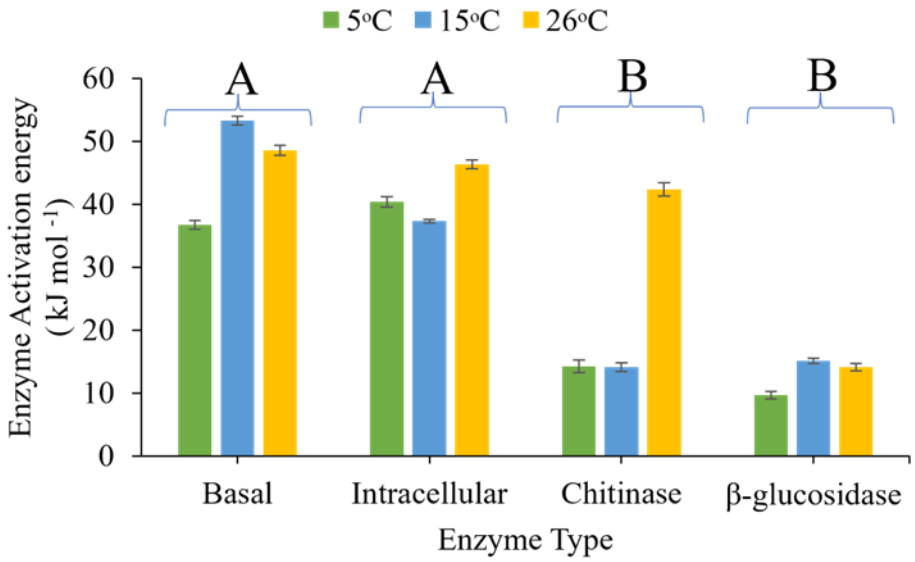
Reviewer 1

I have only one major point to raise:

In line 170 authors wrote "Ea was calculated for two ranges of temperature (5 °C – 26 °C for intracellular enzymes and basal respiration, and 5 °C – 37 °C for the two extracellular enzymes) to ensure that the data used to calculate Ea conformed to the Arrhenius functional form (Schulte, 2015)". Looking at the figures it is not clear why authors decided to use different temperatures to calculate the activation energy of intracellular and extracellular processes. I would suggest to use the same range of temperatures or justify very well why they think the ranges of temperatures should be different. As the authors want to compare the activation energy from these different processes, using distinct temperature ranges creates a problem for their comparison. Please justify your choice or make the required changes in the analysis and corresponding figure.

Our reason for calculating Ea at 5 °C – 26 °C for intracellular metabolic processes and basal respiration, and 5 °C – 37 °C for the two extracellular enzymes was to ensure that the Ea was quantified in the rising phase of the temperature response for each assay (consistent with Arrhenius theory). However, we understand that this can be problematic when making a direct comparison between the intracellular and extracellular processes. We have therefore calculated Ea at 5 °C – 26 °C for both intracellular and extracellular processes in the revised manuscript and presented this in a revised Figure 4. The main difference is lower Ea for chitinase in soils pre-incubated at 5 °C and 15 °C. The p value for the effect of pre-incubation temperature on Ea increases from P = 0.001 to P = 0.002 but the p value for the interaction between pre-incubation temperature and enzyme type decreases from P = 0.046 to P = 0.029. β-glucosidase activity and chitinase activity had a significantly lower Ea than intracellular metabolic activity, and basal respiration.



Reviewer 2

I have gone through the revised manuscript titled "Differential Temperature Sensitivity of Intracellular and Extracellular Soil Enzyme Activities" by Adekanmbi et al. The manuscript has shown improvement, and the authors have addressed all of my comments. However, there are still some points that require correction or clarification.

1/I am surprised that the article titled "The Inflection Point Hypothesis: The Relationship between the Temperature Dependence of Enzyme-Catalyzed Reaction Rates and Microbial Growth Rates" by Erica J. Prentice et al. has not been cited in the present study, as its present theory that explain the results obtain in the present study. Therefore, I suggest that the authors cite and integrate this article into their discussion.

