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Hot experience for cold-adapted microorganisms: Temperature sensitivity of soil enzymes

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ABSTRACT

High latitude and cold ecosystems, which constitute the major environment on Earth, are particularly threatened by global warming. Consequently, huge amounts of SOC stored in these ecosystems may be released to the atmosphere by accelerated enzymatic decomposition. Effects of intensive warming on temperature sensitivity and catalytic properties of soil enzymes were tested in cold-adapted alpine grassland of the Tibetan Plateau. We hypothesized that 1) maximal reaction rate will be insensitive to intensive warming at high temperature range ($V_{max} - Q_{10} = 1$); 2) substrate affinity (K_m) remains constant at elevated temperatures due to expression of enzymes with less flexibility. These hypotheses were tested by examining the kinetics of six enzymes involved in carbon (cellobiohydrolase, β-glucosidase, xylanase), nitrogen (tyrosine-aminopeptidase, leucine-aminopeptidase) and phosphorus (acid phosphomonoesterase) cycles after soil incubation at temperatures from 0 to 40 °C.

Q_{10} and E_a decreased at high temperature (25–40 °C). However, enzymes that degrade low quality polymers remained temperature-sensitive even above 25 °C ($V_{max} - Q_{10} = 2$), which explains the faster decomposition of recalcitrant C compounds under warming. Substrate affinity of all enzymes gradually increased up to 20 °C. At 25 °C, however, K_m increased rapidly, leading to an extreme decrease in catalytic efficiency. Above 25 °C, K_m of C and N cycles remained nearly constant, while V_{max} gradually increased from 0 to 40 °C. These results reveal two important implications of warming: 1) there are some temperature thresholds (here 20–25 °C) that lead to sudden reductions in substrate affinity, decreasing temperature sensitivity and catalytic efficiency, 2) decoupled temperature sensitivity of V_{max} and K_m and the resulting maintenance of stable enzyme systems at high temperatures ensured efficient enzymatic functioning and persistent decomposition of SOM at temperatures much higher than the common adaptation range of the ecosystem. Thus, the temperature thresholds of strong changes in enzyme-based processes should be considered and included in the next generation of models in order to improve the prediction of SOM feedbacks to warming.

1. Introduction

Microorganisms in the natural environment cope with changing conditions that demand a wide range of metabolic adaptations (Neidhardt et al., 1990). Among the most challenging environments are high latitude and cold ecosystems, which are threatened by global warming (Davidson and Janssens, 2006). Warming has a fundamental impact on microbial activity, metabolism and enzyme activities (Allison et al., 2010; Van Gestel et al., 2013; Zimmermann and Bird, 2012). Enzymes are essential to microbial metabolism and soil functioning, as they depolymerize large organic compounds and generate soluble oligomers and monomers that can be transported into the cells (Blagodatskaya et al., 2016; Wallenstein et al., 2010). Three mechanisms have been proposed to explain thermal adaptation of enzyme catalyzed processes: 1) change in the enzyme systems 2) the alterations in soil microbial biomass and enzyme expression at higher temperatures and 3) changes in quantity and quality of substrate, affecting reaction rates (Blagodatskaya et al., 2016).

Enzyme activity is a saturating function of substrate concentration and is described by the Michaelis-Menten relationship (Michaelis and Menten, 1913). Enzyme saturation occurs when all

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the enzyme active sites are already occupied by substrate. In this case adding more substrate will not increase the overall rate of the reaction. Both parameters of the Michaelis-Menten equation – the maximal catalytic reaction rate at a given temperature (V_{max}) and the half-saturation constant (K_m), are temperature-sensitive (Davidson et al., 2006; Davidson and Janssens, 2006) and usually increase with temperature (Stone et al., 2012). Various enzymes have different temperature sensitivities and changes in soil temperature may also alter the relative rates of decomposition of different components of organic matter (Koch et al., 2007; Wallenstein et al., 2010; Stone et al., 2012; Razavi et al., 2015). This may affect nutrient availability, for instance it has been observed that N availability may be decoupled from C and P cycling under warming conditions (Allison and Treseder, 2008). Therefore, the temperature sensitivity of enzymes responsible for organic matter decomposition is the most crucial parameter for predicting the effects of global warming on the nutrient and C cycles (Davidson et al., 2006; Davidson and Janssens, 2006).

The temperature sensitivity of V_{max} is directly related to the activation energy for enzyme reaction (Davidson and Janssens, 2006). Activation energies are parameters that mechanistically link enzyme kinetics and temperature response through the Arrhenius equation (Wallenstein et al., 2010). Based on the Arrhenius law, when activation energy is low, the exponential term will tend to 1 and consequently the reaction will become temperature independent (Marx et al., 2007). In the other words, the lower the activation energy, the lower the temperature sensitivity of the reaction rate. Enzymes catalyze biochemical reactions by lowering their activation energy (Gerlt and Gassman, 1993). Thus, a super-efficient enzyme will bring the activation energy to zero (Marx et al., 2007). This is important because, in the context of cold-adapted microorganisms, one way to maintain decomposition processes at low temperatures would be to develop enzymes that are temperature-independent (Marx et al., 2007).

