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Hydrolysis of cellobiose by β-glucosidase from *Aspergillus niger* in the presence of soil solid phases: minerals, biochar and activated carbon

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SUMMARY

The degradation of biomass in soils is a key process in terrestrial biogeochemical cycles. The degradation rate largely determines the organic matter content of soils and the release of nutrients which are essential for plant growth. The soil carbon pool amounts to 1550 Pg, about twice the atmospheric pool, and several studies show that it decreased during the last decades. Also, several models predict a decrease of this pool in the future and this may have significant undesired consequences for the atmospheric CO_2 concentration and for soil properties.

The biomass entering the soil for degradation mainly consists of macromolecular polymers. Saprophytic microorganisms, mainly fungi and bacteria, excrete extracellular enzymes to break down these macromolecular compounds into smaller molecules which can be transferred into the cell where they enter the intracellular metabolism. This first extracellular enzymatic attack on macromolecular biomass entering the soil takes place in a complex and heterogeneous environment where it is strongly affected by the soil solid phase (i.e. different minerals and organic materials originating from biological and chemical transformation of the original biomass material). The adsorption of an organic compound to the soil solid phase slows down its degradation by reducing its bioavailability. However, this process also stabilizes extracellular enzymes when they are adsorbed to the soil solid phase. It therefore may foster the degradation of freely dissolved substrates since these enzymes remain active for a longer time.

This study focused on the effects of soil solid materials on an extracellular enzymatic reaction which is central in the degradation of cellulose: the hydrolysis of cellobiose by β-glucosidase. The isoenzyme used in this study is produced by *Aspergillus niger*, a fungus which is ubiquitous in soils where it grows aerobically on organic matter. The adsorption of cellobiose and β-glucosidase to montmorillonite, kaolinite, goethite, wood charcoal and activated carbon

was quantified under laboratory conditions, the effects of these adsorption processes on the enzymatic kinetics were measured with the natural substrate and mechanistic explanations for the observed effects were proposed.

The experiments show that montmorillonite did not adsorb cellobiose. This mineral adsorbed approximately 10 % of β-glucosidase; the effect of this solid on the reaction rate was negligible. Kaolinite and goethite also did not adsorb cellobiose, but adsorbed approximately 70 % of β-glucosidase; these minerals slowed down the reaction by approximately 18 % relative to controls in the supernatants of the minerals. Also wood char did not adsorb cellobiose. However, it adsorbed more than 99 % of β-glucosidase; the enzymatic reaction was slowed down by approximately 30 %. Activated carbon adsorbed more than 99 % of β-glucosidase and more than 97 % of cellobiose. This completely inhibited the reaction. These results show that: i) the adsorption of β-glucosidase to montmorillonite, kaolinite and goethite is controlled by the electric charges on the surfaces of protein and mineral, ii) the catalytic activity is reduced by enzyme sorption to the materials studied, and, if the substrate is freely dissolved, this activity loss is ≤ 30 %, iii) wood char has a higher adsorption capacity for β-glucosidase than the minerals but the activity loss caused by adsorption is comparable, iv) if cellobiose is completely adsorbed, as in the case of activated carbon, the reaction does not take place.

The interaction of biocatalysts with solid materials is central also for the industrial use of enzymatic processes, since the immobilization of enzymes in solid matrices is desired to prevent washout of the catalyst and thus to increase the specific productivity. Cellobiose hydrolysis may be the final step of an industrial enzymatic conversion of cellulose to glucose and therefore the suitability of agarose, alginate, Eupergit® C and biosilica particles for β-glucosidase immobilization was tested.

The experiments show that most of the enzyme leaked from agarose and alginate beads after 6 h: these materials are unable to efficiently encapsulate the enzyme since the protein is not

adsorbed and the dimensions of the pores are too big to prevent its diffusion. In contrast, the immobilization of the enzyme by covalent bonding to Eupergit® C led to a stable association between β-glucosidase and the matrix, but the formation of the covalent bonds caused an activity loss of approximately 76 %. The encapsulation of β-glucosidase from *Aspergillus niger* in biosilica particles was not successful because the enzyme was expelled from the forming particles, most likely due to electrostatic repulsion or steric hindrance caused by the carbohydrates which are present on the surface of the protein. The encapsulation in biosilica particles of a β-galactosidase from *Escherichia coli*, which is a carbohydrate-free protein, was successful and the activity of the particle-associated enzyme was approximately 62 % of the control.

The effects of the different soil solids on sorption processes of substrate and enzyme and on the hydrolysis rates suggest that a soil microenvironment dominated by a material which does not adsorb cellobiose nor β-glucosidase, like montmorillonite, would tend to slow down the reaction since the enzyme is not stabilized against degradation. A soil microenvironment dominated by a material which does not adsorb cellobiose but adsorbs β-glucosidase to a significant degree, like kaolinite, goethite and wood charcoal, would tend to accelerate the reaction since the small activity loss caused by enzyme adsorption would be compensated by a longer activity period. Finally, the abundance of a material similar to activated carbon, which entirely adsorbs both cellobiose and β-glucosidase, may completely inhibit the reaction at environmentally relevant concentrations since the substrate is not bioavailable and cannot interact with the enzyme.

The results concerning the charred materials also suggest that the amendment of soils with biochars, which is suggested as a strategy to mitigate climate change, may also allow controlling the rate of several soil enzymatic reactions by carefully adjusting properties of the chars such as the specific surface area and the porosity.

ZUSAMMENFASSUNG

Der Abbau von Biomasse im Boden ist ein Schlüsselprozess in biogeochemischen Stoffkreisläufen. Die Abbaurate bestimmt weitgehend den Gehalt an organischer Substanz des Bodens und die Freisetzungsrate von Nährstoffen, die für das Pflanzenwachstum notwendig sind. Der Kohlenstoffvorrat in Böden ist mit 1550 Pg etwa doppelt so groß wie der atmosphärische Vorrat, und mehrere Studien zeigen eine Abnahme dieser Bodenvorräte in den letzten Jahrzehnten. Verschiedene Modelle prognostizieren eine Abnahme der Kohlenstoffvorräte in Böden auch in der Zukunft. Dies könnte bedeutende und unerwünschte Folgen für die atmosphärische CO_2-Konzentration und die Bodeneigenschaften haben. Die Biomasse, die in den Boden eingetragen und mikrobiell abgebaut wird, liegt vorwiegend in der Form von makromolekularen Polymeren vor. Saprophytische Mikroorganismen, hauptsächlich Pilze und Bakterien, scheiden extrazelluläre Enzyme aus, die diese makromolekularen Verbindungen in kleinere Teile abbauen. Diese kleineren Moleküle können von den Zellen aufgenommen werden und im intrazellulären Metabolismus verwertet werden. Dieser initiale extrazelluläre enzymatische Prozess im Abbau der Biomasse findet in einem komplexem und heterogenem Umfeld statt, und wird stark von den Bodenfeststoffen (d.h. verschiedenen Mineralen und organischen Substanzen, die durch biologische und chemische Umwandlungen der ursprünglichen Biomasse entstehen) beeinflusst. Die Sorption einer organischen Verbindung an Bodenfeststoffen verlangsamt ihren Abbau durch eine Verringerung ihrer Bioverfügbarkeit. Allerdings werden auch extrazelluläre Enzyme durch Sorption stabilisiert. Dies kann zu einer Beschleunigung des Abbaus von gelösten Substraten führen, da diese Enzyme länger aktiv bleiben.

Diese Studie untersucht die Auswirkung von Bodenfeststoffen auf eine extrazelluläre enzymatische Reaktion, die wesentlich am Abbau von Cellulose beteiligt ist: die Hydrolyse von Cellobiose durch β-Glucosidase. Das Isoenzym, das in dieser Studie verwendet wurde, stammt von *Aspergillus niger*, ein Pilz der in Böden ubiquitär ist und der organische Substanz aerob abbaut. Die Sorption von Cellobiose und β-Glucosidase an Montmorillonit, Kaolinit, Goethit, Holzkohle und Aktivkohle wurde unter Laborbedingungen quantifiziert, die Effekte dieser Sorptionsprozesse auf die Enzymkinetik wurden gemessen und mechanistische Erklärungen für die beobachteten Effekte vorgeschlagen.

Die Ergebnisse zeigen, dass Cellobiose nicht an Montmorillonit sorbierte. Dieses Mineral sorbierte nur etwa 10 % der β-Glucosidase; die Effekte auf die Reaktionsrate waren vernachlässigbar. Auch Kaolinit und Goethit sorbierten Cellobiose nicht. Sie sorbierten jedoch etwa 70 % der β-Glucosidase; diese Mineralien verlangsamten die Reaktion um etwa 18 % im Vergleich zu den Kontrollen in Überständen der Mineralien. Holzkohle sorbierte ebenfalls keine Cellobiose. Sie sorbierte jedoch mehr als 99% der β-Glucosidase; die Reaktionsrate wurde um etwa 30 % verringert. Aktivkohle sorbierte mehr als 97 % der Cellobiose und mehr als 99 % der β-Glucosidase und hemmte die Reaktion vollständig. Diese Ergebnisse zeigen, dass: i) die Sorption von β-Glucosidase an Montmorillonit, Kaolinit und Goethit von den elektrischen Ladungen auf den Oberflächen des Proteins und der Mineralien kontrolliert wird, ii) die enzymatische Aktivität der sorbierten Fraktion zwar reduziert wird, aber nicht vollständig zum Erliegen kommt iii) Holzkohle hat für β-Glucosidase eine höhere Sorptionskapazität als die Mineralien, aber die Reduktion der enzymatischen Aktivität durch die Sorption ist vergleichbar, iv) wenn Cellobiose vollständig sorbiert ist, wie im Fall der Aktivkohle, findet die Reaktion nicht statt.

Die Wechselwirkung zwischen Biokatalysatoren und Feststoffen ist auch für industrielle Herstellungsprozesse, die enzymatisch katalysiert werden, wesentlich. In diesem Fall ist die Immobilisierung des Enzyms in eine feste Matrix gewünscht, um Auswaschung des Enzyms

zu verhindern und damit die spezifische Produktivität zu erhöhen. Die Hydrolyse von Cellobiose könnte die letzte Stufe einer industriellen enzymatischen Umwandlung von Cellulose in Glucose darstellen, und deswegen wurde die Eignung von Agarose-, Alginat-, Eupergit® C- und Siliziumoxid-Partikeln für die Immobilisierung dieses Enzyms untersucht.

Die Ergebnisse zeigen, dass der Großteil des Enzyms nach 6 Stunden aus den Agarose- und Alginat-Partikeln in die freie Lösung diffundiert war: diese Materialien sind nicht in der Lage das Enzym effektiv zu immobilisieren, da das Protein nicht sorbiert wird und die Dimensionen der Poren zu groß sind, um die Diffusion zu verhindern. Dagegen ergab die Immobilisierung durch kovalente Bindung von β-Glucosidase an Eupergit® C eine stabile Assoziierung, aber die kovalenten Bindungen verursachten einen Aktivitätsverlust von etwa 76 %. Die Immobilisierung von β-Glucosidase aus *Aspergillus niger* in Siliziumoxid war nicht erfolgreich, da das Enzym, wahrscheinlich aufgrund elektrostatischer Abstoßung oder sterischer Hinderung, die von den Kohlenhydraten auf der Oberfläche des Proteins verursacht werden, nicht in die sich bildenden Silikat-Partikeln eingebaut wurde. Die Immobilisierung von β-Galactosidase aus *Escherichia coli*, einem kohlenhydratfreien Enzym, in Siliziumoxid-Partikeln war erfolgreich, und die enzymatische Aktivität des partikelgebunden Enzyms betrung etwa 62 % der Aktivität des freien Enzyms.

Die Effekte verschiedener Bodenfeststoffe auf die Sorption des Substrats und des Enzyms, sowie auf die Raten der enzymatischen Hydrolyse von Cellobiose, deuten darauf hin, dass in Mikrobereichen des Bodens, die von einem Material wie Montmorillonit (das weder Cellobiose noch β-Glucosidase sorbiert) dominiert ist, der Cellulose-Abbau verlangsamt werden könnte, da das Enzym nicht stabilisiert wird. In Bereichen, die von einem Material dominiert sind, das Cellobiose nicht, aber β-Glucosidase in erheblichen Mengen sorbiert (wie Kaolinit, Goethit und Holzkohle), ist zu erwarten dass der Abbau des Substrates beschleunigt wird. Der geringe durch sorption verursachte Aktivitätsverlust des Enzyms wird dann durch eine Stabilisierung des Enzyms gegen Abbau und damit einer längeren Aktivitätszeit

kompensiert. Die Abundanz eines Materials wie Aktivkohle, das sowohl Cellobiose als auch β-Glucosidase vollständig sorbiert, verhindert die Reaktion bei umweltrelevanten Konzentrationen wahrscheinlich vollständig, da das Substrat nicht bioverfügbar ist und nicht mit dem Enzym wechselwirken kann.

Die Ergebnisse bezüglich der verkohlten Materialien deuten darauf hin, dass die Rate verschiedener enzymatische Prozesse beim Zusatz von Biokohle zu Böden über die entsprechende Einstellung von Eigenschaften der Biokohle, wie spezifische Oberfläche und Porosität, kontrolliert werden könnte. Dies ermöglicht eine gezielte Steuerung der Wirkung des Biokohlezusatzes zu Böden, welcher als Strategie für die Verringerung des Klimawandels vorgeschlagen wird.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. Introduction

1.1. Degradation of biomass in soils

Soil can be de defined as “a natural body, differentiated into horizons of mineral and organic constituents, usually unconsolidated, of variable depth, which differs from the parent material below in morphology, physical properties and constitution, chemical properties and composition, and biological characteristics” (Joffe, 1936). It is a complex environment comprising solid phases (altered rock and organic matter), liquid phase (from rain or groundwater) and gaseous phase (a mixture of the same gases that form air but different in the relative amounts of each gas). In terrestrial ecosystems soils are central in the production of biomass, since the primary productivity of plants relies on nutrients, water and mechanical anchorage which all are provided by soils (Hilgard, 1914). The degradation of dead biomass, and the related release of inorganic nutrients and carbon into the biogeochemical cycles, is a key process in soils (Janzen, 2006). The rate at which biomass is degraded in soils has profound consequences on the productivity of ecosystems and on the atmospheric concentration of CO_2. If the input of biomass exceeds degradation, soil organic matter (SOM) is accumulated, which has positve consequences for the physical and chemical properties of soil (for instance cation exchange capacity, pH buffering capacity, water holding capacity, stability of aggregate structure and thus permeability to groundwater and gases; Sequi and Nannipieri, 1989) and carbon is removed from the atmosphere. However, in absence of anthropogenic fertilization, the fertility of the soil depends on SOM being degraded, since a complete inhibition of microbial activities implies that no nutrients are made available for plants (Janzen, 2006). If the degradation rate exceeds the input of biomass, nutrients are made available for plants, but in the long term the SOM stock is depleted and soils tend to become

acidic and to lose their organization in aggregates. The progressive brakeup of soil aggregates tends to accelerate the SOM degradation process even further, since the aggregates themselves can play a major role in SOM storage (Kögel-Knabner and Ziegler, 1993; Rumpel and Kögel-Knabner, 2010). The soil organic carbon pool is estimated to amount to 1550 Pg (Eswaran et al., 1995; Batjes, 1996), about twice the atmospheric pool. Since trends towards a decrease in the soil carbon stocks were observed (Sleutel et al. 2003; Bellamy et al. 2005) and predicted by models (Vleeshouwers and Verhagen, 2002; Wan et al., 2011), a net tranfer of carbon from soils to atmosphere is to be expected. Agricultural practices normally lead to a decrease in the SOM content by reducing the input of biomass to soils (much of the primary production is harvested and thus removed from the ecosystem) and to an increase in the degradation rate of the pre-existent pool of SOM by ploughing (Conant et al., 2006) and irrigation (Wang et al., 2010). The rate at which a specific organic compound is degraded in a soil depends on many factors: aeration, water activity and the partitioning of the compound among the different solid phases play a major role, whereas it seems that the importance of the intrinsic recalcitrance of primary biogenic compounds has been overestimated (Marschner et al., 2008). The intrinsic degradability of a bioavailable organic compound seems to be inversely proportional to its aromaticity degree: among the materials that show a relatively high persistence in soils even if not associated with minerals there are lignin (Kögel-Knabner, 2002) and black carbon (Marschner et al., 2008). Thus, the association of biogenic organic compounds with the mineral components of the soil, particularly clays and iron oxides, plays a major role in the determination of the degradation rate, generally leading to a relevant stabilization of the compounds (Sollins et al., 1996; Kiem and Kögel-Knabner, 2002; Eusterhues et al., 2003; Eusterhues et al., 2005; von Lützow et al., 2007; Kögel-Knabner et al., 2008; Rumpel and Kögel-Knabner, 2010; Rumpel et al., 2010). Even compounds of high nutritional value and high degradability like amino acids and DNA can be remarkably

persistent in soils presumably because of the association with the solid phase (Fan et al., 2004; Kindler et al., 2006; Miltner et al., 2009). Most of the organic compounds that enter the soil ecosystem are polymeric (for instance cellulose, lignins, proteins etc.) and cannot be transported into the cells of degrading microorganisms because their molecular weight is too high. Therefore, microorganisms excrete extracellular enzymes into their environments in order to break up macromolecular substrates and to provide the microorganisms with smaller molecules which can be transported into the cells and enter the intracellular metabolism (Quiquampoix and Burns, 2007). This first enzymatic attack on organic molecules, which takes place in the harsh extracellular environment, is the main topic of this thesis, which will focus particularly on enzyme - solid matrix and substrate - solid matrix interactions and on how these interactions affect enzymatic degradation rate.

1.2. Enzymatic activities in soils

The number of reactions catalysed by soil enzymes is huge. Furthermore, a specific reaction may be catalysed by different isoenzymes which have very different properties in terms of their tendency to be adsorbed to different soil solid surfaces, activity retained in adsorbed state, maximum reaction rate, affinity for the substrate, substrate range, resistance to proteolysis and denaturation, pH range and optimum, temperature range and optimum, minimum water activity required for catalysis, sensitivity to inhibitors and enhancers, etc. Soil enzymes that have attracted the attention of researchers include amylases (enzymes catalyzing the hydrolysis of starch), arylsulphatases (hydrolysis of sulphate esters), cellulases (hydrolysis of cellulose), chitinases (hydrolysis of chitins), dehydrogenases (oxidation of soil organic matter), phosphatases (hydrolysis of esters of phosphoric acid), proteases (hydrolysis of proteins) and ureases (hydrolysis of urea).

Many soil enzymatic activities can be measured, and they are strongly affected by changes in soil conditions. Thus it has been suggested to use them as indicators of soil quality (Nannipieri et al., 2002; Garcia-Ruiz et al., 2008). However, due to the complexity and diversity of soil systems, the results from studies on the effects of disturbances on soil enzymes are often contradictory, in particular if non-natural substrates are applied that are easy to measure. Ploughing, the application of mineral and organic fertilizers, mowing and the presence of grazing animals in pastures, or mechanized preparation of soils before reforestation have all been reported to cause both increases and decreases of biochemical activities (Trasar-Cepeda et al., 2008).

The total enzymatic activity of a soil is comprised of activities of enzymes of various origins and at various locations, such as the cytoplasm of living cells, cell debris, or exo-enzymes sorbed to surfaces of clay minerals and humic colloids (Burns, 1982).

According to Sequi and Nannipieri (1989), soil enzymes fall into two main cathegories: enzymes associated with proliferating cells and accumulated enzymes. The first group comprises intracellular enzymes (for instance enzymes involved in glycolysis, Krebs cycle, oxidative phosphorylation etc. and all the enzymes that require cofactors like NAD or FAD for activity), ectoenzymes (enzymes associated with the cellular membrane and with the active site directed outside the cell or enzymes associated with the cell wall) and non-protected exoenzymes which are rapidly degraded. The second group comprises enzymes protected by clays and humic substances, enzymes temporarily associated with substrates, active enzymes in dead cells or cell debris and enzymes in living but not proliferating cells (i.e. spores).

However, the activity of enzymes associated with proliferating cells is difficult to distinguish experimentally from that of accumulated enzymes, since it is problematic to inhibit the metabolic activities of cells without causing cell lysis and therefore increase the

pool of accumulated enzymes. Furthermore an enzyme can be assigned to different categories in different moments: in theory an enzyme may be localized in a living but not proliferating cell which subsequently starts proliferation. Then this enzyme may remain active in the dead cell, then be transported into the soil solution after cell lysis and finally be adsorbed by a clay surface and become an accumulated enzyme.