We thank reviewer 2 for highlighting this article to us. We have integrated this into our discussion. However, if we understand correctly, we think that it does not explain our results. The theory indicates that the optimum growth rates of organisms occur at the inflection point of the rising phase of the thermal response of intracellular enzymes. However, we don't see any consistent relationship between pre-incubation temperature and the inflection point and we observe that the inflection point is consistently at a higher temperature for extracellular enzymes than intracellular processes. Of course, this interpretation relies on the assumption that it is the same organisms producing the extracellular enzymes who also carry out the intracellular processes.

We expanded the following sentence in the previous version:

> *"Our finding that intracellular catabolism increased with increasing assay temperature up to an optimal temperature between 26 °C and 37 °C, followed by a significant decline thereafter, is very likely due to the inability of the microbial population to function optimally above 37 °C due to impairments in their physiological processes (Todd-Brown et al., 2012; Maire et al., 2013)"*

So that is now reads:

> *"Our finding that intracellular catabolism increased with increasing assay temperature up to an optimal temperature between 26 °C and 37 °C, followed by a significant decline thereafter, is very likely due to the inability of the microbial population to function optimally above 37 °C due to impairments in their physiological processes (Todd-Brown et al., 2012; Maire et al., 2013) and uncoupling of relative rates of constituent enzymes leading to regulatory compromise (Prentice et al. 2020)."*

We have also added the following passage of text to our discussion:

> *"Prentice et al., (2020) identify a relationship between the inflection point of the rising phase of the thermal response of intracellular enzyme activities and the growth rate of the organism. This inflection seems to occur between 15 °C and 26 °C for intracellular metabolic processes and between 26 °C and 37 °C for extracellular enzymes in our experiment and pre-incubation temperature does not consistently affect the temperature at which this inflection point occurs (Figure 2)."*

Please also correct the title based on reviewer 1 and my comment about "intracellular enzyme" vs "intracellular metabolic processes'".

We have corrected the title to "*Differential Temperature Sensitivity of Intracellular Metabolic Processes and Extracellular Soil Enzyme Activities*"

2/I appreciate the authors' clarification on the reason for calculating Q10 at different temperatures and presenting graphs of the relationship between the natural log of enzyme activity/respiration and temperature. The presented graphs should be included in the article, either in the main text or as supplementary information.

We have added these figures to the supplementary material and included the following sentence in our method:

> *"The primary reason why we calculated Q10 at different temperatures is because we found that temperature sensitivity was different at different temperature ranges. This meant that there was not a good linear relationship between the natural log of enzyme intracellular metabolic processes or extracellular enzyme activity and temperature, apart for β-glucosidase, as demonstrated in the supplementary material."*

It seems that beta glucosidase follows the Arrhenius function, and respiration thermal sensitivity presents a bell-shaped response similar to the one proposed by the MMRT. However, I am more concerned about the thermal response of Chintinase, which appears to be more chaotic. Therefore, I suggest that the authors add a couple of sentences that precisely describe the observed temperature relationship of Chintinase and provide arguments or hypotheses to explain why the Chintinase temperature does not follow what is generally observed.

In the previous version of the manuscript we already provided quite a long discussion of the chitinase thermal response as follows:

> *"The potential activity of chitinase also increased with temperature, but, in contrast to β-glucosidase, the response, over the range of assay temperatures, was non-monotonic, reaching a maximum activity between 37 °C and 45 °C. This observed non-monotonic response to increasing temperature is interpreted in terms of three distinct phases: (i) a rising phase where temperature increases lead to increasing reaction rate due to thermodynamic effects, (ii) a plateau which represents the optimum temperature and (iii) a steep falling phase where rate declines beyond the optimum temperature (Schulte, 2015) attributed to thermal denaturation of proteins. Our optimum for chitinase (37 to 45 °C) is relatively consistent with the report of a maximum activity for soil chitinase of 45.5 °C (as assayed through quantification of N-acetyl glucosamine released from added chitin; (Rodriguez-Kabana, et al., 1983)) but contrasts to the optimum of ~63 °C reported in the study by (Parham and Deng, 2000) using the same p-nitrophenol-based assay as used here. Differences in these optimum temperature-activity responses between soils may be due to differences in microbial composition (and thus microbial-produced chitinase isozymes) between soils. Optimum temperatures varying between 40 °C and 60 °C have been recorded for chitinases (partially) purified from soil microorganisms (Gao et al., 2008; Alster et al., 2016; Du et al., 2021; Thakur et al., 2021). Additionally, soil-type dependent stabilization of enzyme structure against thermal denaturation through interaction with soil surfaces might also mediate differential temperature responses (Sarkar et al., 1989). It is presumed that β-glucosidase activity in our study soil had a temperature optimum beyond the maximum tested and our finding that the optimum temperature for chitinase activity was lower than that of β-glucosidase is likely due to between-enzyme family differences in protein structural*