Microbial physiology is evolutionarily selected for the most efficient enzyme systems (Allison et al., 2010; Hochachka and Somero, 2002). Moreover, the activities of hydrolytic enzymes could be adapted to different temperature regimes (Baldwin and Hochachka, 1969; German et al., 2012) with the goal of maintaining critical enzymatic functions. There is evidence for biogeographical patterns in enzyme temperature sensitivity (Huston et al., 2000; Feller, 2003; German et al., 2012). Many studies have observed that cold-adapted microorganisms can produce cold-adapted enzymes that catalyze reactions at lower temperatures with lower activation energy and with higher binding affinity (i.e. low K_m) (Fields, 2001; Bradford, 2013) than their mesophilic counterparts (Gerday et al., 1997). Importantly, microbial adaptation and acclimation strategies have physiological costs (Schimel et al., 2007) and can reduce enzyme catalytic efficiency – determined as V_{max}/K_m (Stone et al., 2012; Tischer et al., 2015).

The parameters of enzyme kinetics – specifically K_m, which determines the binding affinity of the enzyme to substrate – are indicative of enzyme flexibility (the capacity for quick conformation change) (Somero, 1975). The increased flexibility would cause the cold-adapted enzyme to spend more time maintaining conformations that are not optimal for substrate binding (Siddiqui and Cavicchioli, 2006). This can be measured as a gradual increase of K_m with temperature (Fields, 2001). Key to effective enzymatic function is the trade-off between functional capacity and enzyme flexibility, which co-vary with habitat temperature (Somero, 1995; Fields, 2001; Tokuriki et al., 2012). Conformational flexibility and enzyme function are closely related, and organisms have evolved to produce enzymes with thermal optima at their habitat temperature. For example, more flexible enzyme systems are expected under cold conditions, while strongly reduced enzyme flexibility (i.e. low temperature sensitivity of K_m) is predicted in warmer climates (Johns and Somero, 2004; Dong and Somero, 2009; Bradford, 2013).

Furthermore, as enzyme systems are altered by climate warming, different sets of isoenzymes (i.e., enzymes with the same function but different conformations and structures) are expected to be expressed at cold and warm temperatures (Somero, 1978; Bradford, 2013; Razavi et al., 2016). Isoenzymes with higher temperature optima can be produced by the same microbial species adapted to warming (Hochachka and Somero, 2002). Alternatively, isoenzymes can be expressed as a result of changes in microbial community structure caused by warming (Baldwin and Hochachka, 1970; Vanhala et al., 2011). In both cases, temperature sensitivity of catalytic reactions is dependent on enzyme isoforms. Nonetheless, all these mechanisms suggest that microbes prefer to produce enzymes that maintain optimal activity under native soil conditions.

Despite intensive discussion on the mechanisms of enzyme temperature sensitivity, it remains unclear how the functional characteristics of enzymes in cold-adapted soil will be altered by temperature increases. This is extremely important because it provides evidence of the response of cold-adapted soil microbes and the fate of huge amounts of SOC stored in these ecosystems by acceleration of enzymatic decomposition in a warmer world. In addition, there is a lack of studies on the catalytic efficiency of soil enzymes in cold ecosystems as affected by warming.

This study was designed to test the effects of intensive warming on the catalytic properties of soil enzymes in a cold-adapted environment. We hypothesized that maximal reaction rate will be insensitive to intensive warming at high temperature range (H1); and that the substrate affinity (K_m) will remain constant at elevated temperature (H2). To test our hypothesis we collected soil from the Tibetan Plateau and incubated the samples for one month over a temperature range of 0–40 °C (with 5 °C steps) and determined the kinetics, temperature sensitivities and activation energy of six enzymes involved in decomposition of soil organics: cellobiohydrolase and β-glucosidase, which are commonly measured as enzymes responsible for consecutive stages of cellulose degradation (German et al., 2011); xylanase, which degrades xylooligosaccharides into xylose and is thus responsible for breaking down hemicelluloses (Chen et al., 2012); acid phosphomonoesterase, which hydrolyzes (mono) ester bonds of organic P to phosphate under acidic conditions (Eivazi and Tabatabai, 1977; Malcolm, 1983; German et al., 2011). Activities of tyrosine aminopeptidase and leucine aminopeptidase were analyzed to assess the hydrolysis of peptide bonds (Koch et al., 2007; Chen et al., 2012).