Therefore, even though most accumulated enzymes are localized outside proliferating cells, some enzymes end up in this category by chance, whereas "truly" extracellular enzymes are synthesized and excreted by cells with the purpose of increasing a specific catalytic potential of their microenvironment.

The essential role of extracellular enzymes in the cycling of carbon, nitrogen, phosphorous and sulphur is well recognized today (Schimel and Bennett, 2004; Quiquampoix and Burns, 2007). Woods (1899) was the first author to propose that enzymes produced by living cells could catalyse chemical tranformations of organic matter outside of the cellular environment. However, researchers were uncertain on whether chemical reactions in soils were caused by inorganic catalysts (for instance iron hydroxides) or enzymes. Among the first authors to demonstrate the presence of enzymatic activites in soils was Rotini (1932), who detected the activity of phosphatases. Esterases and nucleases were detected in soils by Rogers (1942) and phytases by Jackman and Black (1952).

In soils, extracellular enzymes are produced mainly by saprophytic microorganisms such as bacteria and fungi, but they are also present in root exudates or in the gut of soil fauna. For prokaryotes, extracellular enzymes are a necessity caused by the absence of cellular compartmentation: for instance a protein with an essential function in metabolism and a protease capable of hydrolysing it cannot be present inside the cell at the same time. Many chemical transformation processes must therefore take place outside of the cell. However, also eucaryotes produce extracellular enzymes, the essential function of which is to degrade

biopolymers whose dimensions prevent their transport through the cell membrane. Macromolecular compounds essentially need to be transformed into smaller entities that can be recognized and handled by the membrane transport systems (Quiquampoix and Burns, 2007). Once in the cell they are further degraded and can serve as carbon, nutrient and energy sources (Figure 1). In contrast to intracellular enzymes, which are active in defined homeostatic environments, extracellular enzymes are exposed to highly variable environments in terms of chemical composition, water activity, pH, and temperature.

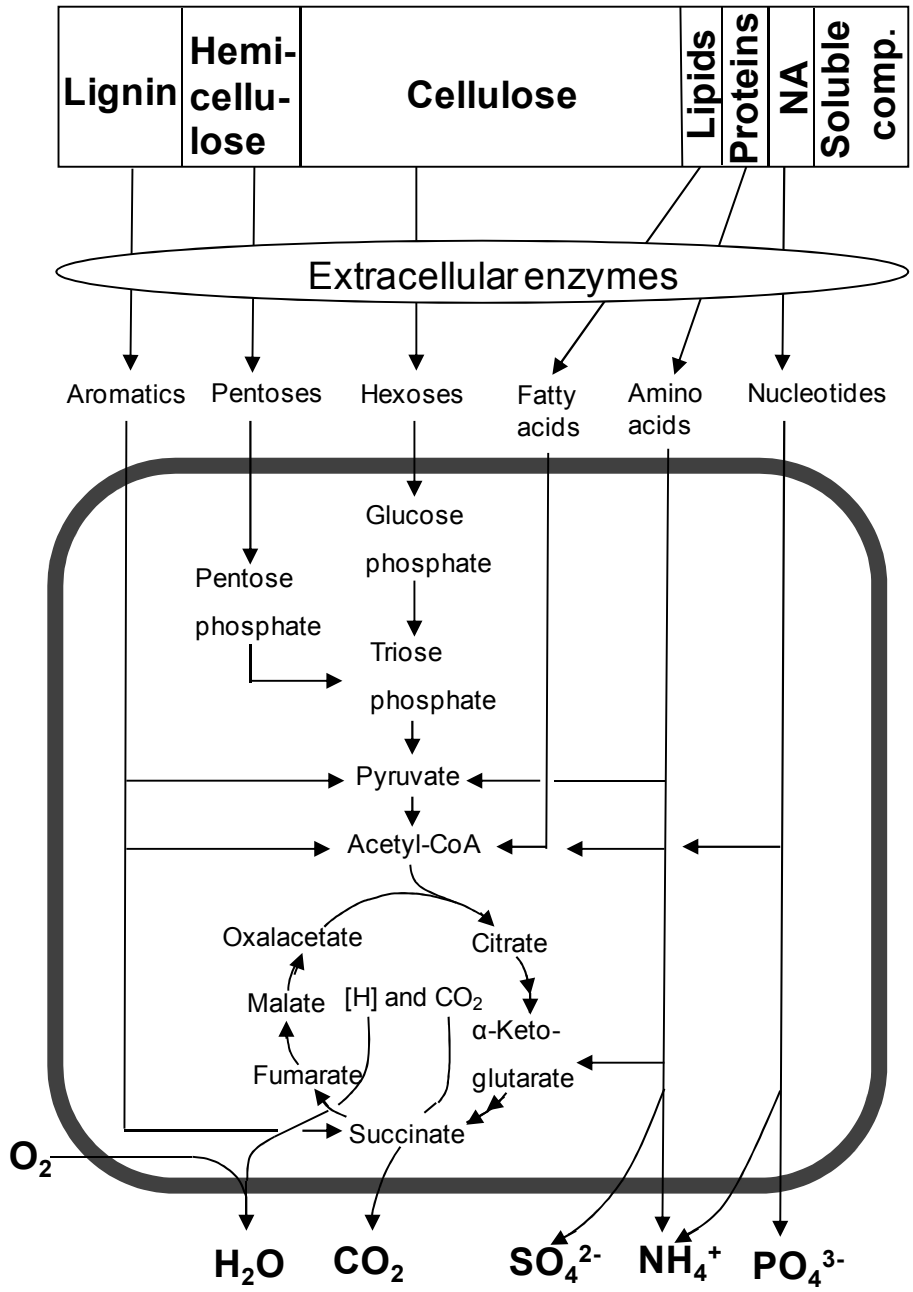

Figure 1. Aerobic mineralization of plant biomass. Extracellular enzymes depolymerize macromolecules and produce smaller molecules which enter the intracellular metabolism (NA = nucleic acids). (Fritsche, 1998).

Enzymes are proteins and therefore molecules of high nutritional value. Once excreted in soils, they would be rapidly degraded if not protected from proteases. Ad- or absorption to

the soil solid phase, which decreases the bioavailability of extracellular enzymes, strongly contributes to this protection. This accumulation of enzymes on, or in, the solid phase (both mineral and organic) generates a pool of stabilized catalytic potential: soil can be considered "as having an indigenous and persistent enzymatic capacity which is independent of current or even recent cell growth" (Burns, 1982).

Adsorbed proteins are therefore more stable than dissolved ones but, in the case of enzymes, ad- or absorption to the soil solid phase can have profound consequences on catalytic activity. For instance, the clay fraction of soils has a large surface area; upon adsorption to these surfaces, enzymes undergo a decrease in mobility and catalytic activity (Quiquampoix, 1987a; Fusi et al., 1989; Tietjen and Wetzel, 2003). In the literature, three main hypotheses can be found to explain the decrease of catalytic activity of enzymes upon adsorption to clays (Quiquampoix and Burns, 2007): 1) limitation of substrate diffusion in the vicinity of the surface, 2) orientation of the protein relative to the surface which hinders the access of the substrate to the active site, 3) modification of structural conformation. The last two hypotheses have been experimentally validated (Quiquampoix and Ratcliffe, 1992; Baron et al., 1999; Servagent-Noinville et al., 2000). The hypothesis that an enzyme adsorbed to the surface of a clay mineral undergoes a change in activity because of a change in the protonation state seems to be confuted by the following argument: even though close to the surface of a negatively charged mineral the pH is lower than in the bulk solution, the proton tendency to react is the same close to the surface and in the bulk solution at proton adsorption equilibrium (Rouxhet, 1990).

The residual activity of adsorbed enzymes is a key factor in determining the extracellular metabolic potential of soils, since the majority of the extracellular enzymes are adsorbed to soil particles (Kandeler, 1990; Lipson and Nasholm, 2001; Nannipieri et al., 2002). Sorption and thus immobilization of an enzyme to a solid phase also reduces the

probability of a contact between enzyme and substrate. This is particularly true for non- or hardly-soluble high-molecular-weight substrates such as cellulose or lignin, which are immobile in soils. However, also the interaction of low-molecular weight substrates with soil constituents can affect their degradation rates. Many organic compounds can be adsorbed to soil constituents because of partitioning, van der Waals interactions, charge transfer, ligand exchange, and ion exchange reactions. If strongly adsorbed, chemicals are often quite persistent in the environment (Scow, K.M, 1993).

1.3. Clay minerals and metal oxides in soils

Clay minerals and iron oxides are major constituents of the mineral fraction of the soil solid phase. Clays are more or less crystalline OH containing aluminosilicates, which are the result of the alteration of the parent rock. The specific surface area (SSA) of clay minerals varies widely depending on the clay type: for instance the SSA range for montmorillonite is 30-800 m^2g^{-1} (data sheet of Clay Minerals Society, Chantilly, USA; Testini and Gessa, 1989) whereas for kaolinites the range is 10-20 m^2g^{-1} (Testini and Gessa, 1989). Even though the type of dominant clay is important, these minerals generally provide the soil with a high SSA and therefore they also provide a huge potential to interact and thus stabilize SOM by forming organo-mineral complexes (Wattel-Koekkoek et al., 2001; Wiseman and Püttmann, 2006). In this study on the effects of soil solids on a typical soil extracellular enzymatic reaction, montmorillonite and kaolinite were chosen among clays. From the chemical point of view, montmorillonite is a 2:1 clay mineral (Figure 2a) whereas kaolinite is a 1:1 clay mineral (Figure 2b). For both minerals the surface charge results from the sum of the permanent charge caused by isomorphic substitutions (more frequent in the first mineral) and the pH-dependant charge caused by –OH groups localized at the edges of the crystal (Sollins et al.,

1988). Among the clays, these two minerals can be considered two extremes of a continuum of variation in SSA and cation exchange capacity (CEC) (see Table 1).

In soils iron oxide minerals are the result of the precipitation of Fe (III), which is released by the alteration of the parent rock. Minerals such as goethite and hematite tend to form very small crystals in soils and therefore their SSA is high (50-150 m^2g^{-1}; Schachtschabel et al. 1989). Iron oxides interact with SOM in similar way to clay minerals but their stabilization effect can be even stronger (Kaiser and Zech, 1999). For this study goethite was chosen among the iron minerals since it is the most common soil iron oxide in all climatic regions (Schachtschabel et al. 1989). This mineral (Figure 2c) generates mainly pH-dependant charge on its surface (Schwertmann and Taylor, 1989).

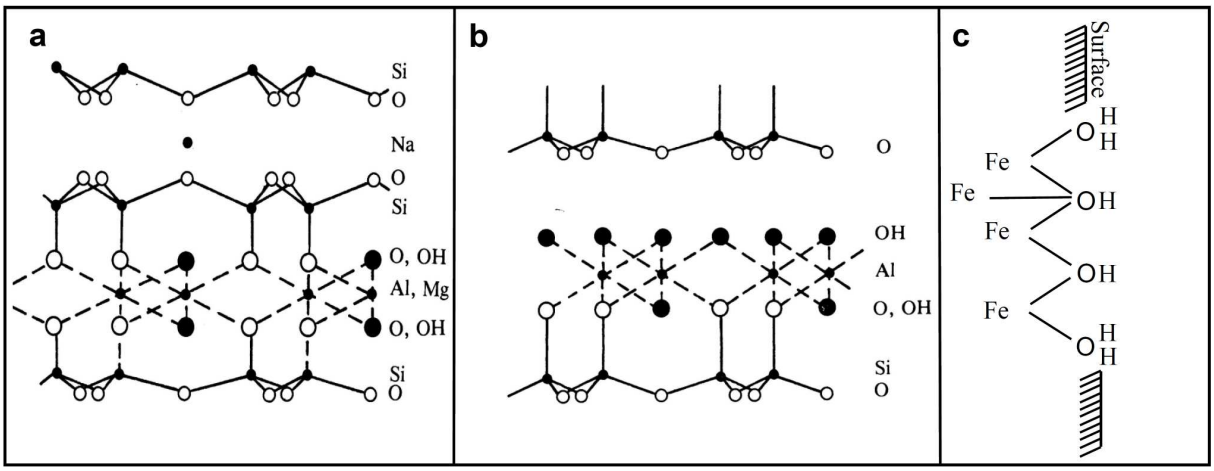

Figure 2. Structures of montmorillonite (a), kaolinite (b), and goethite (c) (Testini and Gessa, 1989, modified; Fontes and Alleoni, 2006, modified).

1.4. Charred materials in soils: occurrence, effects and possible role in climate change mitigation and fertility improvement.

Natural black carbon is produced by incomplete combustion of vegetation during wildfires. It is considered to be ubiquitous in soils (Schmidt et al., 1999; Schmidt et al., 2000;

Cornelissen et al., 2005) and it may represent a significant sink in the global carbon cycle (Kuhlbusch, 1998).

The amendment of soils with anthropogenic biochars has been suggested as a strategy to mitigate global climate change and increase the fertility of soils (Woolf et al., 2010). Since biochars are (bio)chemically more inert than the biomass from which they are obtained, the residence time of biochar-C in soils is considerably longer than that of non-charred plant biomass-C (Schmidt et al., 2000; Lehmann, 2007; Steinbeiss et al., 2009; Woolf et al., 2010). According to the strategy suggested by Woolf et al. (2010), CO_2-C would be removed from the atmosphere by photosynthesis, the resulting plant biomass would be charred and applied to soils where it would form a very stable soil C pool. This strategy of mitigating global climate change by amending agricultural soils with biochar could be considered as a technical exploitation of a natural process.

Many studies show that long term carbon sequestration may not be the only benefit resulting from soil amendment with biochar, since also the fertility/productivity of soils (especially of poor soils) is increased by this treatment (Steinbeiss et al., 2009; Woolf et al., 2010). The model proposed by Woolf et al. (2010), suggests that the use of biomass for energy production *and* biochar production for soil amendments has a larger climate mitigation impact than the complete combustion of biomass for the extraction of the maximum amount of energy. The authors suggest that the advantage of the "energy & biochar" strategy relative to the "energy only" strategy is largely attributable to beneficial feedbacks of soil amendments with biochar on crop yields and soil greenhouse gases fluxes.

The beneficial effects of biochar on soil fertility have already found practical application in pre-Columbian times in the Brazilian Amazon region: the soils in this region are highly weathered and infertile oxisols, but many small areas are known that are characterized by dark and sustainably fertile soils (Glaser et al., 2001; Steiner et al., 2007).

The soil organic matter (SOM) of these Terra Preta soils is characterized by a content of 35 % of black carbon throughout the A horizons (Glaser et al., 2000). Also the SOM in Australian grassland soils under aboriginal fire management is characterized by a content of 30 % of charred organic carbon in the A horizons (Skjemstad et al., 1997).

Understanding the mechanisms leading to increased fertility in soils amended with charred organic materials may have important practical applications. It may allow to predict how different soils would react to biochar amendments and therefore to maximize the fertility increases by manipulating biochar properties (e.g. particle size, porosity, specific surface area) and/or the frequency, abundance and method of application.

Common explanations for the high fertility of soils containing charred organic carbon are high nutrient retention and water holding capacity (Tryon, 1948; Steiner et al., 2008). These factors may indeed be largely responsible for the beneficial effects of charred materials and may also improve the efficiency of fertilisations, reduce fertilizer run-off and reduce the water intensity of crops. However, the effects of biochar amendments may be more extensive, since also biochemical and biological processes, which strongly influence soil fertility, may be affected. For instance charcoal provides a good habitat for microorganisms such as non-symbiotic nitrogen fixing bacteria and mycorrhizal fungi (Ogawa, 1994). Steinbeiss et al. (2009), suggest that the fertilizer effect of biochar may be explained by a stimulation of soil microorganisms resulting in an increased recycling of nutrients derived from biomass residues. The first step in the recycling of nutrients contained in biomass residues is an extracellular enzymatic attack on organic macromolecules (Quiquampoix et al., 2007) and these reactions are prone to effects by the soil matrix. To the best of my knowledge only one study (Bailey et al., 2011) addressed the issue of how biochar could affect enzymatic reactions in soils.

In this thesis the effects of chestnut wood char and activated carbon on a typical soil enzymatic reaction are tested. With a SSA of 2.0 m^2g^{-1} and approximately 900 m^2g^{-1}, respectively, these two materials can be considered as two extremes in a continuum of SSA variation among charred materials. Wood char is characterized by condensed, rigid and aromatic structures with high carbon contents and relatively few polar functional groups (Cornelissen et al., 2005). It consists of randomly oriented stacks of graphitic layers (Schmidt et al., 2000) (Figure 3). Activated carbon is obtained from charcoal by a physical or chemical activation process which increases the SSA by 2-3 orders of magnitude.

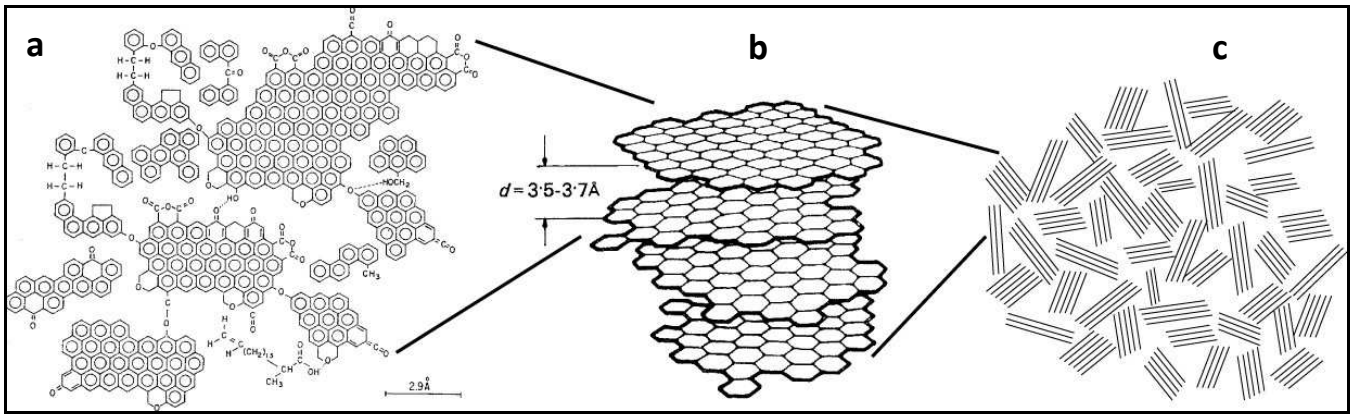

Figure 3. Structure of black carbon: (a) molecular structure of layers**,** (b) basic structural units of three to four layers, (c) randomly oriented basic structural units consisting of a few graphite layers. (Schmidt and Noack, 2000; modified)

1.5. The role of β-glucosidase in the degradation of cellulose

Cellulose is the world's most abundant organic polymer (Kögel-Knabner, 2002): its production amounts to $4 \cdot 10^{10}$ tonnes per year (Goyal et al., 1991) and it represents a major sink of atmospheric CO_2 and a relevant source of soil organic carbon. The most abundant plant tissue types that reach the soil for degradation are parenchyma and wood, and cellulose

is a major structural component of the cells of both of them (Kögel-Knabner, 2002). The degradation of cellulose to glucose is a complex and mainly extracellular process involving three main steps (Eveleigh, 1987): i) attack of amorphous (non-crystalline) regions by endoglucanase (EC 3.2.1.4), ii) cleavage of cellobiose from the exposed chain ends by cellobiohydrolase (EC 3.2.1.91), and iii) hydrolysis of cellobiose to glucose by cellobiase (i.e. β-glucosidase; EC 3.2.1.21). β-glucosidase thus plays an important role in the soil C cycle, and its activity level has been suggested as an indicator of soil quality (Ajwa et al., 1999; Bandick and Dick, 1999; Knight and Dick, 2004; Gil-Sotres et al., 2005; Garcia-Ruiz et al., 2008). The main cellulose decomposing organisms are fungi, but also many eubacteria are capable of degrading it (Kögel-Knabner, 2002). *Aspergillus niger* is a filamentous fungus belonging to the phylum Ascomycota. Its natural habitats are mainly soil and litter where it grows aerobically on organic matter (Schuster et al., 2002). This fungus is equipped with a large complement of extracellular enzymes, including polysaccharide-degrading ones (Hertz-Fowler and Pain, 2007).

Together with endoglucanase and cellobiohydrolase, β-glucosidase enables *A. Niger* to use cellulose, which is a major component of the organic matter input to soils, as a growth substrate. These enzymes perform their catalytic function in the extracellular environment, where the solid matrix can profoundly affect the catalysis rate by interacting with the enzymes and/or the substrates.