properties conferring thermal stability, resulting in differential susceptibility of different enzyme families to thermal denaturation or degree of stabilization in soil."

Instead of adding more to this part of the discussion, we have added a sentence to the end of the paragraph where we introduce the inflection point hypotheses and note the following:

"However, the chitinase enzyme activity does not clearly demonstrate the bell-shaped thermal response expected by macromolecular rate theory. This response may have been observed if assays were undertaken at more temperatures between 26 °C and 45 °C."

3/Was the buffer pre-incubated at different temperatures at the desired temperature? How long did it take for the assay solution to reach the desired temperature (5C or 40C)? As the activity was only assessed based on a 30 min assay, I am wondering if the measure really reflects the activity at 5 degrees or rather an average temperature between room temperature and 5 degrees during the 30 min of cooling. In the latter case, that would mean that extreme temperatures (temperatures that are most different from room temperature, 5 or 40C) are most likely wrong or closer to room temperature than expected (8 C, 35 C, for example). Moreover, did the authors control the temperature of the buffer assay solution during the 30 min, or at least control the temperature at the end?

Overall, the material and method section needs to be much more precise on this point, as the entire article is about the temperature sensitivity of enzymes, and the observed response of one enzyme (chitinase) does not follow proposed/common theory (Arhenius, MMRT).

The buffer was pre-incubated at room temperature rather than at assay temperature. We have made the text more precise by stating "*1 g of soil was weighed into a 50 ml centrifuge tube and mixed with 4ml of room temperature pre-incubated 4-methylumbelliferone (MUB) buffer (pH 6)*"

Unfortunately, we cannot change this and accept that it is likely that during the first few minutes of the assay the temperature of the soil will change from room temperature to incubation temperature. We have added the following sentence to the methodology to acknowledge this shortcoming.

"It is likely that during the first few minutes of the assay the soils were changing temperature from room temperature to the assay temperature."

The temperate during the assay was controlled. The incubators where the incubations took place were set to the desired temperature long before the start of the assay.

4/In the materials and methods section, the authors indicate that the concentration of the substrate used was "1ml 25mM nitrophenyl-β-D-glucopyranoside or 10 mM p-nitrophenyl-N-acetyl-b-D-glucosaminide solution." I suggest that the authors explain why they chose this concentration and whether it was chosen to be most likely in a non-substrate limiting condition (although not tested for the soil under study). In the latter case, this would mean that the authors measured Apparent Vmax.

The protocols are based on published methods. Eivazi and Tabatabai (1988) used 25mM PNG and Parham and Deng (2000) used 10mM NAG.

We have good evidence to suggest that the concentrations chose are not substrate limiting since we carried out a preliminary experiment incubating the soil and the substrates for 15, 30, 45, and 60

minutes and observed a linear response (Figures S-3 and S-4 of the Supporting Information). Had the assay been substrate limiting, we would not have observed a linear response. The time selected for the experimental assay (30 minutes) was in the middle of this linear range.

Eivazi, F and Tabatabai, M A (1988) Glucosidases and galactosidases in soils. Soil Biology & Biochemistry 20, 601-606

J.A. Parham, S.P. Deng (2000) Detection, quantification and characterization of b-glucosaminidase activity in soil. Soil Biology & Biochemistry 32, 1183-1190.