2. Material and methods

2.1. Site description and soil collection

The sampling site is located in the upper Kyi Chu catchment north of Lhasa in Pando County, above the Reting Monastery in Qinghai-Tibetan Plateau (south west of China, 4330 m a.s.l.) (Table 1). The mean precipitation during the growing season (from May to October) is 330 mm. The temperature during the growing season ranges between −4 and +17.7 °C. This site has the largest and most sacred *Juniperus* forest in Tibet, diffusely growing in a carpet-like felty turf of *Kobresia pygmaea* C.B. Clarke (Miehe et al., 2008) which is the dominant and eponymous species (covering up to 98% of the root-mat surface).

Four composites of six soil samples each were collected using soil cores (18.5 cm long 4.5 cm diameter). Each composite sample was collected over a 30 m^2 area. A variable-depth sampling scheme was used to obtain the entire A-horizon. This sampling scheme increases our confidence for minimizing random variation in soil

Table 1
Basic information of the sampling site.

Site	Location	MAP (mm yr^{-1})	MAT (°C)	Dominant soil types	Horizon	Dominant species
Reting, Lahsa	30°18′50″N, 91°30′47″E	549	2.4	Cambisols	A	*Juniperus tibetica, Kobresia pygmaea* C.B. Clarke

properties. Samples were collected in August 2015 when the mean monthly air temperatures were around 2.4 °C.

Once collected, samples were hand-mixed, roots and stones were separated and composite samples were placed in ziplock bags, and kept cold (~4 °C) for transport back to the laboratory (Göttingen University). Thereafter, samples were passed through a 2 mm screen and prepared for incubation.

Extra soil samples were oven-dried under 60 °C for 48 h and used for measurement of soil properties. Soil pH, at the ratio of 1–2.5 (soil to water), was measured using a pH-meter (Metrohm, Herisau, Switzerland). Soils were analyzed for total C and N using an elemental analyzer (Vario Max CN, Hanau, Germany). Soil properties are shown in Table 2.

2.2. Soil incubation and enzyme assays

Enzyme assays were prepared by placing 30 g of soil in air-tight vials (125 ml) equipped with rubber seals. Six enzymes targeting C-, N- and P-containing substrates were investigated after progressively incubating the soil at 0, 5, 10, 15, 20, 25, 30, 35 and 40 °C for one month. During the incubation, soil moisture was checked by weighing and was immediately adjusted to equal 60% of WHC. In order to avoid anaerobiosis, all the samples were regularly aerated by opening the vials for 1 min. Nine climate chambers (SBS C120) were used to regulate the temperature ($< \pm 0.5$ °C).

The kinetics of hydrolytic enzymes involved in C, N and P cycles were measured by fluorimetric microplate assays of 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC) (Marx et al., 2005). Four types of fluorogenic substrates based on MUF and two types based on AMC were used to assess enzymatic activities: 4-methylumbelliferyl-β-D-cellobioside (MUF-C) to detect cellobiohydrolase activity; 4-methylumbelliferyl-β-D-glucoside (MUF-G) to detect β-glucosidase activity; and 4-methylumbelliferyl-β-D-xylopyranoside (MUF-X) to detect xylanase activity. The activities of tyrosine aminopeptidase and leucine aminopeptidase were measured using L-tyrosine-7-amido-4-methyl-coumarin (AMC-T) and L-leucine-7-amino-4-methyl coumarin (AMC-L). 4-Methylumbelliferyl-phosphate (MUF-P) was used to detect acid phosphomonoesterase activity. All substrates and chemicals were purchased from Sigma (Germany).

We determined enzyme activities over a range of substrate concentrations from low to high (0, 10, 20, 30, 40, 50, 100, 200 μmol g^{-1} soil). At each temperature four replicates were incubated. In addition, for all four incubation replicates, the assay of each enzyme at each substrate concentration was performed using three analytical replicates (12 wells in the microplate). To ensure the saturation concentrations of fluorogenic substrates preliminary experiments were performed. Besides, linear increase of fluorescence over time during the assay was properly checked and data, which was obtained after 2 h, was used for further calculation (German et al., 2011).

Suspensions of 0.5 g soil (dry weight equivalent) with 50 ml water were prepared using low-energy sonication (40 J s^{-1} output energy) for 2 min (Stemmer et al., 1998). Then 50 μl of soil suspension was added to 100 μl substrate solution and 50 μl of buffer [MES (pH:6.8) buffer for MUF substrate and TRIZMA (pH:7.2) buffer for AMC substrate] in a 96-well microplate (Koch et al., 2007). During pipetting, the soil suspension was kept agitation. Later each well was homogenized with 2 or 3 aspirations/ejections using a multi-channel micropipette. Fluorescence was measured in microplates at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, slit width of 25 nm, with a Victor 3 1420-050 Multi Label Counter (Perkin Elmer, USA). Right before each measurement each plate was shaken for 1 min. All enzymes were determined and incubated at exact temperature over 2 h. After each fluorescence measurement (i.e. after 30 min, 1 h and 2 h) the microplates were promptly returned to the climate chambers, so that the measurement time did not exceed 2–2.5 min. During assay-incubation, microplates, at all different temperatures, were covered tight to prevent evaporation of solutions within the microplates.