However, the information on the interaction of β-glucosidase from *A. niger* with soil solid materials is very limited and virtually no information is available on how these materials affect the hydrolysis of cellobiose (the natural substrate of β-glucosidase) by this soil enzyme. In a previous study, the interaction between β-glucosidase from *A. niger* and montmorillonite was analysed by using 4-nitrophenyl-3-D-glucopyranoside as substrate; it was found that the

adsorption of the protein increases with decreasing pH below neutrality and that the decrease in hydrolysis rate follows a similar pattern (Quiquampoix et al., 1989).

In order to provide information on the effects of the solids on the natural enzymatic reaction as a whole, rather than on the enzyme alone, the natural substrate needs to be studied how (soil) solid materials (i.e. montmorillonite, kaolinite, goethite, chestnut wood char and activated carbon) affect cellobiose hydrolysis by β-glucosidase from *A. niger*. Indeed, the interactions of the solid phase with the substrate can be as essential as those with the enzyme in determining the reaction rate. In the experimental approach to the enzymatic hydrolysis of cellobiose to glucose by β-glucosidase in presence of potentially sorptive surfaces, the conceptual model used is shown in Figure 4. In theory, substrate molecules (i.e. cellobiose) may be either adsorbed to surfaces or dissolved. Also enzyme (i.e. β-glucosidase) and product (i.e. glucose) molecules may find themselves in one of those two states. In theory, only dissolved substrate may come in contact with enzyme molecules and be transformed into product. However, dissolved substrate can interact with both dissolved and adsorbed enzyme molecules to generate product: usually the catalysis rate is lower for adsorbed enzyme molecules but the amount of catalytic activity lost relative to the dissolved enzyme largely depends on specific properties of the protein and the sorbent.

1.6. Enzymes in industrial processes

Enzymes are used in several industrial processes (van Beilen and Li, 2002), for instance in the production of high fructose syrup (glucose isomerase, EC 5.3.1.5) and the hydrolysis of starch (pullulanase, EC 3.2.1.41). Soil is a vast and still largely unexplored reservoir of enzymes with potential industrial applications. The microbial biodiversity of soil is huge and even organic compounds that are not present in nature are often degraded by entering existing

metabolic pathways or by causing the evolution of new pathways (Copley, 2009). Enzymatic processes could not only lead to new products but also provide an alternative to inorganic synthesis of presently produced chemicals carrying the advantage of mild reaction conditions (for instance low temperatures and pressures, water as solvent) which could reduce the energy intensity of the process and reduce the environmental impact (Gavrilescu and Chisti, 2005). Furthermore enzymes can be produced in large quantities by heterologous expression and, unlike inorganic catalysts, they can be produced based on renewable sources. The applicability of an enzyme which catalyses a commercially interesting reaction largely depends on the cost of producing and purifying the biocatalyst, and on the amount of product that can be obtained with a certain amount of it. The product/biocatayst ratio can be increased by immobilizing the biocatalyst in a suitable matrix: this allows the re-use of the catalyst for several batch reactions or the construction of continuous-flow reactors. Among the materials tested as enzyme immobilization matrices (i.e. agarose, alginate, Eupergit ® C, biosilica particles), biosilica deserves particular attention since the particle size and shape can be controlled by manipulating the conditions of in vitro formation (Naik et al., 2003) and because the enzymes immobilized in it often retain a very high fraction of their activity (up to 90 %) (Luckarift et al., 2004; Betancor et al., 2007). Also, the use of biosilica as a matrix to immobilize enzymes is an example of how technology can be developed from insights into natural biological processes (Kröger et al., 1999; Gill and Ballesters, 2000; Kröger et al. 2002; Foo et al., 2004). β-glucosidase from *A niger* may be used in the industrial hydrolysis of cellulose to glucose, which may then be transformed in a range of different valuable products such as polyols or polyesters (van Dam et al., 2005). Therefore different matrices which are, or coud be used in industrial processes were tested for their suitability for β-glucosidase immobilization: the leakage of enzyme molecules from the matrices and the residual activity of immobilized enzyme molecules was measured.

1.7. Aims of the study

The goal of the present work was to apply a new approach for studying the interactions of extracellular enzymes with (soil) solid materials. By using the natural substrate instead of a chromogenic one, the results have a wider ecological significance since they refer to a biochemical reaction that actually occurs in nature rather than to a model reaction that only provides information on potential activity, as is the case when chromogenic substrates are used. This potential activity may indeed be radically different from actual activity under many circumstances, such as when the sorption of the natural substrate to soil materials (sorption isotherm, reversibility) is significantly different from the sorption of the chromogenic one. This approach was applied to an ecologically important extracellular enzymatic reaction (i.e. hydrolysis of cellobiose by β-glucosidase from *A. niger*) to explore how it is affected by solid (soil) materials such as montmorillonite, kaolinite, goethite, chestnut wood charcoal and activated carbon. Sorption of cellobiose and β-glucosidase by these materials, and the resulting impacts on the hydrolysis rates were quantified (Figure 4). Mechanistic explanations of the sorption processes and of the variations in the reaction rates are proposed. Furthermore, several matrices of potential interest for the industrial application of enzymatic processes were tested for their suitability for β-glucosidase immobilization.

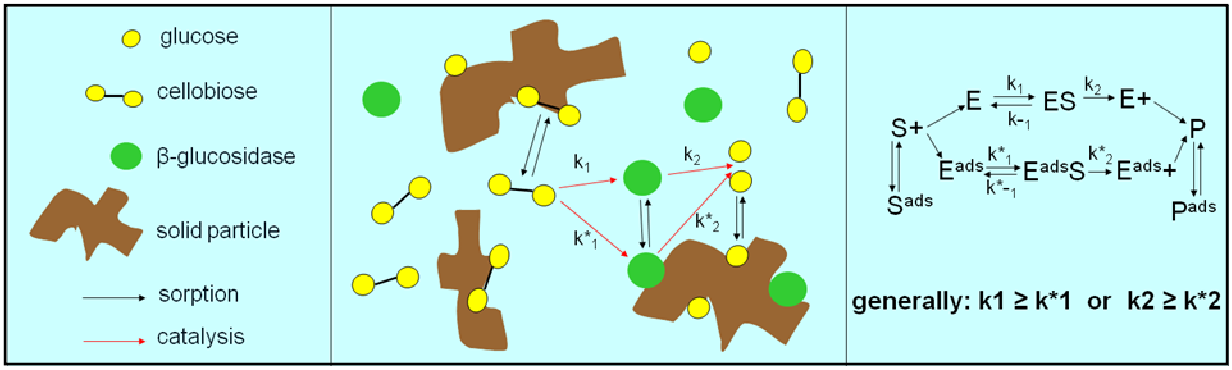

Figure 4. Conceptual model of cellobiose hydrolysis in presence of potentially sorptive solid surfaces. Dissolved cellobiose molecules can interact with both adsorbed and dissolved β-glucosidase molecules. Adsorbed β-glucosidase molecules tend to catalyse at a lower rate than dissolved ones (Lammirato et al., 2010; Lammirato et al., 2011).

2. Materials and methods

2.1. Buffer and solid phases

The experiments were performed in buffer solutions to keep the pH constant. To avoid possible dissolution of the iron mineral goethite, sodium phosphate buffer (67 mM, pH 5.0) was used instead of the citrate buffer used in previous experiments (Quiquampoix, 1987a; Quiquampoix, 1987b; Quiquampoix et al., 1989). Citrate chelates iron, which can increase the solubility of goethite (Essington et al., 2005). In addition, phosphate buffer does not interfere with the enzymatic reaction. The phosphate buffer used in the experiments was autoclaved immediately after preparation in order to preserve it.

pH 5.0 was chosen because this is approximately the optimum for activity of β-glucosidase from *A. niger* in presence of montmorillonite (Quiquampoix et al., 1989). In addition, β-glucosidase extracted from various soil fractions show activities between 80 and 100% of maximum activity at pH 5.0 (Busto and Perez-Mateos, 2000).

Three minerals were used in this study: Na-montmorillonite (SWy-2; Clay Minerals Society, Chantilly, USA), kaolinite and goethite (both from Sigma-Aldrich; St. Louis, USA). For the determination of the specific surface areas of the minerals, a BELSORP-mini volumetric adsorption analyser (BEL Inc., Osaka, Japan) was used for N_2 sorption isotherm measurement. A multipoint BET (i.e. Brunauer, Emmett, Teller) plot (Brunauer et al., 1938) was used to evaluate the monolayer N_2 adsorption capacity of the materials and from this the specific surface areas were calculated (Brunauer et al., 1938; Dollimore et al., 1976). For each of the minerals used, the electrical charge at the surface of the double layer in the described sodium phosphate buffer was analysed by measuring the ζ- potentials with a Zetasizer Nano

from Malvern Instruments (Malvern, UK). To increase the reproducibility of the measurements, the concentration of the solid phase in suspension was reduced to 0.2 mg ml^{-1}.

Chestnut wood char was obtained from Michael Schmidt, University of Zürich, Department of Geography, Zürich, Switzerland. This material was obtained from debarked chestnut wood pyrolized at 450°C (a common temperature for natural forest fires; Turney et al., 2006) for 5 h under N_2 atmosphere (Hammes et al., 2006). If compared with other biochars (Qiu et al., 2008), the specific surface area (2.0 m^2g^{-1}) of the chestnut wood char is low, even though the material was ground in a ball mill to a fine powder (Hammes et al., 2006). Its porosity is therefore likely to be negligible. Some properties of the chestnut wood char are shown in Table 1 (Hammes et al., 2006; modified)

Activated carbon (Darco G-60) was obtained from Sigma-Aldrich (St. Louis, USA); its BET specific surface area is approximately 900 m^2g^{-1}according to Zajac et al. (1997) and ca 762 m^2g^{-1} according to Qiu et al. (2008). Data from the literature on the properties of the activated carbon Darco G-60 are shown in Table 1 (Zajac et al., 1997; Qiu et al., 2008).

Table 1. Physicochemical properties of chestnut wood charcoal[1] and activated carbon (Darco G-60)[2,3].

	Wood charcoal[1]	Activated carbon[2,3]
BET surface area [m^2 g^{-1}]	2.0	761.6[2] - 923[3]
S_{ext}[a] [m^2 g^{-1}]	n.a.	182[3]
Polar surface area[b] [m^2 g^{-1}]	n.a.	53[3]
Apolar surface area[c] [m^2 g^{-1}]	n.a.	860[3]
Porosity [d] [cm^3 g^{-1}]	n.a.	0.716[2]
Micropore volume[e] [cm^3 g^{-1}]	n.a.	0.31[3]
C [g kg^{-1}]	682	884[2]
N [g kg^{-1}]	1.6	n.a.
H [g kg^{-1}]	39.8	8.8[2]
O [g kg^{-1}]	270.6	80.2[2]

[1] all data from Hammes et al. (2006)
[2]Qiu et al. (2008)
[3] Zajac et al. (1997)
[a] mesopore (> 0.5 nm in diameter) and external surface area
[b] estimated from the enthalpy of adsorption of n-butanol from n-heptane
[c] estimated from the enthalpy of adsorption of n-butanol from water
[d] total pore volume of pores less than 77.05 nm in diameter
[e] pores less than 0.5 nm in diameter

The solid phases were washed with the phosphate buffer: after 24 h of shaking at room temperature, the solid phases were separated by centrifugation (10 min. at 11 180 g with a 17 RS centrifuge, Heraeus Sepatec Instruments, Osterode, Germany) and suspensions were prepared by resuspending 20 mg ml^{-1} of each solid in the same buffer. Small deviations from

pH 5.0 were corrected with addition of phosphoric acid. The reactions took place under non-sterile conditions, but with sterilized solutions, because significant microbial activity was not expected in the short experimental period (4.5 h for the adsorption processes and 60 min for the hydrolytic processes).

2.2. Substrate and enzyme

D-(+)-cellobiose from Sigma-Aldrich (St. Louis, USA) was used as substrate for β-glucosidase. The β-glucosidase preparation from a submerged fermentation of *A. niger* was supplied by Sigma-Aldrich (St. Louis, USA). The preparation was used without further purification after removal of suspended particles by centrifugation (15 700 g for 10 min. with a 5415R centrifuge, Eppendorf, Hamburg, Germany).

A Bradford protein assay (Bio-Rad Laboratories; Munich, Germany) using BSA as standard showed that the protein concentration of the preparation was 43.4 ± 1.1 mg ml^{-1}. In order to determine the number of reactive β-glucosidase isoforms and to provide an approximate estimation of their abundance in comparison to other proteins, a native gel electrophoresis (polyacrylamide gel electrophoresis: PAGE) was performed using a Biorad Mini PROTEAN® 3 system (Bio-Rad Laboratories; Munich, Germany) following the manufacturer's instructions. The chromogenic substrate 4-nitrophenyl β-D-glucopyranoside (PNPG) from Sigma-Aldrich (St. Louis, USA) was used as an indicator of the hydrolytic activity of β-glucosidase. For staining of total protein in the preparation, Coomassie Brilliant Blue (Bio-Rad Laboratories; Munich, Germany) was used. The lanes (Figure 5) with the two different stainings where compared to identify the active components of the preparation. In order to determine the molecular weight of β-glucosidase, a denaturing gel electrophresis (SDS-PAGE) was performed using a Biorad Mini PROTEAN® 3 system (Bio-Rad

Laboratories; Munich, Germany) following the manufacturer's instructions. A protein marker (New England Biolabs; Ipswich, USA) was used as reference for the molecular weight.

2.3. Quantification of glucose and cellobiose concentrations and determination of enzymatic activity

The progress of cellobiose hydrolysis was monitored by measuring the increases in glucose concentration over time. Glucose concentrations were measured with a kit from Sigma-Aldrich (St. Louis, USA) which was modified for use in microtiter plates. The test is based on the activities of hexokinase (glucose + ATP → glucose-6-phosphate + ADP) and glucose-6-phosphate dehydrogenase (glucose-6-phosphate + NAD → 6-phosphogluconate + NADH; this second step makes the test specific for glucose). The absorbance at 340 nm, caused by the NADH produced by the reactions, was measured with a Victor^{2}™ 1420 Multilabel Counter (PerkinElmer®, Waltham, USA). The glucose concentrations of the samples were calculated from the measured absorbances by means of a standard curve.

In order to measure the adsorption of cellobiose to the minerals, it was necessary to determine the residual cellobiose concentrations in the supernatants. These residual cellobiose concentrations were quantified by hydrolysing the cellobiose in the supernatant samples to glucose with β-glucosidase (protocol: after dilution 1/3, 4 µl of β-glucosidase preparation were added to 650 µl of supernatant samples; these samples were then incubated at 45 °C for 3 h and finally boiled for 3 min.). The glucose concentrations resulting from cellobiose hydrolysis were then measured with the method described above and correlated to the pre-hydrolysis cellobiose concentrations by means of a standard curve. The standard curve (standard cellobiose concentrations: 0.5, 1, 1.5 and 2 mM) was highly linear and the amount of cellobiose converted to glucose was 75 ± 1 %.

In all experiments on β-glucosidase adsorption and activity in the presence of solids, enzymatic activity was defined as initial reaction rate. The concentration of glucose produced by the reaction was measured after 0, 10, 20, 60 min. The initial cellobiose concentration was 2 mM. The data points were fitted to a quadratic equation (Eq.1) and the first derivative of this equation at time zero was taken as a measure of the initial reaction rate (Eq. 2):

$$f(t) = at^2 + bt = [G] \qquad \text{(Eq. 1)}$$

$$f'(t) = 2at + b, \text{ thus } f'(0) = b \qquad \text{(Eq. 2)}$$

where [G] = glucose conc. [mM]; t = time [min.]; b = initial reaction rate [mmol l^{-1} $min.^{-1}$].

The Michaelis-Menten constant (K_M) of β-glucosidase for cellobiose was quantified by adding 1.5 µl of enzyme preparation to 250 ml cellobiose samples (0.1, 0.5, 1, 2, 4 mM) in phosphate buffer. Samples were taken after 0, 5, 10, 15, 20 min. and boiled for 3 min.; the glucose concentrations were then measured and the initial reaction rates calculated. The Lineweaver-Burk method (Lineweaver and Burk, 1934) was applied to quantify K_M.

2.4. Adsorption of glucose, cellobiose and β-glucosidase to the minerals and the charred materials

In order to determine the equilibrium and kinetics of glucose/cellobiose adsorption to the solid materials, the residual concentration in the supernatants after removal of the solid phase was quantified: 1ml of glucose/cellobiose solution (150 mM) was added to 74 ml of the suspensions of the solids, resulting in a final concentration of 2 mM. The solid phase was kept in suspension by gently mixing on a shaker at 25 °C; samples were collected at various times over a period of approximately 4 h and were filtered through 0.2 µm PTFE syringe filters

(Acrodisic PSF Syringe Filters, Pall Corporation, USA) to remove the solid phase. Residual glucose and cellobiose concentrations were measured by the methods described above.

Measurements of dissolved enzymatic activity were used to quantify β-glucosidase adsorption. The residual activity in the supernatants after removal of the solid phase (and thus the adsorbed enzyme) was quantified and the fraction of missing activity relative to the negative controls (i.e. β-glucosidase added to supernatants instead of the suspensions of the minerals) was considered equal to the adsorbed fraction of β-glucosidase. Briefly, 5.3 µl of enzyme preparation (diluted 1/3) were added to 31 ml of the mineral suspensions and of the supernatants (the average activity of that amount of enzyme in 31 ml of 2 mM cellobiose supernatants was 17.7 ± 1.4 µmol glucose l^{-1} $min.^{-1}$). Triplicate samples were shaken for 20, 40 and 80 min. at 25 °C. After these adsorption times, the samples were centrifuged for 10 min. at 11 180 g (with a 17 RS centrifuge, Heraeus Sepatec Instruments, Osterode, Germany) and 15 ml of the supernatants were collected in Erlenmeyer flasks. Then the residual enzymatic activity in these supernatants was measured: hydrolysis reactions were started with 200 µl of a concentrated cellobiose solution (150 mM) being added to each of the Erlenmeyer flasks (14.8 ml) at final concentrations of 2 mM; samples from the reaction mixtures were taken after 10, 20 and 60 min., boiled for 3 min. and stored at -20 °C. Finally the glucose concentrations in the supernatants were measured and the initial reaction rates were calculated as described above.

During the preparation of the experiments on cellobiose hydrolysis in the presence of activated carbon, it was necessary to equilibrate aliquots of the activated carbon separately with cellobiose and β-glucosidase at twice the experimental concentrations (see below). Therefore, the adsorption of cellobiose and β-glucosidase was also measured at these concentrations (i.e. 4 mM total cellobiose concentration; 10.6 µl of enzyme preparation, diluted 1/3, in 31 ml of suspension) to ensure that sorption equilibria under this conditions do

not differ from those under the "standard" conditions for which the effects of the solids on the reaction rate were measured (i.e. 2 mM total cellobiose concentration; 5.3 µl of enzyme preparation, diluted 1/3, in 31 ml of suspension).

2.5. Effects of the minerals and the charred materials on the reaction rates

The adsorption experiments showed that montmorillonite, kaolinite, goethite and chestnut wood char did not adsorb cellobiose nor glucose. Therefore, in order to analyse how the hydrolysis rate is affected by the presence of the solids, 5.3 µl of enzyme preparation (diluted 1/3) were added to 30.6 ml of the suspensions. After 2 h of adsorption time, hydrolysis reactions were started with 413 µl of cellobiose solution (150 mM) being added to the suspensions in order to reach a total cellobiose concentration of ≈ 2 mM. After 10, 20 and 60 min., samples were collected and filtered. The filtrates (approximately 500 µl) were boiled for 3 min. in order to destroy enzymatic activity and then stored at -20 °C. Negative controls (supernatants taken before enzyme addition) were treated in the same way. For all samples and controls, three independent replicates were run independently. Finally, the glucose concentrations were measured and the enzymatic activity was calculated.

The sorption experiments showed that activated carbon absorbed more than 97% of cellobiose. Since the aim was to measure the effect of activated carbon on the reaction rate under sorption equilibrium conditions (both for substrate and enzyme), it was important to avoid concomitant cellobiose absorption to activated carbon and hydrolysis by β-glucosidase. Indeed, if cellobiose hydrolysis would take place before the cellobiose absorption equilibrium is reached, the (decreasing) dissolved substrate concentrations would determine the reaction rate. To avoid this temporal overlap, cellobiose and β-glucosidase were allowed to reach sorption equilibrium (considered to be reached after 4.5 h from the start of the sorption

processes) with activated carbon in separated suspensions. During this sorption processes the total concentrations of both substrate and enzyme were doubled as described above, whereas all other conditions remained unchanged. The sorption of substrate and enzyme was analyzed also under these conditions and found to proceed to more than 98 %. To determine the effect of substrate and enzyme sorption to (distinct) activated carbon particles on the reaction rate, equal volumes (15.5 ml) of the suspensions containing absorbed cellobiose and adsorbed β-glucosidase were then mixed, resulting in the desired total concentrations of substrate and enzyme. The progress of the reaction was then monitored by means of total glucose concentration determinations.

