactosidase; α-galactosidase; β-glucosidase; β-D-fucosidase while PC2 explained 14.115% and was contributed by β-cellobiosidase, cellulase (Figure. 6.8). When

the principal scores of soil variables tested were plotted against different crop rotations, WSDC and MDCES showed somewhat close relationship with PC1 and DCWG and MDCFS showed close relationship to PC2 while MCS were not clearly related to the principal axes (Figure 6.8).

Table 6.2. Principal Component scores of soil enzyme activiities under different cropping systems

Parameter	PC1	PC2
β-cellobiosidase	-2.580	0.278
β -glucosidase	5.293	0.273
β -galactosidase	4.054	-0.139
α-galactosidase	0.060	-0.476
Cellulase	-1.690	0.315
Invertase	-3.548	0.000
β -D-fucosidase	-1.588	-0.251
Eigenvalue	5.905	0.988
Variability (%)	84.351	14.115
Cumulative %	84.351	98.466

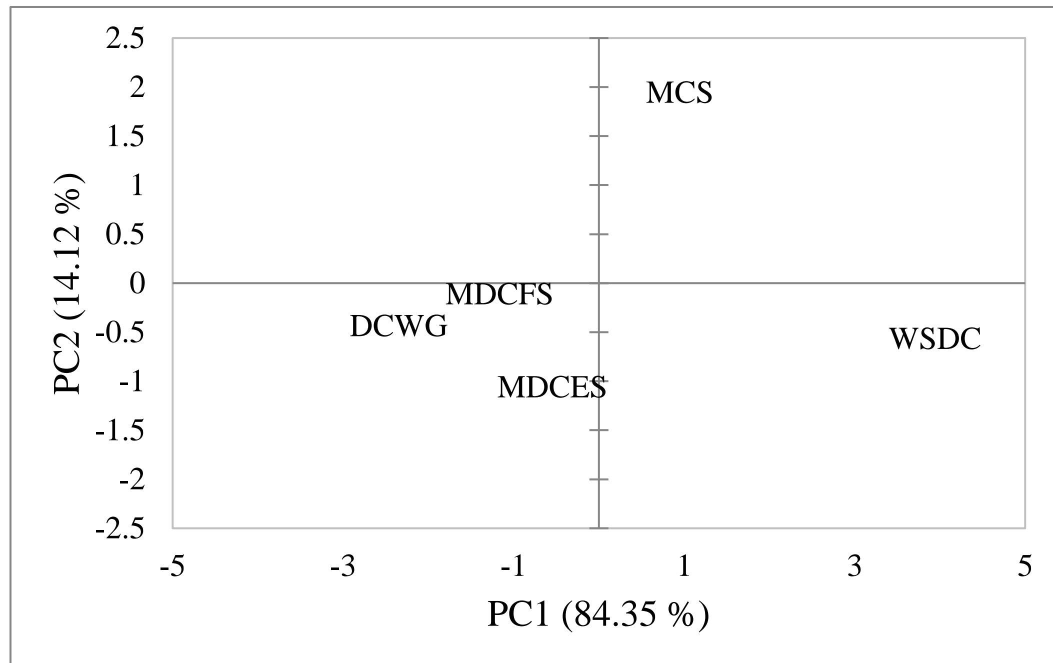

Figure 6.8. Factor scores of different cropping systems regarding soil enzyme activities. WSDC: wheat/soyabean double cropping system; MDCES: modified double crop system early season; DCWG: double crop wheat graze; MCS: monocrop soybean; MDCFS: modified double crop full season.

Discussion

Intense use of tillage for land preparation, improper crop residues management and crop protection along with the practice of monoculture have negative effects on soil physical and biological properties thus affecting the quality and sustainability of the soil. It is urgent to develop such agricultural practices that promote soil quality and ultimately agricultural sustainability. Soil can be protected from degradation through proper residues management, plantation aiming towards minimum soil degradation, along with crop

rotation (Dick, 1984; Kiani et al., 2017; Li et al., 2021). Practicing extensive tillage or improper management of residues modify the soil structure resulting in exposed organic matter which undergo microbial degradation. Balota et al. (2004) found that land preparation and soil management using traditional approaches may reduce the organic matter and microbial activities in soil. Reduced microbial activities are directly related to lower enzymatic activities of the soil. Our results regarding the activities of seven C transforming soil enzymes (β-cellobiosidase, β-glucosidase, β-galactosidase, α-galactosidase, cellulase, β-D-fucosidase and invertase) showed that different crop rotations have significant effect on enzymatic activities.