Enzyme activities were expressed as MUF or AMC release in nmol per g dry soil per hour (nmol product released h^{-1} g^{-1} dry soil). Enzyme activity (nmol product released h^{-1} g^{-1} dry soil) was calculated from the MUF or AMC standard curve following German et al. (2011). We checked possible temperature effects on the chemical decomposition and thermal hydrolysis of the four MUF-substrates and two AMC-substrates, but no significant effects were detected over the range 0–40 °C (Razavi et al., 2015).

The Michaelis-Menten equation was used to determine parameters of the enzyme activity *(V)*:

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where V_{max} is the maximum enzyme activity; K_m represents the half-saturation constant, or the substrate concentration at which the reaction rate equals $V_{max}/2$; and S is the substrate concentration at active site of the enzyme (Michaelis and Menten, 1913; Segel, 1975; Von Lützow and Kögel-Knabner, 2009). Both V_{max} and K_m parameters were approximated by the Michaelis-Menten equation (1) with the non-linear regression routine of STATISTICA. Fitting was performed for the mean of 12 replicates. Analysis of variance (ANOVA) followed by the Tukey HSD at a probability level of $p < 0.05$ was used to define the ranges of temperatures with significantly different K_m ($p < 0.05$). This means that pairwise differences were applied to distinguish the significant differences for each neighboring pair of independent variables (mean values of K_m at 0, 5, 10, 15, 20, 25, 30, 35, 40 °C) (Razavi et al., 2015, 2016). Homogeneity of variance and normality of the values was tested by Levene's test and the Shapiro-Wilk test. We used the routine Q_{10} function (2) to examine temperature sensitivity and to express temperature responses of each enzyme kinetic parameter (i.e., K_m or V_{max} separately).

$$Q_{10} = \left(\frac{R_{(T+10°C)}}{R_{(T)}}\right) \quad (2)$$

where R is the rate of a process or a value of a kinetic parameter and T is temperature (Kirschbaum, 1995).

Table 2
Description of soil properties.

Site	Soil bulk density (g cm^{-3})	C (%)	N (%)	C/N	Soil pH
Reting	1.1	4.4 ± 0.2	0.3 ± 0.01	14 ± 0.3	5.5

The activation energy was calculated according to the classical Arrhenius equation (Eq. (3)):

$$k = A\,exp\left(-E_a/RT\right) \quad (3)$$

where k is the reaction rate constant; A is the frequency of molecular collisions; E_a is the required activation energy in Joules per mole; R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is the temperature in Kelvin. The activation energy was calculated in two steps: once for the low temperature range from 0 to 20 °C and once for the elevated range from 25 to 40 °C. These two steps were selected on the basis of the absolute maximum temperature of the studied area: 24.1 °C (Miehe et al., 2008).

3. Results

3.1. Temperature sensitivity of enzyme activity

The V_{max} values increased with temperature for all enzymes (Fig. 1 and Fig. S1). Changes in V_{max}-Q_{10} were not gradual over the whole range of temperatures tested, and were clearly pronounced between 0 and 15 °C (Fig. 2). The magnitude of the temperature response varied between enzymes, ranging from 1.3 to 3.8, which corresponds to E_a values of 19–53 kcal mol^{-1} (Fig. 3). For all enzymes, E_a was higher in the low temperature range (0–20 °C) and decreased strongly from 25 to 40 °C (Fig. 3). The fitting of V_{max} to the Arrhenius model demonstrated higher E_a values for cellobiohydrolase and xylanase compared to proteases, acid phosphomonoesterase and β-glucosidase.

3.2. Response of substrate affinity to temperature

The changes in K_m-Q_{10} were not gradual over the range of temperatures tested, and were maximal between 0 and 15 °C (Fig. 2, Table S1). The Q_{10} values for K_m varied over a more narrow range of 1.0–2.5 that was 1.5 times lower compared to V_{max}-Q_{10}. The K_m-Q_{10} demonstrated two enzyme-specific patterns: 1. Decrease of K_m-Q_{10} for the whole temperature ranges; this pattern corresponded to enzymes of the C and N cycles. 2. The pattern observed for acid phosphomonoesterase K_m-Q_{10} was nearly constant over the whole temperature range.