At equilibrium, sorption of glucose to activated carbon amounted to 32 ± 2 % in a concentration range from 0.2 to 1 mM. Measurements of total glucose concentrations in presence of activated carbon were corrected to account for this adsorbed glucose pool.

2.6. Enzyme immobilization in matrices of potential industrial interest

For the immobilization of β-glucosidase in agarose beads, 75 ml of 4 % agarose (Sigma-Aldrich, St. Louis, USA) solution was heated in a microwave oven for 5 min. The agarose solution was left at room temperature to cool down and 170 µl of β-glucosidase preparation were added at 55 °C. Then 2.5 ml of β-glucosidase containing agarose solution were dropped through a needle into 75 ml of deionized water at 4 °C. The leakage of the encapsulated enzyme was quantified by leaving the enzyme containing beads obtained from 2.5 ml of 4 % agarose in 75 ml of sodium phosphate buffer (67 mM, pH 5.0) for 6 h and by determining the increase in the enzymatic activity in the solution during this period. After 6 h also the residual activity of the alginate beads was measured after washing the particles with deionized water. The initial cellobiose concentration for the enzymatic activity tests was 2

mM in a volume of 75 ml. In the controls, an amount of β-glucosidase equal to that contained in the alginate beads used for the leakage experiment (i.e. 5.6 µl of β-glucosidase preparation) was present in freely dissolved state.

For the immobilization of β-glucosidase in alginate beads the method used by Busto et al. (1995) was used with modifications. Alginic acid sodium salt for the immobilization of β-glucosidase in alginate beads was obtained from Sigma-Aldrich. 2.5 g of Na-alginate were dissolved in 60 ml of deionized water (4% alginate) and then 135 µl of β-glucosidase preparation were added. Then 2.5 ml of Na-alginate + enzyme were dropped through a needle into 70 ml of 0.1 M $CaCl_2$ stirred solution and the beads were left there for hardening for 1 h 20 min. The leakage of the encapsulated enzyme was quantified by the same method used for the agarose beads.

In order to covalently bind β-glucosidase through oxirane groups to the external and internal surfaces of macroporous Eupergit® C particles (Sigma-Aldrich, St. Louis, USA), the manufacturer´s instructions were followed. Briefly, 0.37 g of Eupergit® C particles were added to 7.5 ml of sodium phosphate buffer (pH 7, 1 M) containing 1 % glucose and 1 % hydrobenzioc acid. Then 115 µl of enzyme preparation was added to the suspension and incubated for 20 h in a shaker at room temperature. After incubation the particles were separated from the supernatant and washed 4 times with deionized water. The leakage of the enzyme from Eupergit® C particles was determined by shaking the particles in 75 ml of sodium phosphate buffer (67 mM, pH 5.0) for 6 h, separating the particles by centrifugation and then measuring the enzymatic activity of the supernatant and the particles separately (initial cellobiose concentration 2 mM in 75 ml). In the controls, an amount of β-glucosidase equal to that contained in the Eupergit® C particles used for the leakage experiment (i.e. 115 µl of β-glucosidase preparation) was present in freely dissolved state. In the controls this amount of enzyme generated a fast reaction which could not be well described by the

quadratic equation used to describe all other reaction kinetics. In this case the initial reaction rate was considered to be equal to the average reaction rate in the first 10 min.

For the immobilization of β-glucosidase in biosilica particles, the method described by Luckarift et al. (2004) was used. Briefly, tetramethyl orthosilicate (TMOS) was obtained from Sigma-Aldrich (St. Louis, USA) and the R5 peptide (H2N-SSKKSGSYSGSKGSKRRIL-COOH) from New England Peptides (Gardner, USA). Biosilification was obtained by sequentially adding the following solutions into a reaction tube: a) 80µl of β-glucosidase preparation (diluted 9 times in sodium phosphate buffer 0.5M, pH 7); b) 10µl TMOS 1 M in HCl 1 mM (hydrolysis time 30 min); c) 10µl of R5 peptide dissolved in deionized water (100 mg ml^{-1}). The mixture was gently shaken and after 25 min the supernatant was removed by centrifugation (1 min at 14000 rpm; 5415R centrifuge, Eppendorf, Hamburg, Germany). The particles were washed 2 times with 100 µl of sodium phosphate buffer (16.5 mM, pH 5). The amount of activity in the biosilica particles and supernatant + wash fractions was measured immediately after the immobilization protocol in 70 ml sodium phosphate buffer (67 mM, pH 5.0) with an initial cellobiose concentration of 2 mM.

For the immobilization of β-galactosidase (EC 3.2.1.23) from *E. coli* (lyophilized powder; Sigma-Aldrich, St. Louis, USA) in biosilica particles, 1.5 mg of protein powder was dissolved in 1 ml of phosphate buffer (0.1M, pH 8) and 80 µl of the obtained enzyme solution were used for the immobilization protocol described for the immobilization of β-glucosidase in biosilica particles. The activities of the particles and of the supernatant + wash fractions were measured with initial lactose concentrations of 5 mM under the same conditions described for β-glucosidase in biosilica particles.

2.7. Statistics

In order to test the statistical significance of the difference between the mean initial hydrolysis rates in presence and absence of the minerals and the charred materials, a t-test was performed with Sigma Plot 11.0 (Systat Software INC., USA). The threshold p-value for statistical significance (α) was set at 0.05.

3. Results

3.1. Characterization of β-glucosidase

In order to estimate the effect of the solids on the substrate turnover, the enzyme preparation was first analysed by means of protein electrophoresis for the relative amount of reactive protein and the number of β-glucosidase isoforms.

The native gel stained with PNPG (Figure 5) showed one well-defined activity band supporting the thesis that only one isoform of β-glucosidase was present in the preparation. A corresponding band was visible in the other half of the same native gel stained with Coomassie Brilliant Blue. In addition a non-reactive smear of proteinaceous material preceded the β-glucosidase, which possibly derived from degradation products of the enzyme. Reactive β-glucosidase appeared to be the most abundant protein in the preparation.

From the denaturing gel (SDS-PAGE; Figure 6) the molecular weight of β-glucosidase was estimated to be approximately 110 kDa. Its K_M for cellobiose was determined to be 1.26 mM.

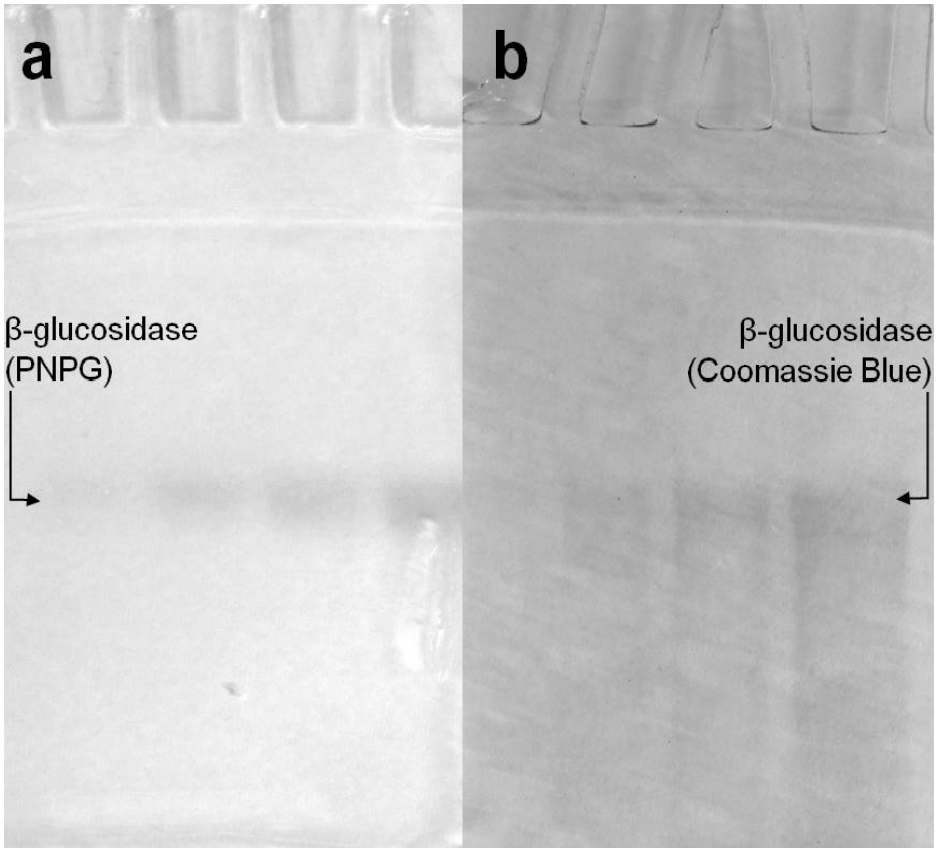

Figure 5. Native PAGE of the β-glucosidase preparation. Different stainings of the same gel: a) activity (PNPG), b) total protein (Coomassie Brilliant Blue). In both (a) and (b) the dilution of the enzyme preparation decreases from left (1/12) to right (1/4).

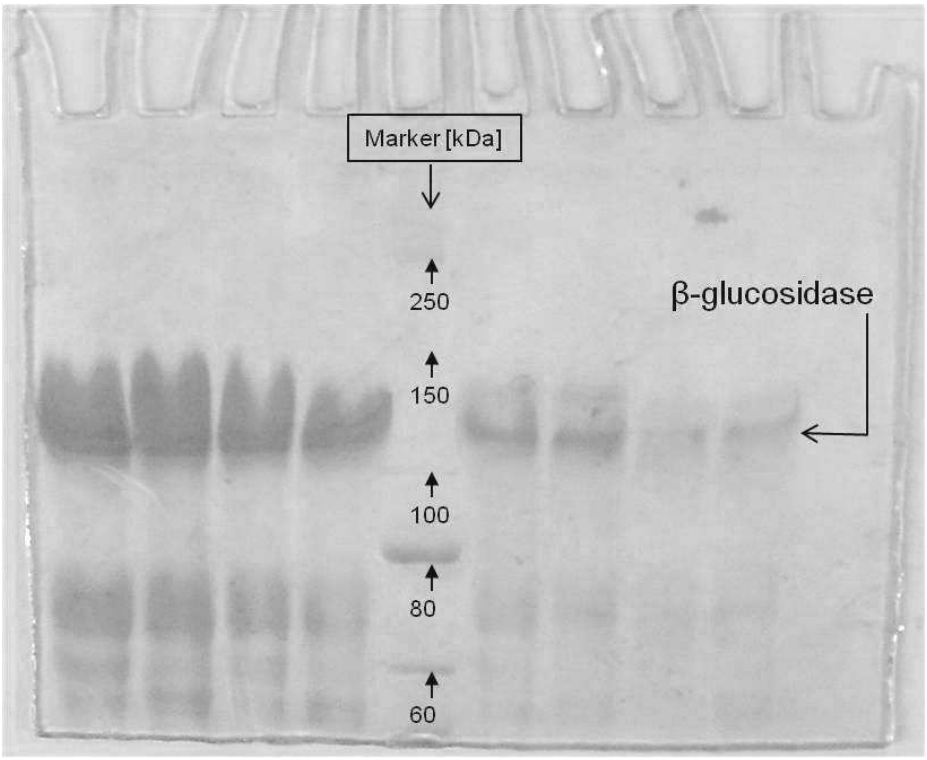

Figure 6. Denaturing (SDS) PAGE of the β-glucosidase preparation. Increasing dilution of the enzyme preparation from left (1/4) to right (1/32).

3.2. Specific surface area and ζ-potential of the minerals

The results of the specific surface area (SSA) and ζ-potential measurements are shown in Table 1 together with literature data on the point of net zero charge (PZC) and cation exchange capacity (CEC).

At pH 5.0, montmorillonite and kaolinite are above their PZC and therefore their surfaces are negatively charged; goethite, on the other hand, is below its PZC and its surface is positively charged.

The ζ-potential of a particle (i.e. the electric potential in the interfacial double layer at the location of the slipping plane versus a point in the bulk fluid away from the interface) is widely used for quantification of the magnitude of the electrical charge at the double layer (Delgado et al., 2005) and it largely determines the degree of repulsion/attraction between charged particles. Under the experimental conditions, the measured ζ-potentials were all

negative. The sign of the ζ-potential of a particle is supposed to be the same as that of the surface charge but the measurements showed that in the phosphate buffer this is not true for goethite. The negative ζ-potential of goethite was likely caused by adsorption of phosphate anions, resulting in charge reversal of the positively charged surface of goethite (Quiquampoix, 1987a). According to these measurements, all three materials are expected to behave as negatively charged surfaces in the adsorption processes at the given experimental conditions.

Table 2. Properties of the minerals used in this study: specific surface area (SSA), point of zero charge (PZC), cation exchange capacity (CEC), ζ-potential in sodium phosphate buffer (67 mM, pH 5.0).

	Montmorillonite	Kaolinite	Goethite
SSA [$m^2 g^{-1}$]	34.3	7.3	82.1
PZC [pH]	3.2 - 3.6[(1)]	2.7 - 3.2[(2)]	7.4 - 8.2[(2)]
CEC [meq/100 g]	76.4[(3)]	2 - 3.3[(3)]	NA
ζ-potential [mV]	-38 ± 5	-52 ± 3	-33 ± 2

[(1)] Ijagbemi et al. (2009)
[(2)] Appel et al. (2003)
[(3)] http://www.clays.org

3.3. Sorption of cellobiose and β-glucosidase to the minerals

The sorption processes of cellobiose, β-glucosidase and glucose to the mineral particles in the experimental systems are shown in Figure 7.

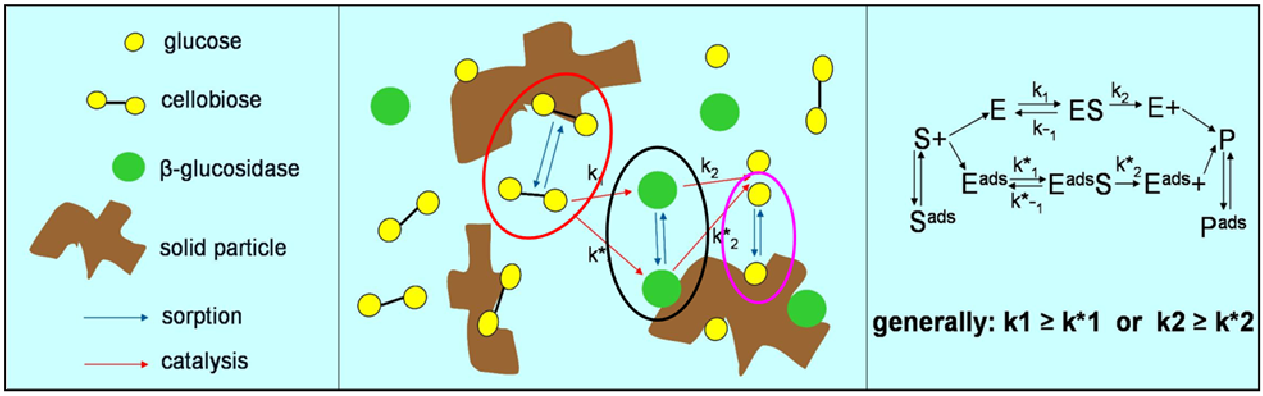

Figure 7. Adsorption of cellobiose ( ), β-glucosidase ( ) and glucose ( ) in the experimental systems.

No significant adsorption of glucose or cellobiose to the minerals was detected; this implies that the tested minerals did not diminish the dissolved substrate concentrations, i.e. they did not limit its availability to the enzyme. Activated carbon was used as a positive control (maximum adsorption). More than 97% of the cellobiose, but only 32 ± 2% of the glucose was sorbed to this material (see below).

Adsorption equilibrium for β-glucosidase was reached after 40 minutes, with adsorbed fractions amounting to 9.7 ± 7.3 %, 70.3 ± 3.1 % and 71.4 ± 1.8 % for montmorillonite, kaolinite and goethite, respectively (Figure 8).

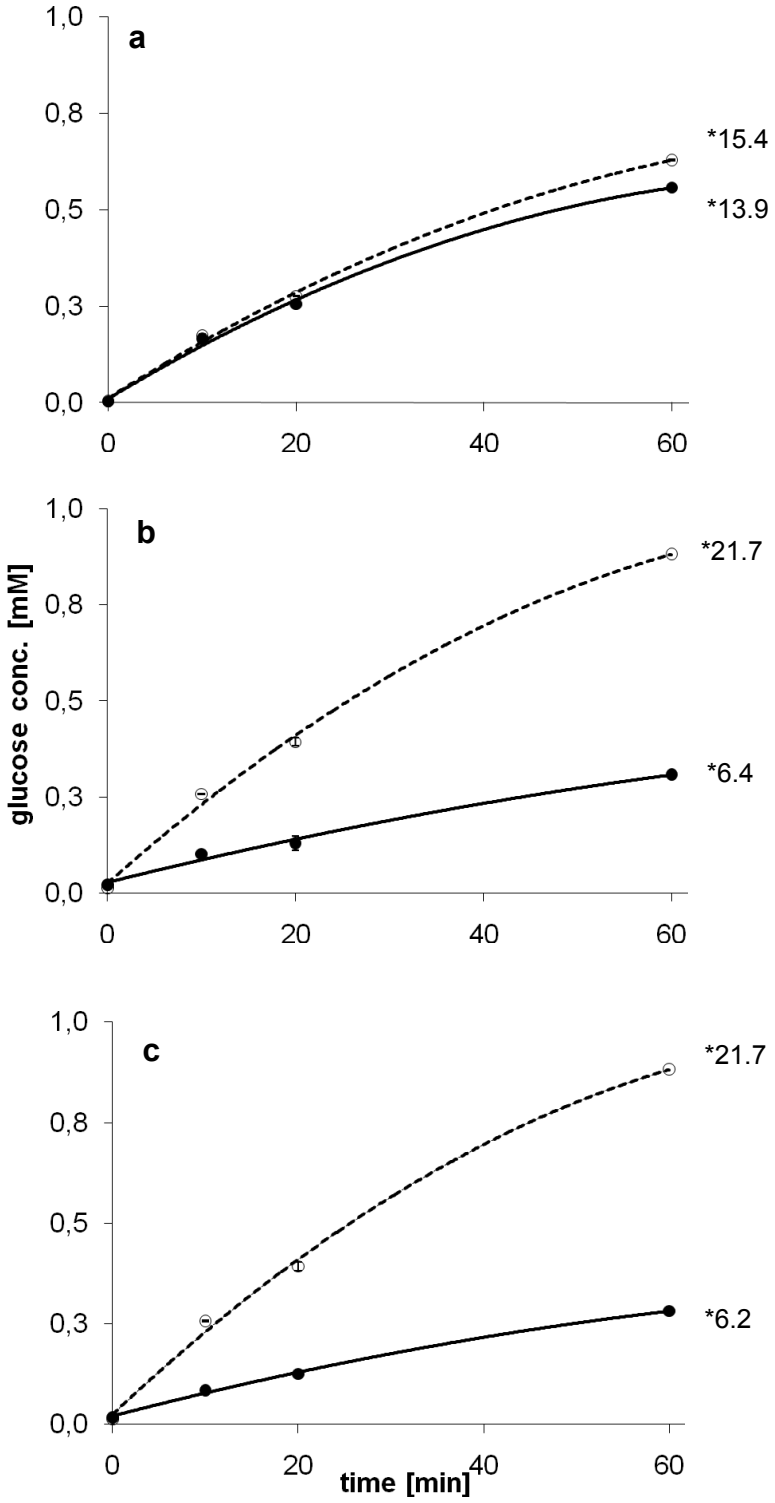

Figure 8. Enzymatic activity in supernatants (—●—) after removal of the solids in comparison to controls (----○----). The solids were removed after adsorption of β-glucosidase had reached equilibrium. The fraction of activity in the supernatants represents the fraction of dissolved enzyme. a) montmorillonite, b) kaolinite, c) goethite. (*initial reaction rate [μmol l^{-1} min^{-1}]). If error bars are not visible, they are smaller than the symbol size.

3.4. Sorption of cellobiose and β-glucosidase to the charred materials

The sorption processes of cellobiose, β-glucosidase and glucose to the charred materials in the experimental systems are shown in Figure 7.