Buildup of crop residues on the surface and provision of organic materials via belowground root residues may assist in enhancing activity of enzymes in the soils. But many researchers observed increased enzyme activities no-tilled soil when accounting for soil C content. It suggested that enhanced enzyme activities in no-tilled soils were accelerated by several other factors. Tillage plays a considerable role in altering the soil characteristics with special emphasis on spatial distribution of different enzymes in soil profile (Deng and Tabatabai1996a; Bergstrom et al. 1998a, b; Ai et al., 2018; Li et al., 2021). Opposite to deeper layers of soil, tilled soils exhibit less enzyme activities on its surface as compared to zero-till soils (Angers et al. 1993; Kandeler and Böhm 1996; Bandick and Dick 1999; Kandeler et al. 1999a, b; Roscoe et al. 2000; Dumontet et al. 2001; Singh et al., 2018; Ai et al., 2018; Li et al., 2021), and the fact is conventionally tilled soils has less microbial activities than zero-till soil.

In the present study, different cropping systems significantly affected the enzymatic activities. Higher enzymatic activities were observed in wheat/soyabean double cropping system. Our results are in accordance with the previous studies where crop rotation had significant impacts on enzyme activities (Klose and Tabatabai, 2000: Dodor and Tabatabai, 2005). It has been reported that crop rotations having different crop sequences improve and maintain the soil quality. This practice also modifies the soil environment owing to variations in root depth, extracted nutrients, remaining residues and their composition in soil (Breakwell and Turco, 1989; Dick et al., 1988; Bonanomi et al., 2008). In contrary to monoculture, crop rotations accelerate biological activities and biodiversity in soil. Monoculture can cause deleterious modifications in soil habitat and limit the crop yield and microbial diversity in soil (Blecharczyk et al., 2004; Chu et al., 2007; Järvan et al., 2014). Continual monoculture of crop plants triggers the soil degradation and reduce the microbial diversity and organic matter (Xiang et al., 2008; Wolna-Maruwka et al., 2009). While comparing monoculture and crop rotation, it has been demonstrated that enzymatic activities in soil are responsive to positive impacts of multiple cropping (Khan, 1970; Blagoveshchenskaya and Danchenko, 1974; Dick, 1984; Bolton et al., 1985; Tian et al., 2011; Chu et al., 2016).

The higher enzymatic activities observed by the previous researchers under no-till can be attributed to the increased microbial activities in response to the less disturbance of the soils. Similarly, Acosta-Martinez et al. (2008) reported that zero-till pasture soil exhibited more enzymatic activities owing to extracellular enzymes and vigorous microbial biomass comprising intracellular enzymes. Non-tilled soils have less oxidizing

biochemical environment because of absence of disturbance as compared to tilled soils (Melero et al., 2009). As a result, non-tilled soils may have more extracellular enzymes (Trasar-Cepeda et al., 2008) thus supporting increased enzymatic activities which indicate that microbial-mediated transformation of plant debris and soil organic matter is favored in no-till systems.

However, biology work did not propose modification in diverse functions of actively growing bacterial components in soil. This indicates that increased enzyme activity described here might be due to abundantly present fungi in non-tilled soil. Previous studies have been reporting the decreased functional diversity of microbes in soil disturbed due to tillage that pose adverse effects on soil microorganisms (for example, breakage of fungal hyphae due to tillage) (Lupwayi et al., 2001; Chu et al., 2016). It has been reported that reduction in the intensity of tillage benefitted the soil with increased soil organic matter (Logan et al. 1991; Cannell and Hawes 1994; Canarutto et al., 1995; Tian et al., 2011; Chu et al., 2016) and resulted in stratified soil (both chemically and physically) having high nutrients and organic matter close to the surface (Hendrix et al. 1986; West and Post 2002; Singh et al., 2018; Li et al., 2021). The researchers explained that tillage type can alter the impacts of cultivation on the soil environment. Conservation tillage systems cause less harms to soil as compared to conventional tillage and exhibit higher activities of enzymes in surface soil (<10 cm depth) (Klein and Koths, 1980; Doran, 1980; Dick, 1984; Chu et al., 2016; Singh et al., 2018).

Conclusion

Crop rotation and residues management are key soil management practices which can limit the soil erosion, maintain soil organic matter and water, and promote the biological activities. Cropping system affect the enzyme activities by changing the composition of microbial population. Moreover, expansion of root system and type of crop also influence the enzyme activities. In the present study, the WSDC system resulted in significantly higher enzyme activities as compared to the rest of the cropping systems while the minimum enzymatic activity of most of the enzymes was recorded in double crop wheat graze system.

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CHAPTER VII

CONCLUSIONS

- An assay method was developed to quantify activities of cellobiosidase in soil.
- Soil pH, sample storage temperature and time, trace elements, and management practice affected stability and/or activity of cellobiosidase in soil.
- The tested enzyme activities had mostly positively correlated each other and with microbial biomass carbon content, with the exception of urease activities which was negatively correlated with microbial biomass.
- Enzyme activities were not significantly correlated with microbial respiration, suggesting that the detected activities are mostly originated from cell-free enzymes that have been stabilized by soil.

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