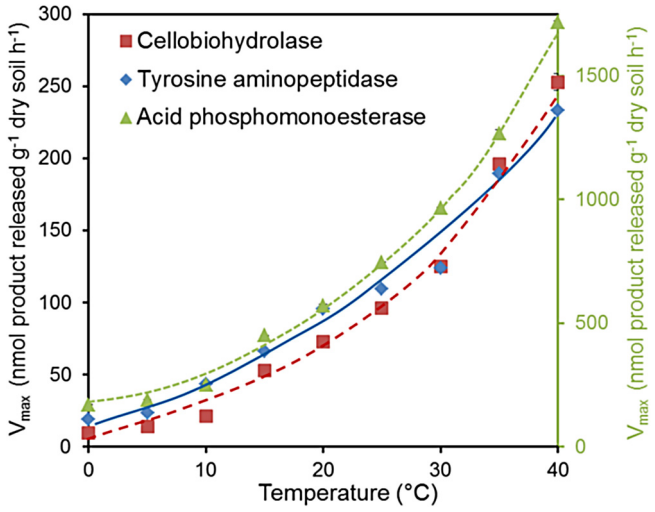

Fig. 1. Enzyme activity as a function of temperature demonstrates a gradual increase for cellobiohydrolase, tyrosine aminopeptidase and acid phosphomonoesterase within the range of nine temperatures. Each enzyme was assayed at a range of substrate concentrations (8 concentrations) at each of 9 temperatures. Error bars stand for standard error. (Activity of the other three enzymes are presented in Table S1).

The temperature effect on K_m revealed a distinct threshold with a significant decrease in the affinity of all enzymes to substrate at temperatures above 25 °C (Fig. 4). Cellobiohydrolase, β-glucosidase and xylanase demonstrated stepwise increases of K_m values at low to moderate temperatures (0–20 °C). The K_m values of these enzymes strongly increased (by around 40%) between 20 and 25 °C (Fig. 4). After such an extreme increase, the K_m values did not change significantly up to 40 °C (Fig. 4). The changes of acid phosphomonoesterase's K_m followed a pattern different to that of the enzymes involved in carbohydrate decomposition and proteases. Acid phosphomonoesterase demonstrated slightly increased K_m values across the whole temperature range (0–40 °C), (Fig. 4).

Thus, the K_m of all C and N cycle enzymes changed significantly within psychrophilic and mesophilic temperatures, while substrate affinity was relatively constant within the elevated range (25–40 °C).

3.3. Catalytic efficiency of enzymes as affected by temperature

The catalytic efficiency of the enzymes (V_{max}/K_m) increased from cold to moderate temperatures (0–20 °C). Further extreme increases in K_m at the 25 °C threshold were always accompanied with a sharp decrease in the catalytic efficiency of enzymes of the C and N cycles (Fig. 4), and leveled off above 25 °C. In contrast, the catalytic efficiency of acid phosphomonoesterase increased gradually from 0 to 40 °C (Fig. 4).

4. Discussion

Most soil studies and models tacitly accept the gradual (according to Q_{10}) increase of reaction rates (and consequently process intensities) with temperature. Both V_{max} and K_m increased with temperature for all tested enzymes, although the increase was not linear and indicated different temperature sensitivities of V_{max} and K_m (Figs. 1 and 4). The Q_{10} values of reaction rates varied from 1.9 to 3.8 within the low temperature range and decreased to 1.3 at higher temperature. Similarly, the activation energy of all tested enzymes was higher at low and moderate temperatures (0–20 °C) compared to elevated levels (25–40 °C). This general reduction of temperature sensitivity confirms theoretical predictions (Davidson and Janssens, 2006) and experimental observations on reduced reaction rate Q_{10} values at elevated temperature (Tjoelker et al., 2001; Razavi et al., 2015). In line with previous studies, activation energy and temperature sensitivity of enzymes responsible for complex C-compound degradation (i.e. xylanase and cellobiohydrolase) were higher compared to β-glucosidase (Craine et al., 2010; Conant et al., 2011). However, contrary to our hypothesis (H1), reaction rates of enzymes that degrade low quality polymers remained temperature sensitive (i.e. $V_{max} - Q_{10} = 2$) even in warm temperature ranges. According to Arrhenius law the higher activation energy associated with the breakdown of recalcitrant substrates could result in a greater temperature sensitivity of decomposition (Knorr et al., 2005; Hartley and Ineson, 2008). This logic appears to be supported by measurements of the temperature sensitivity of leaf litter decomposition (Fierer et al., 2005).