Wood char did not adsorb glucose whereas activated carbon adsorbed 32 ± 2 % in a total concentration range from 0.2 to 1 mM.

Cellobiose (total concentration = 2mM) was not adsorbed by chestnut wood charcoal, whereas activated carbon absorbed more than 97 % after 4.5 h (Figure 9). For activated carbon, fast absorption during the first 20 min was followed by a much slower process, indicating that after saturating binding sites close to the surface of the particles, cellobiose molecules diffused into the inner micropores. With a total cellobiose concentration of 4 mM, as used for the preparation of the experiments on enzymatic hydrolysis in the presence of activated carbon, the solid adsorbed 98 % of the disaccharide after 3 h.

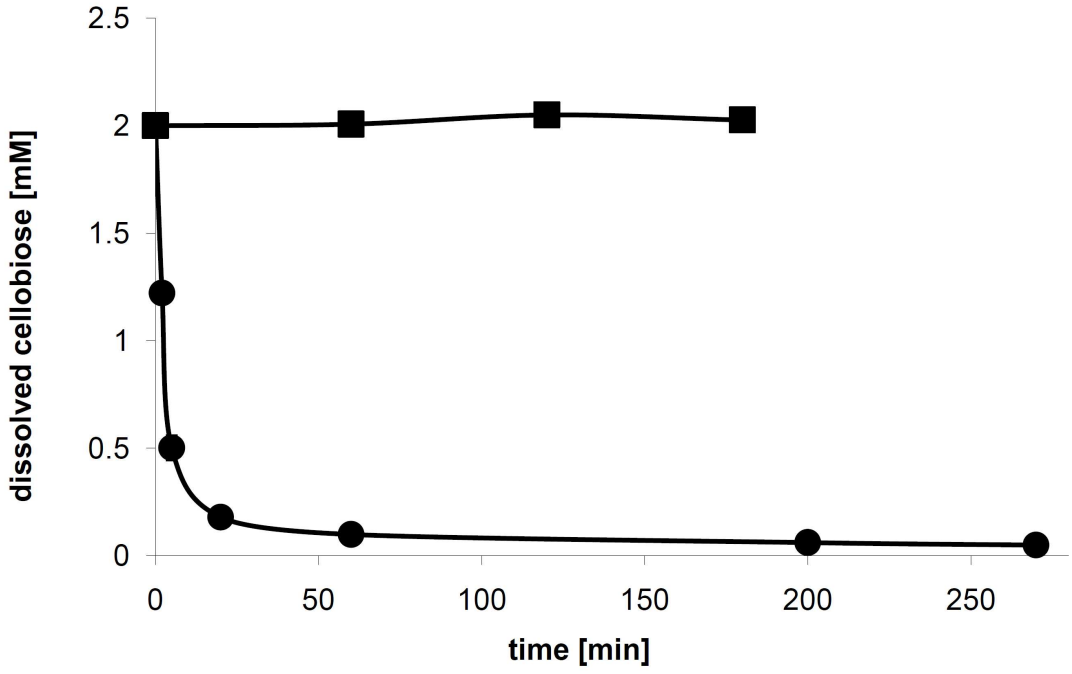

Figure 9. Kinetics of cellobiose sorption to chestnut wood char (—■—) and activated carbon (—●—). Total cellobiose concentration = 2 mM. If error bars are not visible, they are smaller than the symbol size.

Both chestnut wood charcoal and activated carbon adsorbed more than 99% of β-glucosidase in less than 1 h (Figure 10).

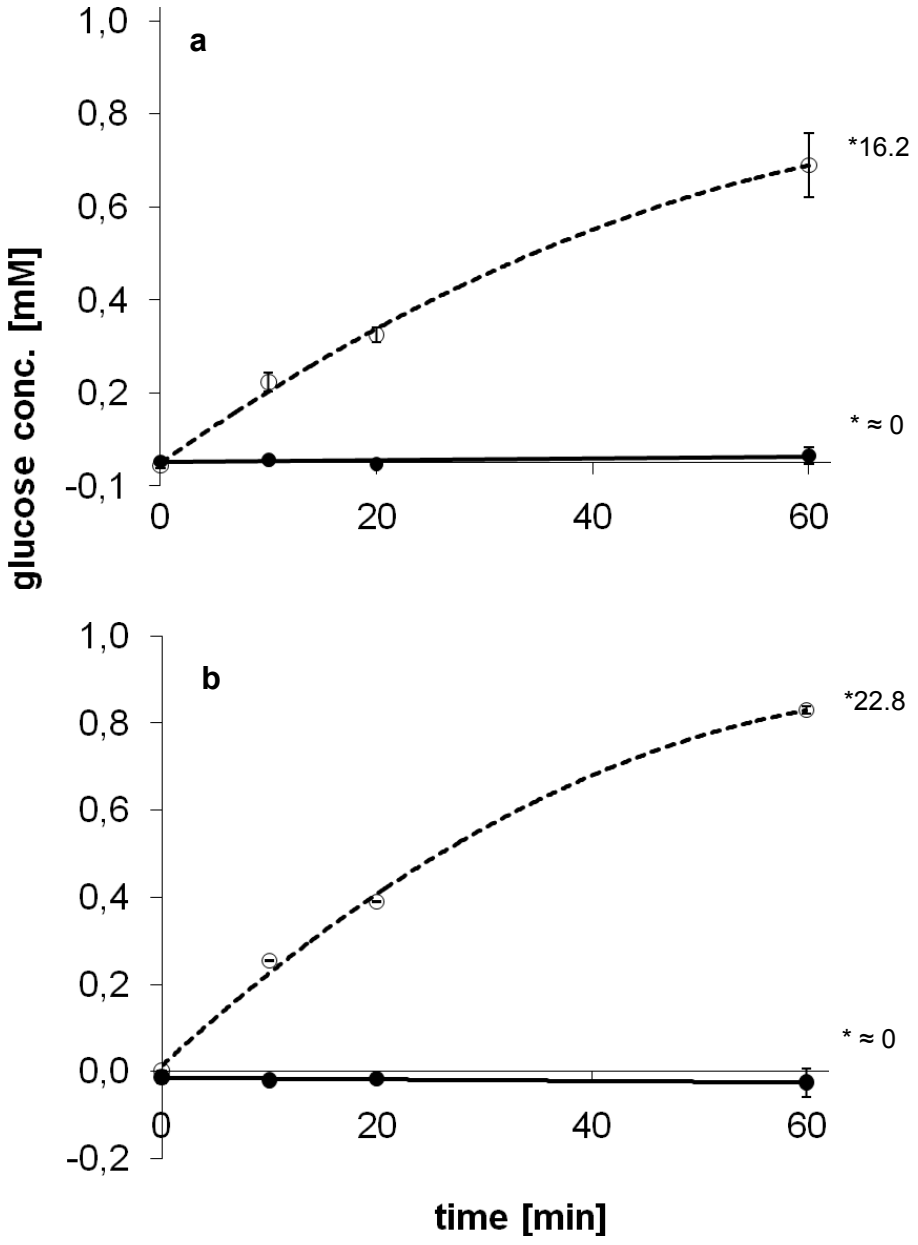

Figure 10. Enzymatic activity in the supernatants (—●—) of a) wood char and b) activated carbon after the removal of the solid phase in comparison to controls(----○----). The solid phase was removed after adsorption of β-glucosidase had reached equilibrium. The fraction of activity in the supernatants represents the fraction of dissolved enzyme. (*initial reaction rate [μmol l^{-1} min^{-1}]). If error bars are not visible, they are smaller than the symbol size.

For activated carbon, more than 99% of the total β-glucosidase was also adsorbed at double the standard concentration, as used for the preparation of the experiments with activated carbon (Figure 11).

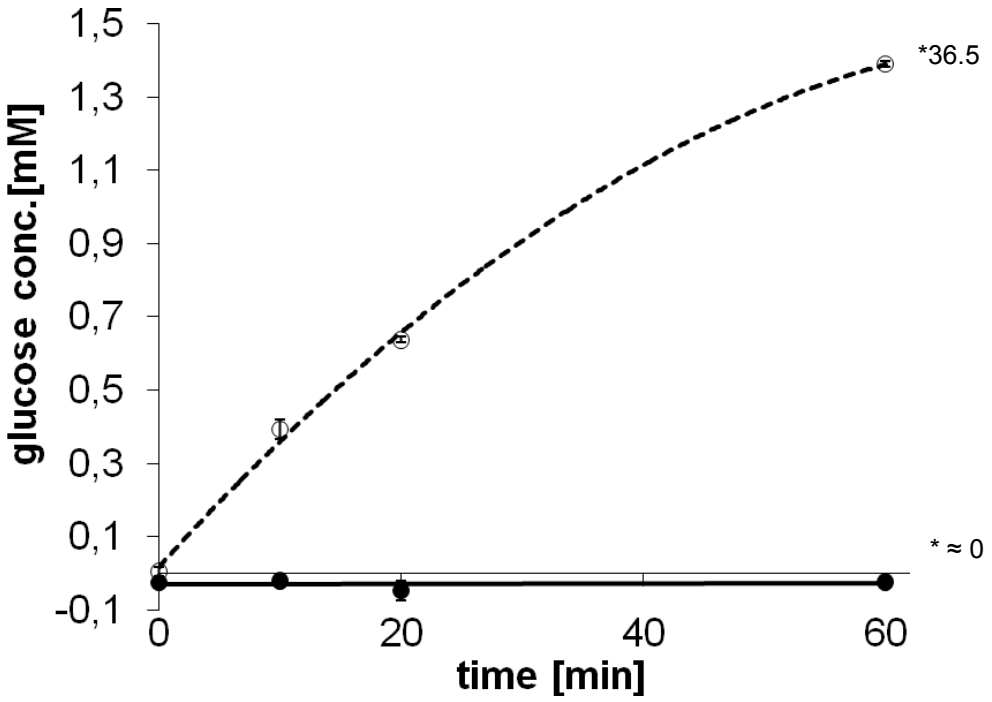

Figure 11. Enzymatic activity in the supernatants (—●—) of activated carbon after the removal of the solid phase in comparison to controls(----○----). The β-glucosidase concentration was twice the standard concentration (i.e. 10.6 µl of enzyme preparation, diluted 1/3, in 31 ml of suspension instead of 5.3 µl). The solid phase was removed after adsorption of β-glucosidase had reached equilibrium. The fraction of activity in the supernatants represents the fraction of dissolved enzyme. (*initial reaction rate [µmol l^{-1} min^{-1}]). If error bars are not visible, they are smaller than the symbol size.

3.5. Enzymatic reaction in the presence of the minerals

The enzymatic hydrolysis of cellobiose in presence of the minerals in the experimental systems is shown in Figure 12.

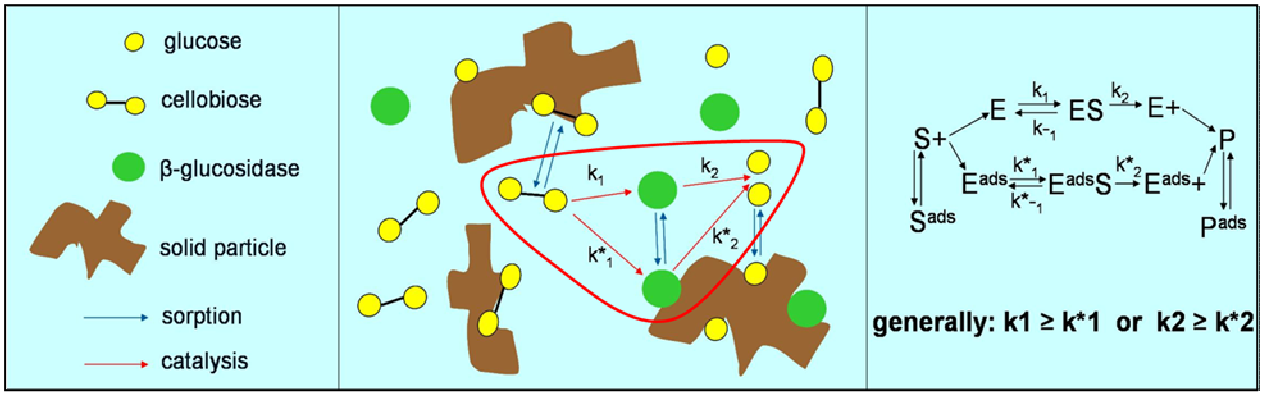

Figure 12. Hydrolysis of cellobiose in the experimental systems.

The adsorption experiments showed that cellobiose is not adsorbed by the minerals whereas β-glucosidase is. Adsorption of β-glucosidase is expected to cause a reduction in the hydrolysis rate of cellobiose. This effect is expected to be stronger for kaolinite and goethite than for montmorillonite, since the amount of adsorbed protein is higher for the first two minerals.

The comparison between total activities in the presence and the absence of minerals is shown in Figure 13. In comparison to the controls, i.e. the same amount of β-glucosidase in supernatants (average enzymatic activity of all controls = 17.7 ± 1.4 µmol glucose l^{-1} $min.^{-1}$), the total activity (TA) decrease caused by each mineral relative to the respective control amounted to 1.7 ± 6.8 % for montmorillonite, 18.8 ± 3.4 % for kaolinite and 17.9 ± 4.7 % for goethite. For kaolinite and goethite the difference of total activity in the samples and the controls was statistically significant ($P = 0.002$ and $P = 0.006$ respectively; $\alpha = 0.05$). For montmorillonite ($P = 0.734 > \alpha$) the hypothesis that the mean total activites in the samples and

the controls were the same cannot be rejected and the observed difference in the means is likely to have been originated by chance.

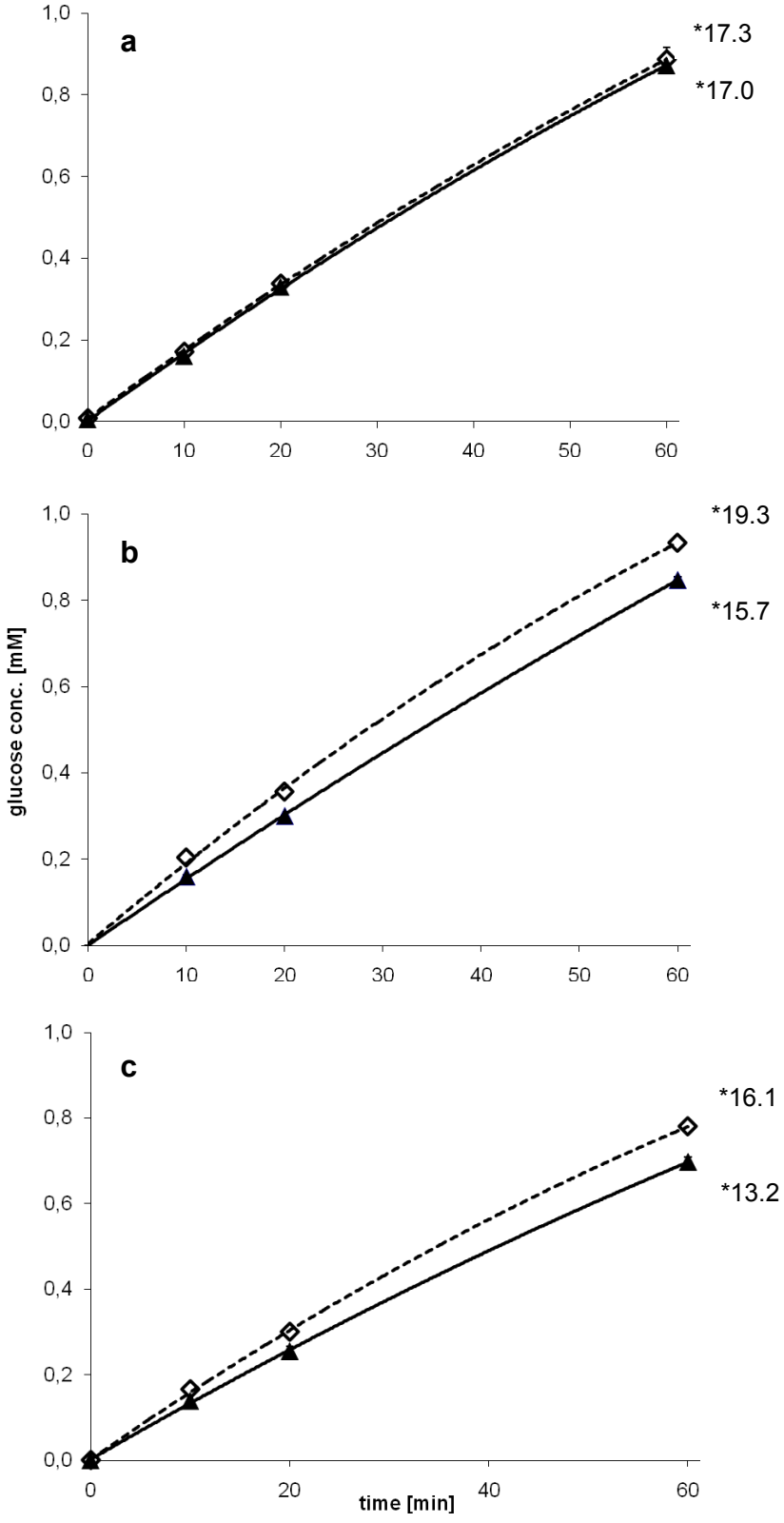

Figure 13. Effects of: a) montmorillonite, b) kaolinite and c) goethite on the β-glucosidase mediated hydrolysis of cellobiose. —▲— = in presence of solid particles; ---◇--- = controls in absence of solid particles. (*initial reaction rate [μmol l^{-1} min^{-1}]).

If error bars are not visible, they are smaller than the symbol size.

The method used to measure the effects of the minerals on the reaction rate shows how the total enzymatic activity (i.e. activity of dissolved and adsorbed molecules) is affected by the solids. It was assumed that the specific catalytic activity of the dissolved pool of enzyme was not affected by the solid particles whereas that of the adsorbed pool underwent a partial reduction**.** Therefore, the total enzymatic activity (TA) in the presence of the minerals was defined as the sum of the activities of adsorbed and dissolved enzyme molecules (Eq.3):

$$TA = (DM \cdot ADM) + (AM \cdot AAM) \qquad \text{(Eq. 3)}$$

where DM = number of dissolved enzyme molecules, ADM = average activity of one dissolved enzyme molecule, AM = number of adsorbed enzyme molecules = total number of enzyme molecules – DM, AAM = average activity of one adsorbed enzyme molecule.

The catalytic activity of the adsorbed pool (AM · AAM) was calculated by rearrangement of Eq. 3 for kaolinite and goethite. Compared with a dissolved molecule, an adsorbed β-glucosidase molecule loses on average 26.8 % of its activity when adsorbed to kaolinite and 25.0 % when adsorbed to goethite. In the presence of kaolinite and goethite, most of β-glucosidase molecules were in adsorbed state. In both cases, the calculated adsorbed activity (adsorbed activity = AM · AAM; 9.9 µmol glucose l^{-1} $min.^{-1}$ for kaolinite and 8.7 µmol glucose l^{-1} $min.^{-1}$ for goethite) outweighed the dissolved activity (dissolved activity = DM · ADM; 5.7 µmol glucose l^{-1} $min.^{-1}$ for kaolinite and 4.7 µmol glucose l^{-1} $min.^{-1}$ for goethite). Thus, most of the activity was adsorbed activity and cellobiose hydrolysis proceeded in the presence of these minerals, although at a lower rate in comparison to the controls.

For montmorillonite, no significant effect of the mineral on the total activity was detected, probably because the adsorbed fraction of β-glucosidase, and thus the effect on TA, was too small. However, an inhibiting effect of montmorillonite on the activity of the small pool of adsorbed molecules cannot be excluded.

3.6. Enzymatic reaction in the presence of the charred materials

The enzymatic hydrolysis of cellobiose in presence of the charred materials in the experimental systems is shown in Figure 12.

The effects of chestnut wood char and activated carbon on the effective total enzymatic activity (eTA; see below) are shown in Figure 14. The presence of chestnut wood char particles (Figure 14a) caused a decrease in the cellobiose hydrolysis rate of approximately 30 % in comparison to the control supernatant; a t-test on the mean initial reaction rates in the suspensions and in the supernatants showed that this difference was statistically significant ($P = 0.009$). In the presence of activated charcoal particles the effective activity was completely inhibited ($P < 0.001$; Figure 14b).