We found a gradual increase of K_m from 0 to 40 °C (acid phosphomonoesterase) and for all other tested enzymes from 0 to 20 °C. This could be a consequence of increased enzyme flexibility, i.e. the capacity for quick conformation changes ensuring a fast rate of catalytic reaction by changing temperature. We also assume that the gradual increase of K_m with increasing temperature may reflect stepwise expression of isoenzymes. Proteases and cellulolytic

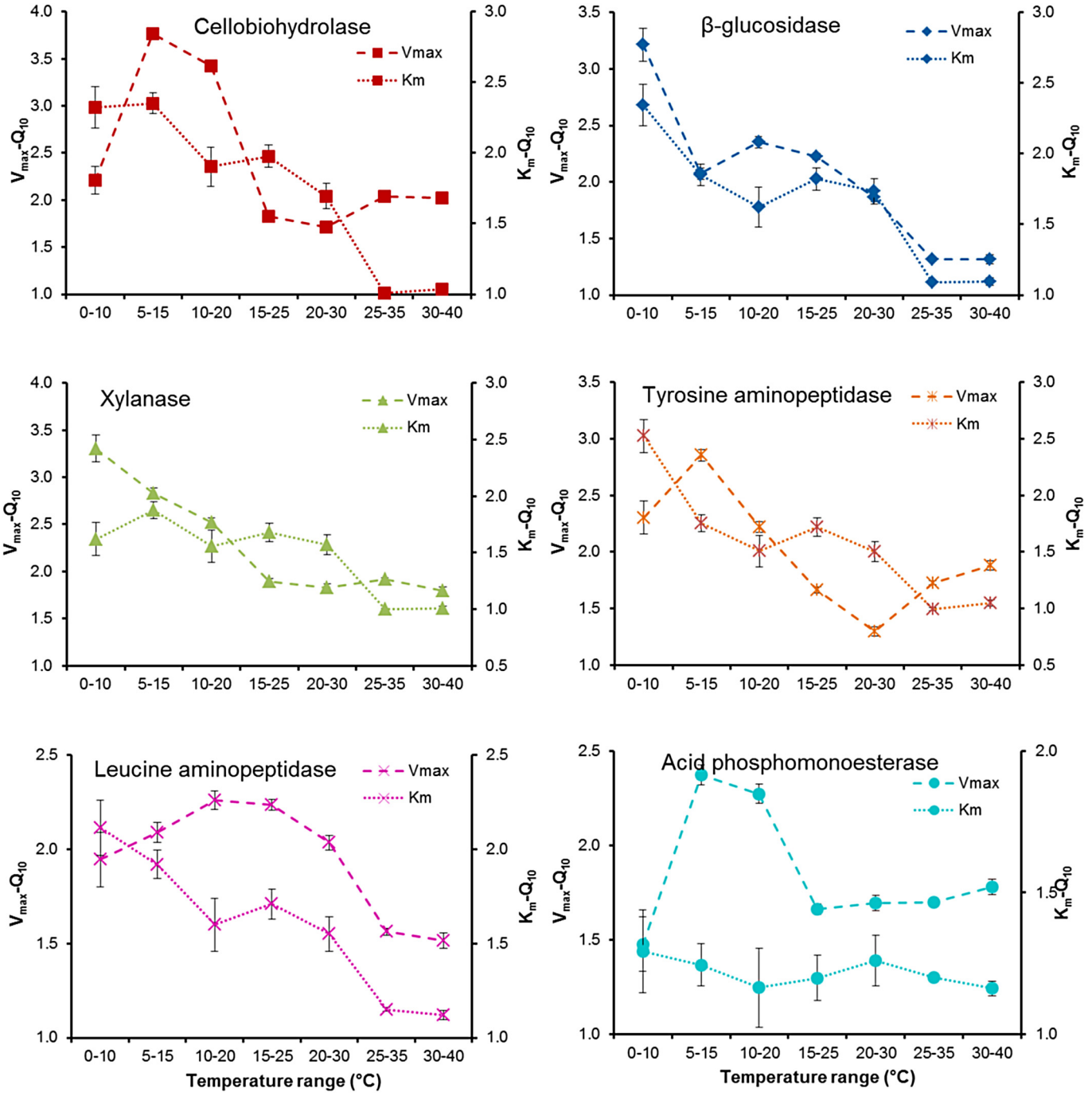

Fig. 2. Temperature sensitivity of maximal reaction rate ($V_{max} - Q_{10}$) and substrate affinity ($K_m - Q_{10}$) of six enzymes as a function of temperature with 5 °C increments.

enzymes demonstrated constant K_m from 25 to 40 °C which is in line with the previous findings of Fields and Somero (1998) and the theoretical prediction of Bradford (2013) regarding the stability of enzyme systems at high temperatures. A strong increase in K_m by 40–50% at high temperatures (25 versus 20 °C) reflected a two-fold reduction of the enzyme-substrate affinities. However, such temperature thresholds seem to be higher in temperate climates (30 °C) (Razavi et al., 2016), compared to highland areas like Tibet (25 °C).