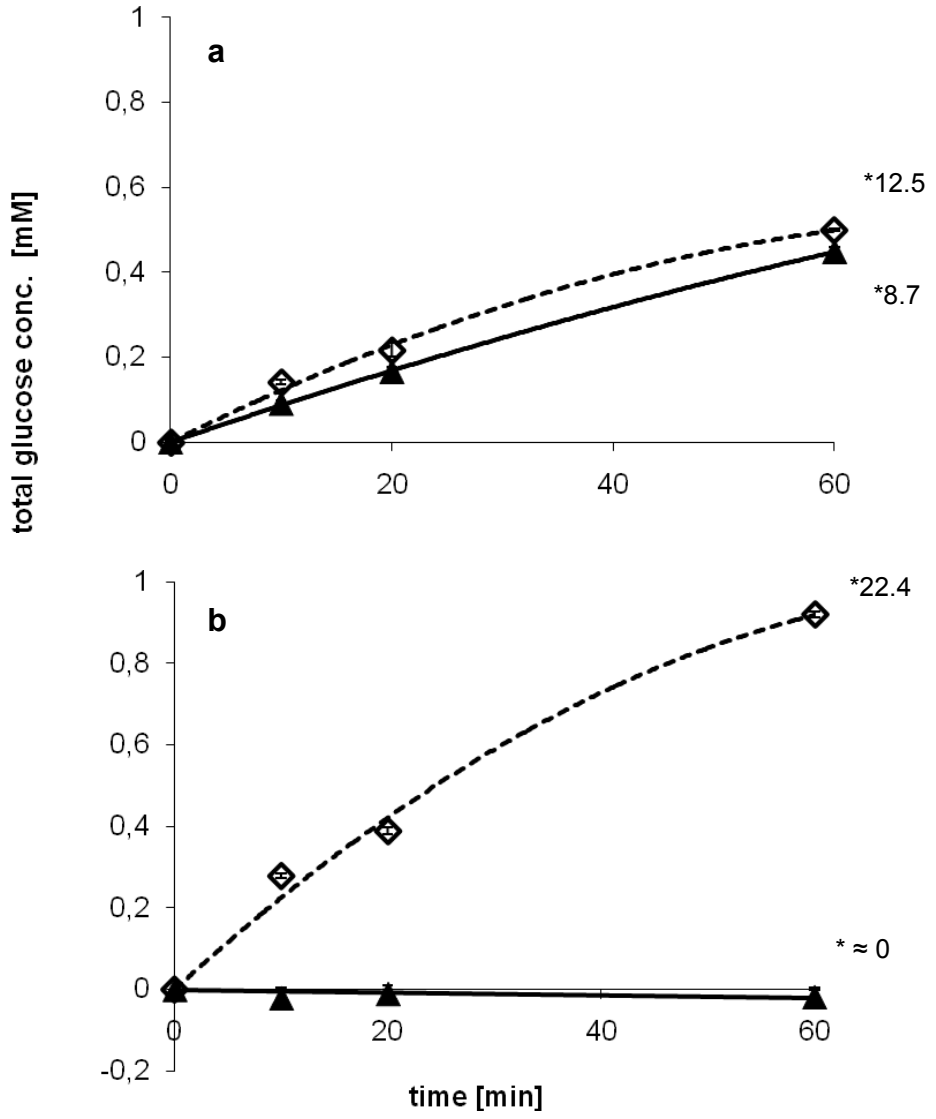

Figure 14. Progress of cellobiose hydrolysis in the presence of chestnut wood char (a) and activated carbon (b) (—▲— = in presence of solid particles;---◇--- = controls in absence of solid particles). Each point represents the average of three independent replicates. If error bars are not visible they are smaller than the symbols. (*initial reaction rate [μmol l^{-1} min^{-1}]). If error bars are not visible, they are smaller than the symbol size.

In the presence of both enzyme and substrate sorption, as can be the case with the charred materials, the effective total enzymatic activity (eTA) should be distinguished from the potential total enzymatic activity (pTA). The effective, total enzymatic activity (eTA) in a

suspension is the observed activity and it results from the sum of adsorbed and dissolved effective enzymatic activities. In analogy with Eq. 3, which is suitable to describe systems where no substrate adsorption takes place, eTA can be described by Eq. 4.

$$eTA = (DM \cdot eADM) + (AM \cdot eAAM) \qquad \text{(Eq. 4)}$$

where DM = number of dissolved enzyme molecules, eADM = average effective activity of one dissolved enzyme molecule, AM = number of adsorbed enzyme molecules = total number of enzyme molecules – DM, eAAM = average effective activity of one adsorbed enzyme molecule.

According to the Michaelis-Menten kinetics, the effective total enzymatic activity depends on the dissolved substrate concentration, therefore the effective dissolved activity (DM · eADM) and effective adsorbed activity (AM · eAAM) are defined by

$$DM \cdot eADM = (V_{max\ DE} \cdot [S_{diss}]) / (K_{m\ DE} + [S_{diss}]) \qquad \text{(Eq.5)}$$

and

$$AM \cdot eAAM = (V_{max\ AE} \cdot [S_{diss}]) / (K_{m\ AE} + [S_{diss}]), \qquad \text{(Eq. 6)}$$

where $V_{max\ DE}$ = maximum enzymatic activity of the dissolved enzyme pool [mmol l^{-1} min^{-1}], $[S_{diss}]$ = dissolved substrate concentration [mmol], $K_{m\ DE}$ = Michaelis-Menten constant of the dissolved enzyme pool [mmol], $V_{max\ AE}$ = maximum enzymatic activity of the adsorbed enzyme pool [mmol l^{-1} min^{-1}], $K_{m\ AE}$ = Michaelis-Menten constant of the adsorbed enzyme pool [mmol].

This model takes into account that the parameters of the Michaelis-Menten equation are different for dissolved and adsorbed enzyme, but it does not consider the possibility of consumption-driven desorption of the substrate from the solid in the case of (partially or completely) reversible adsorption.

The potential total enzymatic activity (pTA) is defined as the hypothetical cumulated activity of the adsorbed and dissolved enzyme pools if all of the substrate was dissolved ($[S_{diss}] = [S_{tot}]$, where $[S_{tot}]$ = total substrate concentration)

In the presence of chestnut wood charcoal, eTA was 8.7 ± 0.7 µmol l^{-1} min^{-1} whereas in the controls without solid particles it was 12.5 ± 1.1 µmol l^{-1} min^{-1}. Since in the experimental systems β-glucosidase was completely adsorbed and all cellobiose was freely available, eTA is equal to pTA and corresponds to the catalytic activity of the adsorbed enzyme molecules (Eq. 7).

$$eTA = AM \cdot eAAM = pTA \qquad \text{(Eq.7)}$$

$$\text{since } DM = 0 \text{ and } [S_{diss}] = [S_{tot}]$$

The loss of catalytic activity of adsorbed enzyme molecules relative to dissolved ones is very similar to those measured for kaolinite and goethite. Since adsorbed enzyme molecules retain most of their activity, chestnut wood charcoal may be well-suited for maintaining an active adsorbed extracellular enzyme pool in soils, presumably also resulting in enzyme stabilisation.

It is noteworthy that in the supernatant of chestnut wood char the enzymatic activity is lower than in the supernatant of activated carbon (Figure 14). The enzymatic activities measured in the controls of the experiments with the minerals (17.7 ± 1.4 µmol glucose l^{-1} min^{-1}) were also higher than those measured in the chestnut wood char supernatant. It is hypothesized that chestnut wood char releases soluble substances with an inhibiting effect on β-glucosidase. However, the difference in the reaction rate between a suspension and the respective supernatant was caused only by the surface effect of the solids present in the suspension, since no relevant differences for enzymatic activity should exist between the liquid phase of the suspension and the respective supernatant.

Activated carbon completely inhibited the enzymatic reaction, whereas in the controls the enzymatic activity was 22.4 ± 0.6 µmol l^{-1} min^{-1} (Figure 14b). It was not possible to

calculate the pTA from these data, because the substrate was completely sorbed and thus not available to the enzyme. It is only possible to state that the average potential activity of adsorbed enzyme molecules (pAAM) was ≥ 0 and that complete absorption of cellobiose, which strongly reduced its availability, results in complete inhibition of the reaction even if the adsorbed enzyme remained active (Eq. 5). Thus, no conclusion about the activity of the adsorbed enzyme is possible.

$$eTA = 0 \leq pTA \qquad \text{(Eq. 8)}$$

since $[S_{diss}] = 0$, DM = 0 and pAAM ≥ 0.

A conceptual model of the enzymatic reaction in the presence of chestnut wood char and activated carbon is shown in Figure 15. The hydrophobic surface of chestnut wood char particles adsorbed all β-glucosidase molecules, which retained most of their potential enzymatic activity and hydrolysed the entirely dissolved cellobiose. Activated carbon particles exposed to the enzyme adsorbed all β-glucosidase molecules and the activated carbon particles exposed to the substrate absorbed all cellobiose molecules; no contact between cellobiose and β-glucosidase can take place, and the unavailability of the substrate for the enzyme is sufficient to completely inhibit the reaction even if the adsorbed enzyme remains active.

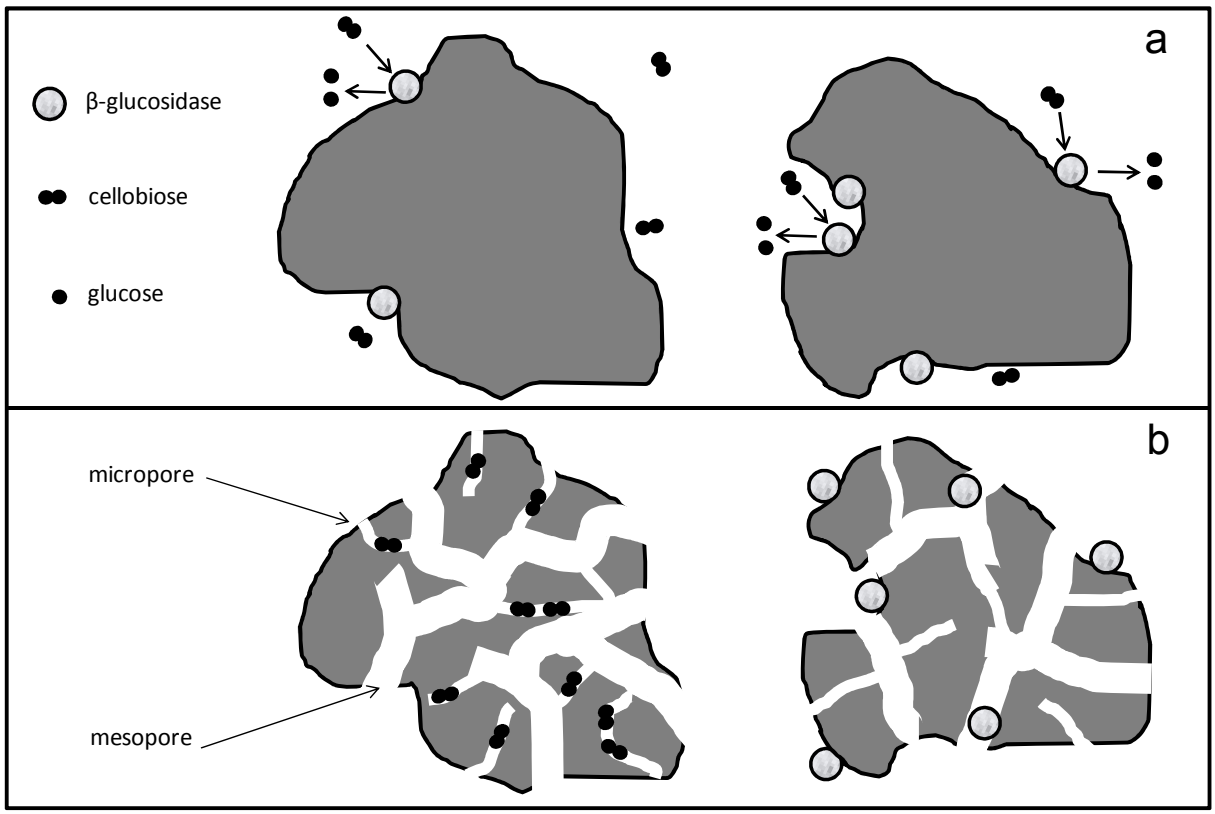

Figure 15. Conceptual model of cellobiose hydrolysis in presence of charred materials: the reaction takes place with chestnut wood char particles (a) whereas activated carbon particles (b) do not allow substrate-enzyme contact. For activated carbon, enzyme and substrate were adsorbed to distinct particles to avoid enzymatic hydrolysis during adsorption.

3.7. Efficiency of enzyme immobilization in matrices of potential industrial interest

The leakage of β-glucosidase encapsulated in agarose and alginate beads and covalently bound to Eupergit C was tested by measuring the increase of the enzymatic activity in the buffer where the enzyme containing particles particles were shaken for up to 6 h. The amount of β-glucosidase (and β-galactosidase from *Escherichia coli*) immobilized by biosilica particles was determined by measuring the activity in the particles and the supernatants immediately after particle formation.

Agarose beads containing β-glucosidase were shaken in phosphate buffer for six hours. During this time the enzymatic activity in the buffer increased and after six hours the activity in the buffer was approximately 22 times the activity of the agarose beads (Figure 16).

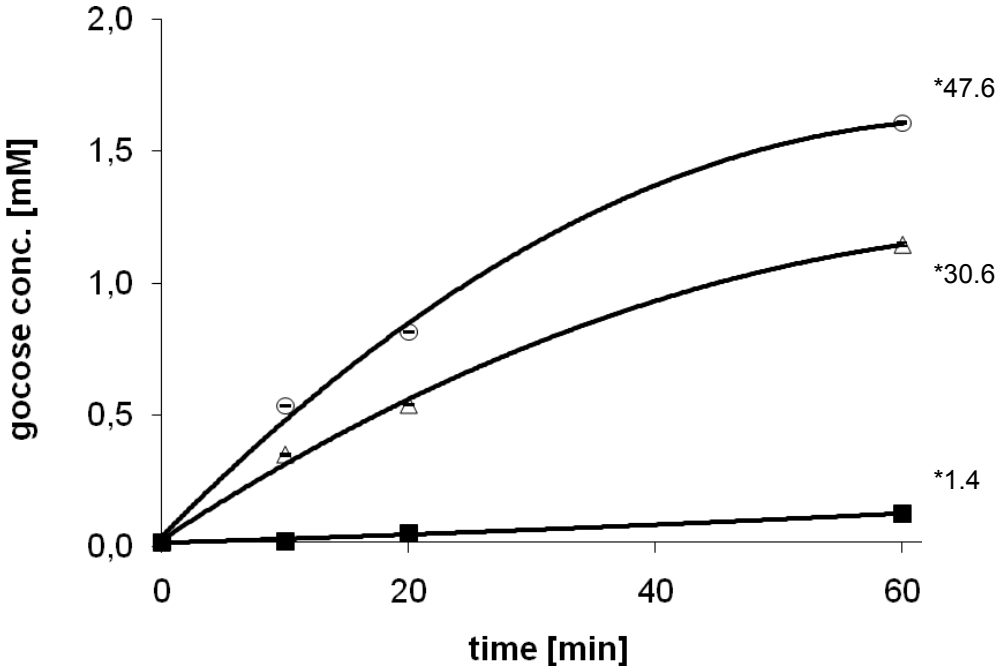

Figure 16. β-glucosidase activity in agarose beads(—■—) and solution(—△—) after 6 h of shaking. The control (—○—) shows the activity in solution of the amount of β-glucosidase that was present in the agarose beads before leakage started. (*initial reaction rate [μmol l^{-1} min^{-1}]). If error bars are not visible, they are smaller than the symbol size.

Agarose is unable to encapsulate β-glucosidase and leakage is fast. Agarose molecules are not supposed to adsorb β-glucosidase since they are non ionic and hydrophilic. Enzyme immobilization could take place by entrapment in the pore space but the results indicate that the pore dimensions are too big in relation to the dimensions of the enzyme: this material is unsuitable for β-glucosidase immobilization.

Also the results obtained with alginate beads show fast enzyme leakage (Figure 17).

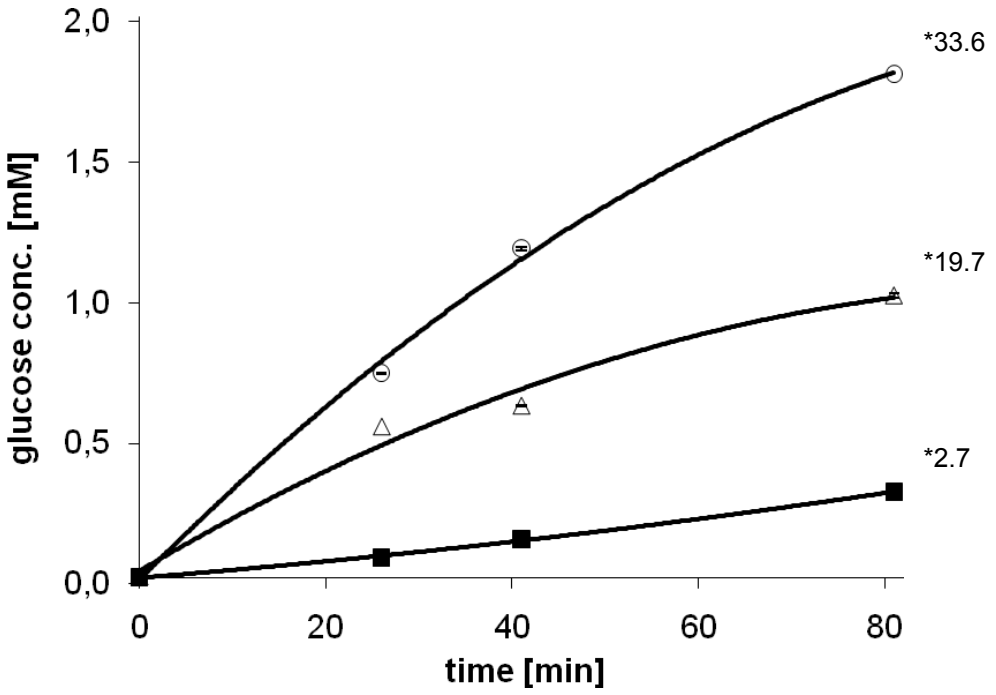

Figure 17. β-glucosidase activity in alginate beads(—■—) and solution(—△—) after 6 h of shaking. The control (—○—) shows the activity in solution of the amount of β-glucosidase that was present in the alginate beads before leakage started. (*initial reaction rate [μmol l^{-1} min^{-1}]). If error bars are not visible, they are smaller than the symbol size.

Similar to agarose, alginate is not appropriate for β-glucosidase immobilization and the excessive dimensions of the pores is the probable cause (Tanaka et al., 1984).

In contrast to encapsulation in agarose and alginate, immobilization by covalent bonding to Eupergit C led to a stable association between enzyme and matrix (Figure 18): after 6 h of shaking the activity in the buffer remained undistinguishable from 0 whereas the activity in the particles was approximately 24 % of the control.

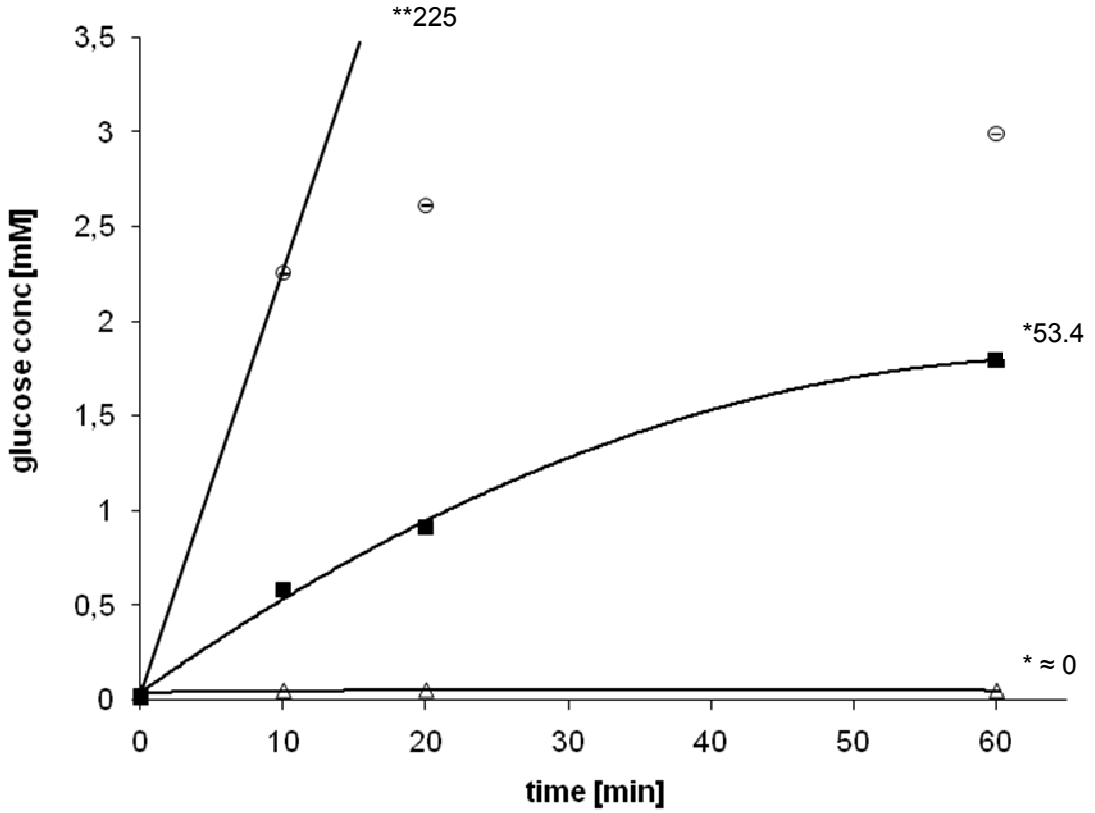

Figure 18. β-glucosidase activity in Eupergit C particles(—■—) and solution(—△—) after 6 h of shaking. The control (—○—) shows the activity in solution of the amount of β-glucosidase that was immobilized in the Eupergit C particles. (*initial reaction rate [μmol l^{-1} min^{-1}]; **initial reaction rate = average reaction rate during the first 10 min). If error bars are not visible, they are smaller than the symbol size.

Immobilization of β-glucosidase in biosilica particles was not successful: after the immobilization protocol the enzymatic activity in the supernatant was only slightly lower than that of the control whereas the activity of the particles was negligible (Figure 19).

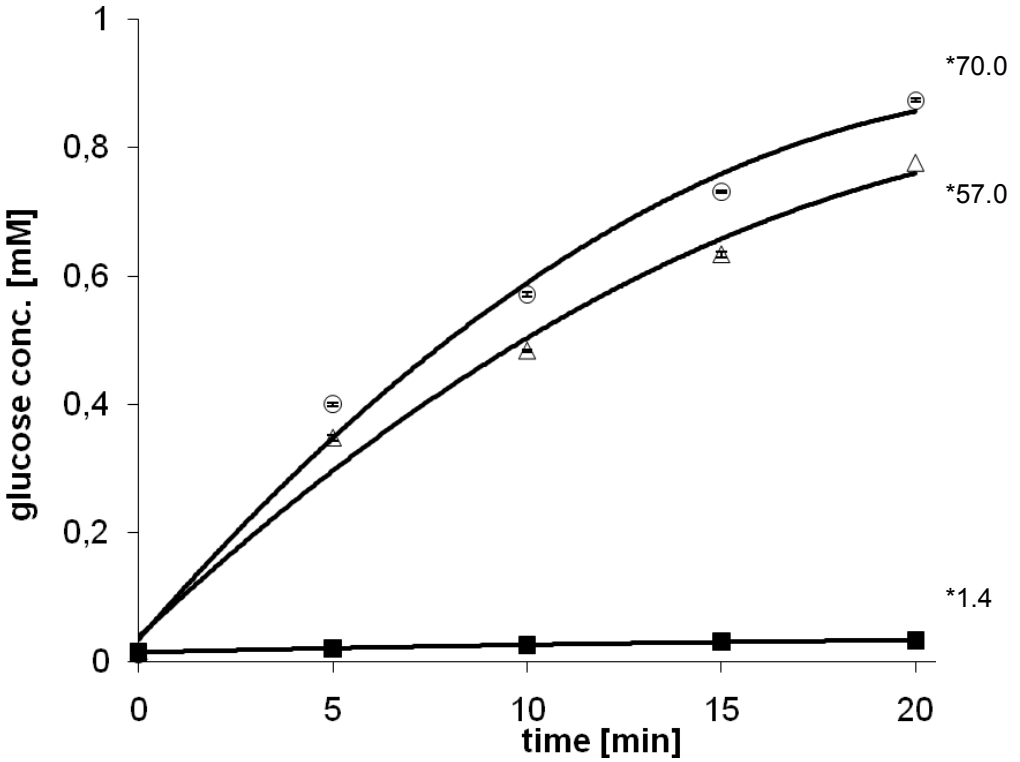

Figure 19. β-glucosidase activity in biosilica particles(—■—) and solution(—△—) after immobilization protocol. The control (—○—) shows the activity in solution of the amount of β-glucosidase that was used in the immobilization protocol. (*initial reaction rate [μmol l^{-1} min^{-1}]). If error bars are not visible, they are smaller than the symbol size.

The results on β-glucosidase immobilization indicate that during the formation of biosilica particles the enzyme is expelled by the forming particles and remains in solution. It is hypothesized that electrostatic repulsion between enzyme and the surface of the forming biosilica particles prevents enzyme immobilization.

To exclude that the failure of β-glucosidase immobilization in biosilica particles was caused by an anomalous formation of the biosilica particles, the immobilization protocol was repeated with β-galactosidase from *E. coli* (Figure 20): in this case immobilization was remarkably successful since the activity of the particles was approximately 62 % of the activity of the control and the activity in the supernatant was close to 0.

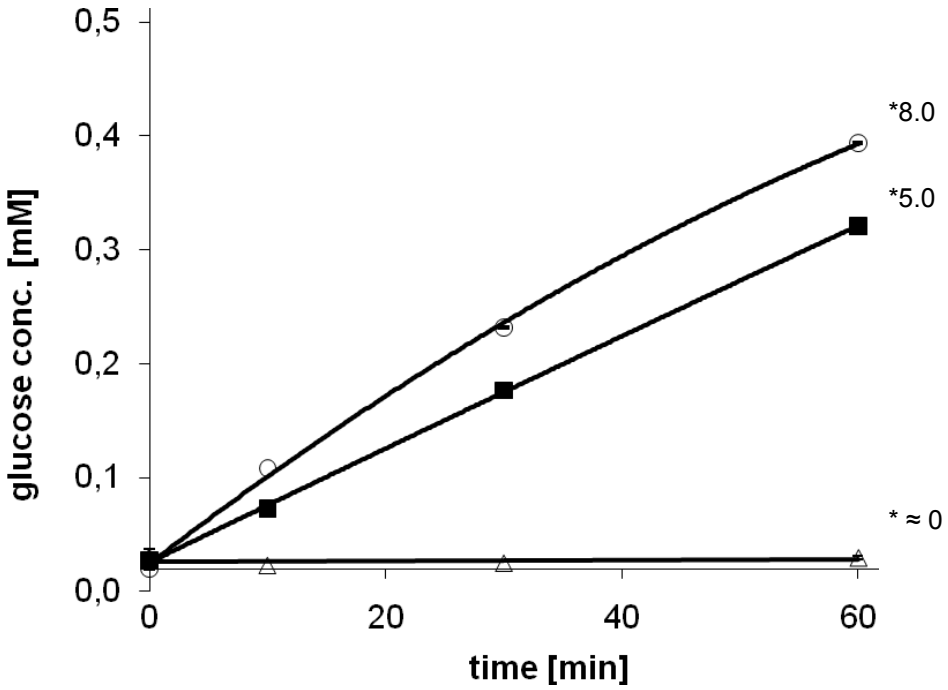

Figure 20. β-galactosidase activity in biosilica particles(—■—) and solution(—△—) after immobilization protocol. The control (—○—) shows the activity in solution of the amount of β-galactosidase that was used in the immobilization protocol. (*initial reaction rate [µmol l^{-1} min^{-1}]). If error bars are not visible, they are smaller than the symbol size.

4. Discussion

4.1. Effects of montmorillonite, kaolinite and goethite on the enzymatic hydrolysis of cellobiose: summary and ecological implications

The present work we shows that, under the experimental conditions, the adsorption of the exoenzyme β-glucosidase from *A. niger* was substantial for the minerals kaolinite and goethite, whereas for montmorillonite most of the protein remained in solution. The adsorption of β-glucosidase was not correlated with the specific surface area, indicating that the interactions are not limited by the availability of sorption sites. Instead, adsorption of the enzymes to the minerals seems to be controlled by the electrical properties of the mineral surfaces. Kaolinite and goethite caused a decrease in the hydrolysis rate of the natural substrate (i.e. cellobiose) due to a decrease of the average activity of adsorbed β-glucosidase molecules, whereas no measurable effect was observed with montmorillonite. Likely due to the high water solubility and the polar, but non-ionic nature of cellobiose, no detectable adsorption was observed under the experimental conditions chosen. This implies that any effect of the tested solids on the reaction kinetics with cellobiose must be attributed entirely to interactions of the minerals with β-glucosidase.

As montmorillonite did not interact with cellobiose and only very weakly with β-glucosidase, the negligible effect on the hydrolysis rate can be explained in a straightforward manner: at pH 5.0 both the mineral and the enzyme, slightly above its isoelectric point (IEP) of pH 4.0 (Mc Cleary and Harrnigton, 1988), have a net negative charge. The cation exchange capacity of montmorillonite (76.4 meq 100 g^{-1}; http://www.clays.org) is high in comparison to kaolinite (2-3.3 meq 100 g^{-1}; http://www.clays.org). Even if the different specific surfaces are taken into account, the surface charge density is higher for montmorillonite (22.2 μeq m^{-2})

than for kaolinite (2.7 – 4.5 μeq m^{-2}). The resulting electrostatic repulsion is very strong in the case of montmorillonite and it outweighs the sum of the attractive non-coulombic forces.

In contrast to montmorillonite, adsorption of β-glucosidase to kaolinite was substantial. Also in this case, both the mineral and the protein have a net negative charge, but the charge density at the mineral surface is lower than for montmorillonite. The resulting electrostatic repulsion between the mineral surface and the protein is weaker than for montmorillonite and it is outweighed by the attractive non-coulombic forces. The interaction of kaolinite with β-glucosidase results in a significant effect on the reaction rate, which is likely to be caused by a modification of the structural conformation of the protein upon adsorption and/or by a steric hindrance of the access of the substrate to the active site (Quiquampoix and Burns, 2007). However, the persistence of the catalytic activity in the adsorbed state shows that the inhibition caused by adsorption is not sufficient to completely suppress cellobiose hydrolysis.

The results obtained for goethite are similar to those obtained for kaolinite, even though its ZPC is > pH 5.0, suggesting that its surface is positively charged at this pH. However, the electrical properties of this mineral in phosphate buffer differ from those in other solutions, because a superequivalent specific adsorption of phosphate anions from the buffer can lead to a surface charge reversal (Quiquampoix, 1987a). This assumption is corroborated by the ζ-potential measurements that show negative values for the goethite-phosphate complex, which this minerals forms in phosphate buffers. Therefore, under the experimental conditions, goethite interacted with β-glucosidase as a material with a negatively charged surface. For all tested minerals adsorption is caused by non-coulombic forces and the similarity of the adsorption mechanism may explain the similarity of the effects of kaolinite and goethite on the enzyme activity level. A conceptual model of β-glucosidase adsorption to the different minerals is shown in Figure 21.

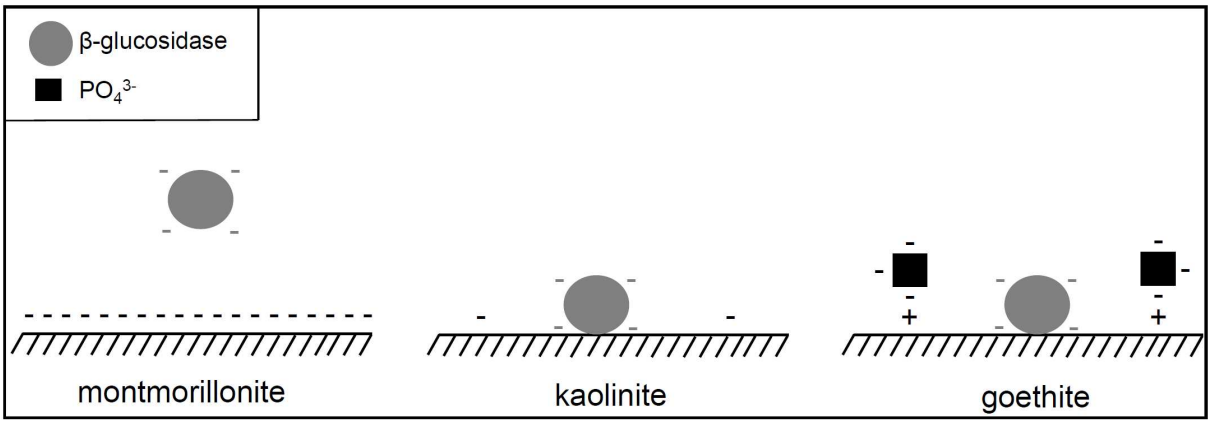

Figure 21. Conceptual model of β-glucosidase - surface interaction: the high charge density of montmorillonite strongly hinders adsorption; the lower charge density of kaolinite is more favourable to protein adsorption by means of attractive non-coulombic forces; the positive charges of goethite are overcompensated by phosphate anions and the mechanism of enzyme adsorption is analogous to that of kaolinite.

These results indicate that, at a pH close to 5.0, soil microenvironments dominated by one of the three different minerals should affect the ecological function of β-glucosidase from *A. niger* in different ways:

- If montmorillonite is dominant then an enzyme recently secreted by a cell will be mostly in solution; the reaction rate will be high in the beginning but since the protection against proteases provided by adsorption is missing, the rate of inactivation of the enzyme molecules may also be high.
- If kaolinite is dominant then most of the excreted enzyme will be rapidly adsorbed: at first the reaction rate will be slightly slower than for montmorillonite but this will be quickly compensated by the higher resistance to proteolysis provided by adsorption.
- If goethite is dominant and phosphate is very abundant (as may happen in heavily fertilized agricultural soils) than the scenario in the microenvironment should be very similar to that of kaolinite. If anions of lower charge would be adsorbed to the goethite surface then the density of the negative charge of the goethite-anion complex

would be lower and more adsorption (and therefore more protection from proteolysis) and lower initial reaction rates would be expected.

According to these hypotheses a preponderance of kaolinite and goethite in a soil microenvironment would maximize the "return" (i.e. flow of metabolizable molecules) on "investment" (i.e. energy consumed for enzyme synthesis and excretion) for the organism on the longer term.

To the best of my knowledge, there is only one study related to the interaction of soil minerals and β-glucosidase from *A. niger* (Quiquampoix et al., 1989). This study tested the effect of montmorillonite, in lower concentrations than in this study, on the hydrolysis of the chromogenic substrate 4-nitrophenyl-3-D-glucopyranose and, at pH 5.0, in the presence of citrate buffer. An important difference to the present study was the amount of other proteins that were introduced into the experimental systems along with the β-glucosidase. In the cited study, β-glucosidase represented a small fraction of the total protein in the enzyme preparation, whereas in the present study it was the most abundant protein in the preparation. This allowed measurable activities to be obtained with a total amount of protein in the systems of 2.45 $\mu g\ ml^{-1}$, whereas in the previous study 385 $\mu g\ ml^{-1}$ was used. Referring the total amount of protein to the amount of montmorillonite, 0.128 $g\ g^{-1}$ was used in the cited study whereas in the present study the concentration was three orders of magnitude lower ($0.123 \cdot 10^{-3}\ g\ g^{-1}$). This low total protein/mineral ratio allows lateral interactions between adsorbed proteins to be safely neglected, since the protein adsorption to the mineral surface is far from saturation. Even though these differences in the experimental procedure are relevant, the divergence of the results is limited. By comparing the activity in the supernatant with the activity in the control at pH 5.0, in the cited study the adsorption can be estimated to be approximately 29 %, whereas in the present study it was 9.7 ± 7.3 %. It is difficult to estimate the extracellular protein content of soils (mainly because of the problem of extracting strongly adsorbed proteins without lysing cells). In Alaskan tundra soils the amount of protein

extractable with 0.1 M $NaHCO_3$ was in a range of approximately $1 \cdot 10^{-3}$ to $4 \cdot 10^{-3}$ g g^{-1} soil (Weintraub and Schimel, 2005), therefore to the amount of protein used in the present study may well be close to values found in soils.

The adsorption of β-glucosidase from *A. niger* to montmorillonite measured by Quiquampoix et al., 1989, was slightly higher than that measured in this study. However, this adsorption to montmorillonite (Quiquampoix et al., 1989) was significantly lower than the adsorption to kaolinite and goethite quantified in this study, and in both cases the effect of montmorillonite on the reaction rate was very close to zero. This raises the question of whether the higher charge density of the surface of montmorillonite relative to kaolinite not only reduces the amount of adsorbed β-glucosidase but also the activity of the adsorbed pool. On the surface of montmorillonite the negative charges may be closer to the adsorbed protein and the stronger interactions with the charges of the protein may cause deformation to be less favoured energetically.

Another study (Quiquampoix, 1987a) characterized the behaviour of the β-glucosidase from the sweet almond plant (*Prunus dulcis*) in the presence of montmorillonite, kaolinite and goethite. The results from this work at pH 5.0 and $[Na^+]$ = 20-40 mM are compared with the results of the present study in Table 3.

Table 3. Comparison of the interaction of β-glucosidase from *A. niger* and β-glucosidase from *P. dulcis** with montmorillonite, kaolinite and goethite: fraction of adsorbed β-glucosidase (AE), loss of total catalytic activity (TA loss), average catalytic activity loss of the adsorbed pool of β-glucosidase [(1-AAE/ADE)*100]. Data on β-glucosidase from *A. niger* are referred to controls with an average activity of 17.7 ± 1.4 μmol glucose l^{-1} min^{-1}; data on β-glucosidase from *P. dulcis** are referred to controls with an average activity of approximately 0.114 μmol 4-nitrophenol min^{-1} (*).

		Montmorillonite	Kaolinite	Goethite
		[%] of control		
AE	*A. niger*	9.7 ± 7.3	70.3 ± 3.1	71.4 ± 1.8
	P. dulcis *	>99	>99	>99
TA loss	*A. niger*	1.7 ± 6.8	18.8 ± 3.4	17.9 ± 4.7
	*P. dulcis**	45	48	11
(1-AAE/ADE)*100	*A. niger*	ND	26.8	25.0
	*P. dulcis**	45	48	11

*(Quiquampoix, 1987a). All data refer to enzymatic activities at pH5.0.

For all minerals, the adsorption of the *P. dulcis* β-glucosidase is much higher than that of the *A. niger* β-glucosidase; this may be caused by a slightly higher IEP of the plant enzyme. The activity loss of the adsorbed enzyme molecules is higher for sweet almond β-

glucosidase in the case of montmorillonite and kaolinite (goethite caused a low activity loss for *P. dulcis* β-glucosidase but citrate and phosphate buffers cause different changes at the surface of the iron mineral and therefore the results are not directly comparable). Differences in the IEP may cause this behaviour to a certain extent, but a better adaptation of the fungal extracellular β-glucosidase to the soil environment can be expected. Actually, the detailed localization of the β-glucosidase from sweet almond is unknown, but it is not a typical soil enzyme and in the closely related species *Prunus avium* it was shown to be mainly cytosolic (Gerardi et al., 2001). It should be noticed that, whereas the contribution of β-glucosidase from *P. dulcis* to the degradation of soil organic matter may be negligible, the isoenzyme from *A. niger* should play a major role.

The comparison of the sorptive behaviour, and of the activity retention in adsorbed state, of the β-glucosidase from *A. niger* and *P. dulcis* shows an important fact: two different isoenzymes of the same enzyme class (E.C. 3.2.1.21) react differently to the same "disturbance" (i.e. presence of a potentially sorptive surface). This fact has profound consequences if considered in the light of using the activity of certain enzyme classes as indicators of soil quality. In fact, when a chromogenic substrate is added to soil samples for measuring the effect of a disturbance on the activity of an enzyme class, what is actually measured is the mean potential activity of all isoenzymes of the class which are present in the sample (Wallenstein and Weintraub, 2008). However, this method does not indicate which specific isoenzymes have been inhibited, enhanced or not affected by the disturbance. This lack of information may be the cause of the contradictory results from studies on the effect of disturbances on enzyme activities. The development of methods to identify the most abundant and most active isoforms of an enzyme class in a soil sample is necessary if a mechanistic understanding of how an enzyme class responds to a disturbance is to be gained.

This part of the study shows that, under the experimental conditions, the hydrolysis of cellobiose by β-glucosidase from *A. niger* is affected by montmorillonite, kaolinite and

goethite to a different extent. The effects are caused by adsorption of the enzyme and their intensity is controlled by the charge density at the surface of the minerals. Adsorption of β-glucosidase causes only minor decreases in the enzyme activity level. The inhibiting effect of the solid surface on the adsorbed pool of the enzyme is quantitatively very similar for kaolinite and goethite in spite of their different properties and we hypothesize that for both minerals adsorption takes place on neutral portions of the surface via non-coulombic interactions (see also Figure 21).