Following the strong increase at 20 °C, the K_m remained nearly constant from 25 to 40 °C, while the maximal enzyme activity (V_{max}) gradually increased with temperature. The accelerated enzymatic activity (V_{max}) by temperature could indicate the increase in enzyme production due to an increase in microbial biomass. Alternatively, constant K_m – accordance with our hypothesis (H2) – can be explained by an expression of multiple isoenzymes each with a different temperature optimum (Somero, 1995; Bradford, 2013). Such isoenzyme expression leads to an optimal balance between the static character of the enzyme (responsible for high efficiency at constant optimal temperature) and functional capacity, under their respective optimal working conditions (Zavodsky et al., 1998; Conant et al., 2011; Razavi et al., 2016).

Sudden and strong changes in K_m at 25 °C indicated a switch

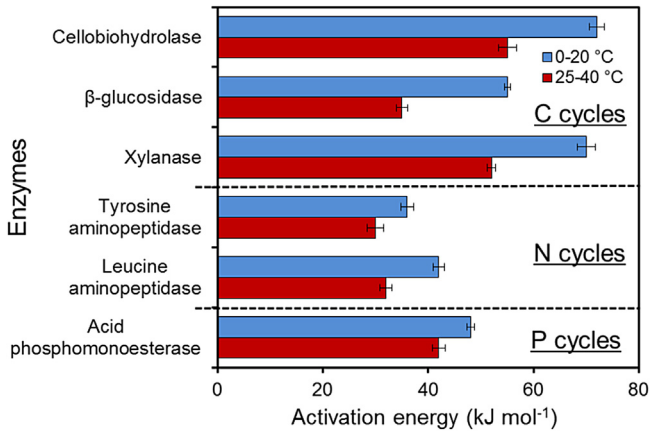

Fig. 3. The activation energy (E_a) of all tested enzymes at two temperature ranges: low (0–20 °C) and high (25–40 °C).

from cold- and moderate-to warm-adapted enzyme systems with decreased substrate affinity. In fact, such an increase was responsible for the reduced temperature sensitivity and catalytic efficiency of overall enzyme function. A constant K_m value from 25 to 40 °C was accompanied by a gradual increase of catalytic efficiency with temperature. From another point of view, production of enzymes with similar substrate affinity and higher efficiency might be a preferred microbial strategy (Stone et al., 2012; Hoang et al., 2016) in the studied soil. Catalytic efficiency demonstrated a general trend of gradually increasing with temperature at both cold and warm temperatures (Figs. 4 and 5). The only remarkable exception occurred at 25 °C, where a strong increase in K_m was accompanied by a significant decrease in catalytic efficiency (Fig. 5). Thus, decoupled responses of V_{max} and K_m to temperature resulted in irregular increases of catalytic efficiency with temperature. Quite simply, if catalytic properties are to be maintained under a particular thermal regime, the "goal" that must be met would be expression of isoenzymes with similar K_m values (Somero, 1978). Thus, maintaining the high binding affinity to substrate (constant K_m) ensured efficient enzyme conformation within the unaccustomed temperate range.

However, to generalize the conclusions based on one soil type from alpine climate, more soils from various zones need to be tested. Therefore, we need more mechanistic work, *in situ* studies along with the studies of pure and isolated enzymes from a range of habitats to verify assumptions regarding temperature responses of specific proteins. Previous studies on pure cultures demonstrated a decline in catalytic efficiency between 20 and 30 °C (Siddiqui and Cavicchioli, 2006). However, the pattern observed here was different which might be due to the complex composition of microbial communities in soil. In addition, as these temperatures are extremely unusual for the original microbial community under the natural climate, with an annual temperature of 2.4 °C (Bárcenas-Moreno et al., 2009), such sharp changes in K_m could be an indicator of isoenzyme expression (Baldwin and Hochachka, 1970) due to a major shift in species dominance above 25 °C or an alteration in enzyme systems (Khalili et al., 2011; Bradford, 2013). However, while such a conclusion has been done for the one soil studied here, the relevance of the observed patterns needs to be proven for soils with contrasting properties (e.g., texture, structure, pH, C content, etc.) in a range of climate zones, e.g., in boreal and tropical environments. Furthermore, thermal denaturation – usually occurring at temperatures much higher than 40 °C (dos Santos et al., 2004; Goyal et al., 2014) – affects the kinetic constants of enzymes and also increases K_m (Dick and Tabatabai, 1978). These indirect