4.2. Effects of biochar and activated carbon on the enzymatic hydrolysis of cellobiose: summary and ecological implications

In this chapter the effects of chestnut wood char and activated carbon on the enzymatic hydrolysis of cellobiose are quantified and a mechanistic explanation of these effects is proposed. At the molecular level wood char and activated carbon are both characterized by condensed, rigid and aromatic structures with high carbon contents (Cornelissen et al., 2005), and by very diverse surfaces containing both polar and apolar regions and both acidic and basic functional groups (Zajac et al., 1997; Qiu et al., 2008). Even though the chemical differences between these two materials may not be negligible, for the purpose of using them as soil amendments the main difference is likely to be the specific surface area. In this respect, chestnut wood char (SSA = 2 m^2 g^{-1}) and activated carbon (SSA = 762-923 m^2 g^{-1}) can be considered as the extremes of a continuum of variation among charred materials. The high specific surface area, and its diversity in terms of physicochemical properties, enables activated carbon to adsorb a great variety and a great amount of substances, whereas chestnut wood char may be quickly saturated by substances which are adsorbed to less extended regions of its surface. For example, even at environmentally relevant (i.e. low) total cellobiose concentrations, the polar regions of chestnut wood char may be completely occupied by a very small fraction of the total cellobiose, most of which would therefore be freely dissolved.

While the adsorption of a non-ionic polar molecule to charred materials is not likely to be strongly affected by variations of the pH in the range that can be found in soils, that of an amphoteric compound like a protein may be strongly affected: Quiquampoix (1987a, 1987b) showed that the interaction of proteins with soil minerals is strongly affected by the pH, which determines the coulombic attraction/repulsion between surface and protein. However, if non-coulombic forces are largely responsible for the adsorption of the protein to the surface of the charred material, the effect of the pH may be weak until the large apolar surface is saturated.

Chestnut wood char did not adsorb the polar cellobiose in the experimental systems. In contrast, β-glucosidase was almost completely adsorbed to chestnut wood char particles: we hypothesize that the enzyme is adsorbed by non-coulombic forces between uncharged regions of the protein and uncharged regions of the char surface. The activity loss caused by β-glucosidase adsorption to this material (approximately 30 %) is comparable to values obtained for inorganic materials such as kaolinite and goethite: the two minerals adsorbed less of the enzyme under similar conditions (approximately 71%) but the activity lost by adsorbed enzyme molecules relative to dissolved ones was approximately 27 % for kaolinite and approximately 25 % for goethite. We therefore hypothesize that, under the experimental conditions, the mechanism of β-glucosidase adsorption is very similar for chestnut wood char, kaolinite and goethite (and also activated carbon), in spite of the different chemical natures of these materials. Indeed, an adsorption-induced activity loss that is relatively low and largely independent from the chemical nature of the adsorbing soil material would be consistent with the ecological role of β-glucosidase from *A. niger*, which consists in supplying the fungus with substrate by providing extracellular depolymerisation activity in a system which is characterized by diverse organic and inorganic surfaces.

The low activity loss of β-glucosidase upon adsorption can be attributed to structural adaptations of this protein, for example its "hardness" (see below) and the specific spatial

distribution of the glycosides associated with it. This aspect deserves attention because glycosylation is common to most fungal extracellular soil enzymes.

Chestnut wood char thus may favour the presence of an abundant adsorbed, and therefore stabilized, β-glucosidase pool. The potential activity of the enzyme is only slightly diminished by adsorption to this type of material. Since cellobiose tends to remain in solution, eTA corresponds to pTA and the substrate is rapidly hydrolysed. The enzymatic activities in chestnut wood charcoal supernatants were lower than those in the supernatants of activated carbon. Similar to Warnock et al. (2010) we hypothesize that chestnut wood char releases aromatic compounds like phenols and polyphenols and that these compounds have an inhibiting effect on β-glucosidase. Hammes et al. (2006) suggest assigning the shoulders in the O-aryl region (160 ppm) of the NMR spectra of chestnut wood char to compounds such as benzo-furans, polycyclic aromatic hydrocarbons or heterocyclic compounds, which may be formed during wood pyrolysis. Smith et al. (2010) found that the soluble C and N released by biochar produced from switchgrass (*Panicum virgatum*) were dominated by aromatic, aliphatic and carboxylate groups. Plant char may therefore release a number of aromatic and aliphatic, potentially inhibitory compounds into the solution. However, the difference in the reaction rate between a suspension and the respective supernatant was caused only by the surface effect, since no relevant differences for enzymatic activity should exist between the liquid phase of the suspension and the respective supernatant. In contrast, we did not detect any decrease in the activity of β-glucosidase in the supernatant of activated charcoal, suggesting that this material did not release any compounds inhibiting the enzymatic reaction.
A fungus excreting β-glucosidase in a microenvironment dominated by wood char similar to the one used in this study would create a microgradient of cellobiose hydrolysis potential and, since both substrate and product are freely available, the “return” (i.e. energy produced by substrate metabolism) on “investment” (i.e. energy used for enzyme synthesis and excretion)

would be immediate. Since a stabilization of the enzyme upon adsorption to chestnut wood char is to be expected, this favourable situation for the fungus may be stable.

In contrast to chestnut wood char, activated carbon absorbed cellobiose almost completely. The specific surface area of activated carbon is approximately 380 - 450 times as high as that of chestnut wood char and this has to be attributed to the large micro- and mesoporosity of this material. We hypothesize that the adsorption of the disaccharide from the solution was caused by hydrogen bonds to polar surface groups of activated carbon (e.g. COOH, SO_4H, PO_4H), since it was shown that the surface of activated carbon can be very reactive for small polar molecules (Julien et al., 1998). Unsurprisingly, β-glucosidase was also completely adsorbed. Since the chemical structure of activated carbon is very similar to that of chestnut wood char, the mechanism of enzyme adsorption and the effect on the potential activity may also be very similar. The data obtained in this study do not allow to decide whether β-glucosidase from *A. niger* retained its catalytic activity or not when adsorbed to activated carbon in the experimental systems. Indication that catalytic activity may have been retained comes from a previous study (Daoud et al., 2010) which showed that cellulase from *A. niger* (which contains endoglucanase - E.C. 3.2.1.4, cellobiohydrolase - E.C. 3.2.1.21 and β-glucosidase - E.C. 3.2.1.21), retains 70 % of its native catalytic activity (30 °C, pH 4.8) when adsorbed to activated carbon. The amount of enzymatic activity retained by cellulase from *A. niger* adsorbed to activated carbon in the cited experiment is remarkably consistent with the data produced by this study for β-glucosidase from *A. niger* adsorbed to wood char and by a previous study on the adsorbed pool of the same enzyme to kaolinite and goethite (Lammirato et al., 2010). Daoud et al. (2010) used methylcellulose as substrate and a commercial activated carbon with a specific surface area of 1073 m^2g^{-1} and a total pore volume of 0.596 cm^3g^{-1}, which are comparable values to those of the activated carbon used in this study. The amount of protein in the experimental systems used by Daoud et al. (2010) was much higher than that used in the present study (1565 mg cellulase g^{-1} activated carbon

vs. 0.123 mg β-glucosidase g^{-1} activated carbon, respectively). The most plausible conclusion from the results presented by Daoud et al. (2010) is that β-glucosidase from *A. niger* was in direct contact with the surface of activated carbon and that the enzymatic activity was largely retained, since the final product of methylcellulose hydrolysis was glucose. Also, literature on enzyme technology shows residual activity of various enzymes immobilized on activated carbon (Ho et al., 1993; Kennedy et al., 2007; Kandasamy et al., 2010; Kumar et al., 2010). Our data show also that activated carbon does not release soluble substances which inhibit β-glucosidase. Therefore strong destabilizing effects of this material on the structure of proteins seems to be unlikely.

We therefore assume that the complete inhibition of cellobiose hydrolysis in the presence of activated carbon can be attributed to the unavailability of the substrate for the enzyme. If this is the case, a soil microenvironment dominated by a carbonaceous material with properties similar to those of activated carbon (i.e. high specific surface area, high micro- and mesoporosity) may favour the presence of a relevant pool of adsorbed and potentially active β-glucosidase ($pTA \geq 0$) in a very similar way to chestnut wood char. Such a material may also remove compounds inhibiting the enzymatic reaction from the solution, which would even increase pTA. However, eTA may be extremely low even if pTA is high, since the availability of the substrate may be close to zero. In such an environment, a fungus excreting β-glucosidase would most likely create a microgradient of potential activity, since the substrate would only be available if its total concentration were to exceed the sorption capacity of the carbonaceous material, which is rather unlikely at environmentally relevant cellobiose concentrations. It also has to be considered that a fraction of the product (i.e. glucose) can be absorbed by the carbonaceous material and therefore become unavailable for the fungus. Thus, the "returns" on the energy invested in enzyme synthesis and excretion may be lower, postponed and less certain.

Based on the results obtained for chestnut wood char and activated carbon, which are charred organic materials characterized by extremely low and high, respectively, specific surface area and porosity, we hypothesize that both materials may be capable of adsorbing soil enzymes without complete loss of their potential activity and that substrate sorption would largely determine the effective enzymatic activity. The extracellular enzymatic degradation rates of highly-soluble and low-molecular-weight substrates may indeed be manipulated by amending soils with charred materials of different micro- and mesoporosities (relevant for sorptive properties) and different particle sizes (relevant for residence time in soils). These materials could stabilize the degradation rate by absorbing substrate during input peaks and then slowly releasing it to an active and stabilized enzyme pool. This substrate release may be caused by consumption-driven desorption (more likely at high saturation of the charred particles with substrate) and by the slow degradation of the charred particles. Since charred materials also tend to increase the cation exchange capacity (CEC) of soils (Liang et al., 2006), the nutrients which are released by the process described above would tend to remain in the system and would enhance soil fertility, which may start a positive feedback resulting in further carbon sequestration.

4.3. Adaptation of fungal enzymes to the extracellular environment: some hypotheses

Many of the adaptations which are responsible for the high activity level of the fungal β-glucosidase in adsorbed state may be very common among extracellular enzymes and therefore be general properties which largely determine the rate of CO_2 release from soils. We hypothesize that the adaptation to the soil environment of typical fungal extracellular enzymes is the result of one or more of the following structural properties of the (glyco)protein: i) structural rigidity (“hardness”) of the protein which resists adsorption induced deformation, ii) spatial distribution of the surface charges which orientates the protein towards the surface in

such a way that the active site is exposed to the bulk solution, iii) spatial distribution of the glycans on the protein surface which leads to proper orientation towards the surface and/or limits deformation (Figure 22). When considering these three factors of activity retention in adsorbed state it should be noticed that the spatial distribution of the glycans (see iii) should not be significantly affected by the pH of the soil solution and that therefore this adaptation may be effective in broad range of micro-environmental conditions.

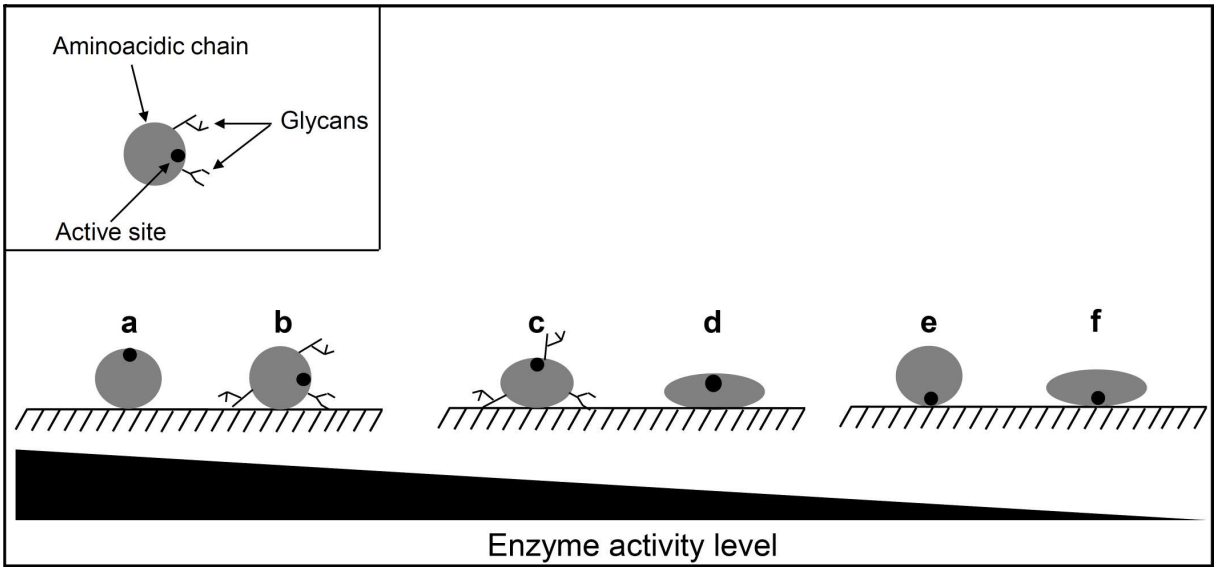

Figure 22. Effects of enzyme "hardness", spatial distribution of surface charges and spatial distribution of glycans on activity levels in adsorbed state. a) "hard" protein properly oriented by spatial distribution of charges; b) "hard" protein properly oriented by distribution of glycans; c) "soft" protein whose deformation as well as orientation is controlled by proper spatial distribution of glycans; d) "soft" protein properly oriented by spatial distribution of surface charges; e) "hard" protein adsorbed with unfavorable orientation; f) "soft" protein adsorbed with unfavorable orientation (modified after Quiquampoix and Burns, 2007).

It has been estimated that more than half of all naturally occurring proteins are glycosylated (Apweiler et al., 1999) and there is convincing evidence of the importance of glycosylation in enzyme activity/specificity, secretion and thermostability (Skropeta, 2009).

For instance, β-glucosidase from *Aspergillus niger* has a carbohydrate content of 7.2-9.4 % [w/w] (Abdel-Naby et al., 1999). Thus, the high activity retention of the adsorbed pool of β-glucosidase from *A.niger* should be attributed to structural adaptations of this protein to the extracellular soil environment, such as a specific spatial distribution of glycosides; this aspect deserves more attention since such adaptations may be common to most fungal extracellular enzymes in soil.

4.4. Enzyme immobilization in matrices of potential interest for industry

The interactions of enzymes with solid surfaces have profound consequences on natural degradation processes in soil. These intractions are also critical in the development of industrial enzymatic processes, since the immobilization of the biocatalyst on a solid matrix is desired to increase its specific productivity.

The results obtained for agarose and alginate show that these matrices are not able to effectively encapsulate β-glucosidase molecules. Leakage takes place because agarose and alginate molecules do not adsorb the enzyme, and because the dimensions of the pores of the gels are excessive to immobilize the protein by preventing its diffusion (Tanaka et al., 1984). The immobilization to Eupergit ® C particles through covalent bonding, on the other hand, led to a stable association between the enzyme and the matrix. The adsorption through a covalent bond prevents enzyme leakage from Eupergit ® C particles, but only 24 % of the catalytic activity is retained. An unfavourable orientation of the bound β-glucosidase, or a strong adsortpion induced protein deformation may strongly contribute to this activity loss. The experiments on the enzyme immobilization in biosilica particles showed that the same immobilization protocol leads to very different results for two different proteins: the fungal glycoprotein β-glucosidase seems to be expelled during the fromation of the silica particles (the activity of the particles is close to zero) whereas the non-glycosylated bacterial β-

galactosidase is efficiently included in the silica particles, the activity of which is 60 % of that of the control with free enzyme. The difference in the outcome of the immobilization experiments of β-glucosidase from *A. niger* and β-galactosidase from *E. coli* has to be attributed to different properties of the proteins, such as the net charge at the pH of the immobilization environment, which could lead to electrostatic repulsion between protein and surface. Furthermore, while glycosylation is common among fungal extracellular enzymes such as β-glucosidase from *A. niger,* this posttranslational modification is much rarer among procaryotic proteins (Benz and Schmidt, 2002) such as β-galactosidase from *E. coli*. The presence of carbohydrates on the surface of β-glucosidase may have sterically hindered the interaction of the enzyme with the forming biosilica particles, therefore reducing protein inclusion. The determination of the isoelectric point of β-galactosidase from *E. coli* and an experiment on the immobilization of a β-glucosidase from *A. niger* deglycosylated under native conditions could contribute to clarify the reasons for the different behaviour of these two enzymes. The size of the two proteins is less likely to play a role since β-galactosidase from *E. coli* is a dimeric enzyme with a molecular weight of 232 kDa (Pilipenko et al., 2007), roughly twice that of β-glucosidase from *A. niger*: a smaller enzyme may leak from the matrix more easily than a bigger one but its inclusion during particle formation should not be hindered.

4.5. Conclusions and outlook

The data presented in this thesis show that, under the experimental conditions, β-glucosidase from *A. niger* retains a large fraction of its catalytic activity when associated with common soil minerals such as montmorillonite, kaolinite and goethite. The electrical properties of the surfaces of the minerals and the protein largely determine the amount of enzyme that is adsorbed. Even though chemically very different, the effect of wood char on

the enzyme activity is very similar to that of kaolinite and goethite: more β-glucosidase is adsorbed relative to the minerals but adsorption causes a remarkably similar reduction in the catalytic activity. Montmorillonite, kaolinite, goethite and wood char do not adsorb the natural substrate of β-glucosidase and therefore do not limit its bioavailability. In a soil environment the abundance of kaolinite, goethite and wood char may favour a stabilization of β-glucosidase and therefore slightly decrease the cellobiose hydrolysis rate at first but then cause a lasting acceleration in the process in the longer term since the excreted enzyme molecules remain active for a longer time. The abundance of montmorillonite may have opposite effects: the much larger fraction of dissolved β-glucosidase does not undergo surface-induced activity losses and therefore hydrolyses cellobiose at a higher rate at first. However, the dissolved enzyme will be quickly degraded and the catalytic activity of the microenvironment will rapidly decrease unless new enzyme is excreted by the soil microorganisms. The case very is different for activated carbon: because of its huge specific surface area and porosity, this materal entirely adsorbs cellobiose alongside β-glucosidase and the reaction is completely inhibited. It is hypothesized that the enzyme remains active when adsorbed to activated carbon but that the unaivailability of the substrate inhibits the reaction; therefore the concepts of potential and effective catalytic activity of soil enzymes were introduced. The comparison between the effects of wood char (low specific surface area and porosity) and activated carbon (extremely high specific surface area and porosity) on the reaction rate leads to the hypothesis that the rate of some soil enzymatic reactions involving soluble substrates may be manipulated by soil amendments with charred materials: charred materials with low specific surface area and porosities may mainly increase the pool of accumulated enzymes and therefore accelerate degradation whereas charred material with very high specific surface area and porosity may strongly reduce the bioavailability even of highly water soluble substrates and therefore slow down degradation. Formulating predictions on how degradation processes in soils would react to increases in the relative amounts of one

of the cited solid materials is a very speculative exercise since soils are highly heterogeneous systems. However, finding methods to control the rates at which soil organic matter is degraded may have huge practical applications since it may contribute to increase crop yealds, reduce the environmental impact of intensive agriculture and limit (or even reverse) the worringly increasing trend in atmospheric CO_2 concentrations. Therefore efforts should be directed towards investigating how different solid materials affect the rate of specific soil enzymatic reactions in laboratory experiments. Chromo- or fluorogenic substrates should be avoided in these tests whenever possible and the natural substrates should be used instead: the efforts necessary to monitor enzymatic reactions using the natural substrate would be rewarded with higher ecological significance of the results, since the sorptive properties of the substrate can be of crucial importance in determining reaction rates. Among the solids to be tested, priority should be given to biochars since these materials can be produced in huge amounts and with extremely different properties and may also stock carbon in a rather stable and useful pool. Among the enzymes priority should be given to those involved in wood degradation, such as cellulases and ligninases since these enzymes play a major role in the carbon cycle. Also, these enzymes should be tested for their applicability for industrial purposes in terms of heterologous expression and immobilizability: huge amounts of glucose could be produced from wood and this molecule may be the precursor of a range of different chemical products.

Finally, these experiments should be complemented with further studies on the techniques that generated Brazilian Terra Preta soils, on the mechanisms responsible for their sustainable fertility and with field experiments aimed at understanding the environmental consequences of biochar amendments to soils in temperate climates.

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