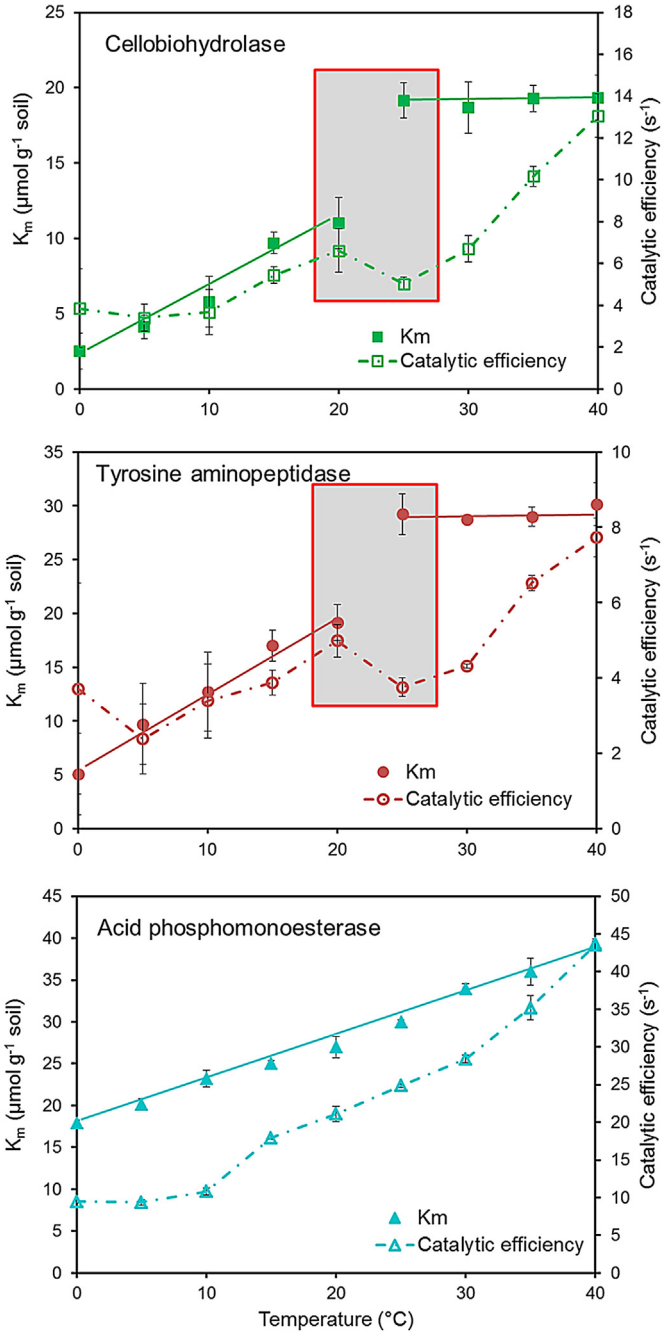

Fig. 4. K_m and catalytic efficiency (V_{max}/K_m) of cellobiohydrolase, tyrosine aminopeptidase and acid phosphomonoesterase. Shading indicates temperature ranges with extreme K_m increases accompanied by decreases in catalytic efficiency. (K_m and catalytic efficiency of the other three enzymes are presented in Table S1).

mechanisms of K_m increase with temperature due to interactions of enzymes with soil particles.

Overall, i) enzymes that degrade low quality polymers are temperature-sensitive over the whole range of temperatures (0–40 °C); ii) soil microorganisms are able to maintain stable or flexible enzyme systems with low or high substrate affinity within wide temperature ranges to ensure efficient enzymatic functioning under diurnally and annually varying temperatures. This ensures the easier adaptation of microbially driven decomposition to changing climate. Thus, acclimation may involve the expression of enzymes at a warmer temperature, potentially with the same K_m

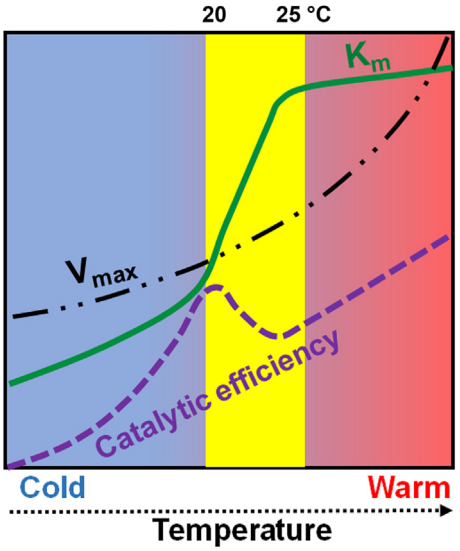

Fig. 5. Generalized thermal responses of enzyme catalytic properties to a temperature increase. The scheme explains that catalytic efficiency gradually increases with temperature at both cold and warm temperatures except at 25 °C, where a strong increase in K_m occurs.

but not necessarily. We conclude that consideration must be given to the temperature thresholds of strong changes in enzyme-based processes and that this is crucial to modeling the consequences of warming for C, N and P cycles and predicting the fate of soil carbon stocks in a warmer world.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.11.026.

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